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Journal of Nutritional Biochemistry 23 (2012) 640–645

Journal of Nutritional Biochemistry

Continuous intake of a high-fat diet beyond one generation promotes lipid accumulation in liver and white adipose tissue of female mice

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Received 29 December 2010; received in revised form 21 February 2011; accepted 7 March 2011

Abstract

Lipid metabolism in a child may be altered when the mother has a high-fat diet (HFD), but it is unclear whether the lipid metabolism of future offspring (grandchildren) is also changed under these circumstances. In this study, we examined the influence of intake of an HFD beyond one generation on offspring in normal mice. Parent mice fed an HFD were bred and the resultant second and third generations were also fed an HFD. The diets used in the study had approximately 20% more energy than a standard chow diet. Changes in lipid metabolism were examined in each generation. Intake of an HFD from generation to generation promoted lipid accumulation in the white adipose tissue of female mice, increased lipid, glucose and insulin levels in the serum, increased the activities of enzymes associated with fatty acid metabolism in the liver, promoted lipid accumulation in hepatocytes and adipocytes and increased the mRNA levels of Cdkn1a in the liver and white adipose tissue. These results suggest that activation of Cdkn1a promoted lipid accumulation in the liver and white adipose tissue of third-generation female mice that were offspring from earlier generations fed HFDs. Moreover, intake of a high-energy diet beyond one generation led to offspring with obesity, fatty liver and hyperinsulinemia.

Keywords: Cdkn1a; Offspring; High-fat diet; Obesity; Fatty liver; Insulin resistance

1. Introduction

There has been a recent increase in obesity worldwide [1], and this condition is likely to increase further, especially in women [2,3]. Obesity is strongly related to the pathogenesis of lifestyle diseases such as type 2 diabetes, hypertension and ischemic heart disease and is also associated with cancer [1,4,5]. These diseases cause a marked decrease of quality of life, which emphasizes the importance of research into the mechanism of obesity.

The main cause of obesity is excessive intake of a high-energy diet and calorific intake is 120%–130% of the recommended level in regions where obesity has increased (FAO Statistical yearbook 2007–2008). The influence of a high-energy diet in parents on the characteristics of offspring has been examined in several studies [4,6–10]. For example, maternal obesity during pregnancy promotes obesity of the child [6], and a high-energy diet during pregnancy and lactation changes lipid and glucose metabolism in the child, increases the risk of obesity and leads to hypertension and insulin resistance [4,7–10]. These results suggest that increases in obesity are accelerated by a high-energy diet in previous generations, but this hypothesis has not been examined. That is, there are few studies of the effects of a high-energy diet over two generations on the features of the third generation. Therefore, in this study, changes in the lipid metabolic system were examined in normal mice to examine whether intake of a high-energy diet over two generations affects obesity in the third generation.

ICR mice fed a high-fat diet (HFD) were bred to the third generation. The diet used in the study had approximately 20% higher energy than a standard chow diet, based on the increase in calorific value in the diet in regions where obesity has increased (FAO Statistical Yearbook 2007–2008). The effect of the HFD on the third generation was evaluated. We examined growth parameters,

Abbreviations: Acly, ATP citrate lyase; ACO, acyl-CoA oxidase; Acox1, acyl-CoA oxidase 1; Acox2, acyl-CoA oxidase 2; CPT, carnitine palmitoyltransferase; Cpt1a, carnitine palmitoyltransferase 1 a; Cfi, complement factor I; Cdkn1a, cyclin-dependent kinase inhibitor 1A; Cyp7a1, cytochrome P450, family 7, subfamily A, polypeptide 1; DTNB, 5,5′-dithio-bio-(2-nitrobenzoic acid); Fas, fatty acid synthase; FAS, fatty acid synthase; FFA, free fatty acid; G6pdx, glucose-6-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gadd45γ, growth arrest and DNA-damage-inducible 45γ; Igfbp2, insulin-like growth factor binding protein 2; ME, malic enzyme; Me1, malic enzyme 1; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; PL, phospholipids; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; Srebf1, sterol regulatory element-binding protein 1; TC, total cholesterol; p53, transformation related protein 53; TG, triacylglycerol.

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doi:10.1016/j.jnutbio.2011.03.008

biochemical parameters in serum and liver and the histology of the liver and white adipose tissue. Changes were compared from generation to generation, and the mechanism of these changes was examined by measuring gene expression levels using DNA microarray analysis and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The results showed that lipid accumulation in the liver and white adipose tissue in third-generation female mice was promoted by activation of Cdkn1a. Moreover, obesity, fatty liver and hyperinsulinemia were caused by intake of an HFD beyond one generation.

2. Materials and methods

2.1. Animals and diets

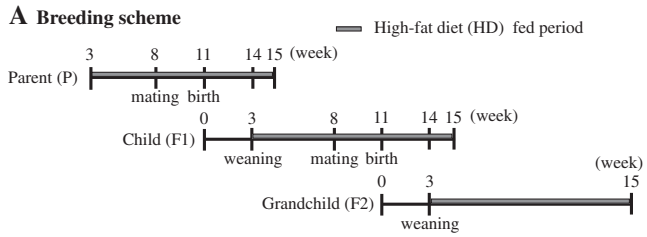
All procedures were performed in accordance with the Animal Experiment Guidelines of Tohoku University. The animal protocol was approved by the Animal Use Committee at Tohoku University [11]. Male and female ICR mice (3 weeks old) were obtained from CLEA Japan (Tokyo, Japan). After acclimatization to a commercial diet (CD) (CE-2; CLEA Japan, Tokyo, Japan) for 3 days, the mice were fed an HFD (Quick fat; CLEA Japan) and mating at 8 weeks old [P; male (n=10), female (n=10)] (Fig. 1). The children were weaned from breast at 3 weeks old and fed HFD [F1; male (n=10), female (n=10)]. By the same token, we breed to grandchild [F2; male (n=10), female (n=10)]. Male and female mice were housed in separate cage and free access to diet and distilled water in a temperature and humidity were maintained room with light cycles of 12 h on and 12 h off. The CD or HFD composition (g/kg diet) was nitrogen-free extract, 500 or 465; crude protein, 251 or 242; crude fat, 48 or 136; crude ash, 67 or 52; crude fiber, 42 or 30; moisture, 93 or 75. The CD or HFD calorie (kcal/100-g diet) was 341.1 or 405.5. Energy of HFD is about 20% higher than that of CD. At the 15 weeks old, the mice were weighed and then sacrificed by decapitation, and the liver, kidney, epididymal or periovular adipose tissue (white adipose tissue) and serum were collected and stored at −80°C until performance of assays.

2.2. Biochemical analyses in serum and liver

The lipid compositions in the liver and serum were measured as described previously [12,13]. Triacylglycerol (TG) and total cholesterol (TC) levels in serum and liver and phospholipid (PL), free fatty acid (FFA) and glucose levels in serum were measured using commercial enzyme kits (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's protocol. Insulin and adiponectin levels in serum were determined using ELISA kits (Shibayagi, Shibukawa, Japan). Phospholipid levels in liver were determined using the method described by Rouser *et al.* [14].

2.3. Enzymatic activity analysis

The activities of hepatic enzymes [fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), carnitine palmitoyltransferase (CPT), acyl-CoA oxidase (ACO)] were basically measured, as described previously [13,15]. Liver was homogenized with 0.25 mol/l sucrose containing 1 mmol/l EDTA and 10 mmol/l Tris–HCl buffer (pH7.4) and then centrifuged at 700×g for 10 min. The supernatant fractions (homogenate) were collected and the remains were centrifuged at 125,000×g for 60 min to obtain a mitochondrial fraction. The homogenates were used for FAS and G6PDH and ME, while the mitochondrial fractions were used for CPT and ACO. All enzymatic activities were spectrophotometrically measured. The FAS activity was measured by the reduction of NADPH, using acetyl CoA and Malonyl CoA as substrates. The G6PDH and the ME activity was measured by the increase of NADPH, using glucose-6-phosphate and malic acid as substrates, respectively. The CPT activity was determined by the reaction of DTNB (5,5′-dithio-bio-(2-nitrobenzoic acid)) and CoA released after adding palmitoyl CoA and L-carnitine. The ACO activity was estimated as the level of hydrogen peroxide produced during the co-oxidation of palmitoyl CoA when peroxidase was added with palmitoyl CoA.



B Difference of group

	grandparent	parent	postweaning
P	CD	CD	HD
F1	CD	HD	HD
F2	HD	HD	HD

Fig. 1. Breeding scheme. (A) Male and female ICR mice were fed an HFD from 4 weeks old and mated at 8 weeks old: first generation (P). Offspring were weaned at 3 weeks old and fed a high-energy diet: second generation (F1). These mice were then bred to produce the third generation (F2). (B) Differences among the groups. Mice in group P were fed a high-energy diet postweaning, whereas the parents and previous generation of these mice were fed chow diets. Mice in group F1 were fed a high-energy diet postweaning, similarly to their parents. Mice in group F2 were fed a high-energy diet postweaning, similarly to the P and F1 generations.

2.4. Histological analysis of the liver and the adipose tissue

The cell form of the liver and adipose tissue was observed. Each mouse liver and adipose tissue were fixed in 10% formalin and embedded in paraffin [16]. Vertical sections (5 μm) were cut, mounted on a glass slide, stained with hematoxylin and eosin, and observed using a microscope (BZ-9000; Keyence, Osaka, Japan). The size of adipocyte was calculated by counting the cell number of constant view that was chosen at random (ten times per one mouse).

2.5. mRNA expression analysis

For DNA microarray and qRT-PCR analysis, total RNA was isolated from liver and white adipose tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA) [17], eluted with 30 μl RNase-free water and stored at −80°C until use. Total RNA was pooled every one group and analyzed for DNA microarray analysis. DNA microarray analysis (GeneSQUARE, Multiplex Assay DNA Microarray Lifestyle Diseases Gene Expression for Mouse) using the total RNA was performed by Kurabo Industries (Osaka, Japan). The expression level of genes was quantified. The mRNA levels for ATP citrate lyase (*Acly*), acyl-CoA oxidase 1 (*Acox1*), acyl-CoA oxidase 2 (*Acox2*), cyclin-dependent kinase inhibitor 1A (*Cdkn1a*/*p21*), complement factor I (*Cfi*), carnitine palmitoyltransferase 1 a (*Cpt1a*), cytochrome P450, family 7, subfamily A, polypeptide 1 (*Cyp7a1*), fatty acid synthase (*Fas*), glucose-6-phosphate dehydrogenase (*G6pdx*), growth arrest and DNA-damage-inducible 45γ (*Gadd45γ*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), insulin-like growth factor binding protein 2 (*Igfbp2*), malic enzyme 1 (*Me1*), transformation-related protein 53 (*p53*), peroxisome proliferator-activated receptor α (*PPARα*), peroxisome proliferator-activated receptor γ (*PPARγ*) and sterol regulatory element-binding protein 1 (*Srebf1*) in liver were determined with a 7300 Real-Time PCR System (Applied Biosystems). This system allows real-time quantitative detection of PCR products by measuring the increase in fluorescence caused by binding of SYBR green to double-stranded DNA [11]. In brief, cDNA was made using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare, UK) from the total RNA in liver. The cDNA was subjected to PCR amplification using SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Otsu, Japan) and gene-specific primers for *Acly*, *Acox1*, *Acox2*, *Cdkn1a*, *Cfi*, *Cpt1a*, *Cyp7a1*, *Fas*, *G6pdx*, *Gadd45γ*, *Igfbp2*, *Me1*, *p53*, *PPARα*, *PPARγ*, *Srebf1*, or *GAPDH* (Table 1). The PCR conditions were 95°C for 10 s and then 95°C for 5 s and 60°C for 31 s over 40 cycles for each gene. Melting curve analysis was performed following each reaction to confirm the presence of only a single reaction product. The threshold cycle (C_T) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. The ratio between the GAPDH content in standard samples and test samples was defined as the normalization factor.

2.6. Statistical analysis

Results are expressed as means±S.E. Data were analyzed by a one-way ANOVA, this being followed by inspecting all differences by Tukey's Honest Significant Difference test. A difference was considered to be significant at P<.05.

3. Results

3.1. Growth parameters

The effect on growth parameters of intake of an HFD from generation to generation was examined in mice (Fig. 2). Body weight and genital white adipose tissue weight significantly increased in females of the F2 group compared with females of the P group (Fig. 2A, B). No significant differences in weight gain and genital white adipose tissue weight were found among the male groups, and no significant differences in food intake (male: P, 4.80 g/day; F1, 4.47 g/day; F2, 4.44 g/day; and female: P, 4.16 g/day; F1, 4.10 g/day; F2, 4.01 g/day), liver weight and kidney weight were found among the male

Table 1
Primer pairs used for the quantitative RT-PCR reaction

Gene name	Primer sequences (5′ to 3′)	
	Forward	Reverse
Acly	CAGCCAAGGCAATTTCAGAGC	CTCGACGTTTGATTAACTGGTCT
Acox1	TAACTTCCTCACTCGAAGCCA	CTGGGCGTAGGTGCCAATTA
Acox2	AACCCAGGGGATCGAGTGT	CGCAGCTCAGTGTTTGGGAT
Cdkn1a/p21	CGAGAACGGTGGAACTTTGAC	CAGGGCTCAGGTAGACCTTG
Cfi	CTTGGCTCTCCACTTGAGTTC	GGAGCGATGCGTGTATTTCTG
Cpt1a	CCTGCTCGCTCAGGATAAACA	GTGTCTTCAGAAACCGCACTG
Cyp7a1	GGGATTGCTGTGGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
Fas	CCTGGATAGCATTCCGAACCTG	TTCACAGCCTGGGGTCATCTTTGC
G6pdx	AGCCACATGAATGCCCTGC	CCACGATGATGCGGTTCCA
Gadd45γ	TTTCACGTTGATTCAGGCGTT	AAATGAGGATGCAATGCAGGT
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Igfbp2	CAGACGCTACGCTGCTATCC	CCCTCAGAGTGGTCGTCATCA
Me1	GAAAGAGGTGTTTGCCCATGA	AATTGCAGCAACTCCTATGAGG
p53	GCGTAAACGCTTCGAGATGTT	TTTTTATGGCGGGAAGTAGACTG
PPARα	AGAGCCCCATGTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
PPARγ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
Srebf1	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG

Acly, ATP citrate lyase; *Acox1*, acyl-CoA oxidase 1; *Acox2*, acyl-CoA oxidase 2; *Cdkn1a/p21*, cyclin-dependent kinase inhibitor 1A; *Cfi*, complement factor I; *Cpt1a*, carnitine palmitoyltransferase 1 a; *Cyp7a1*, cytochrome P450, family 7, subfamily A, polypeptide 1; *Fas*, fatty acid synthase; *G6pdx*, glucose-6-phosphate dehydrogenase; *Gadd45γ*, growth arrest and DNA-damage-inducible 45γ; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Igfbp2*, insulin-like growth factor binding protein 2; *Me1*, malic enzyme 1; *p53*, transformation related protein 53; *PPARα*, peroxisome proliferator-activated receptor α; *PPARγ*, peroxisome proliferator-activated receptor γ; *Srebf1*, sterol regulatory element-binding protein 1.

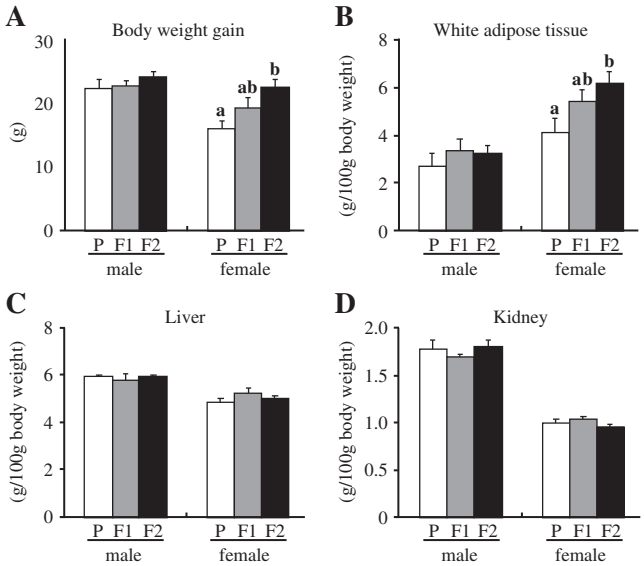


Fig. 2. Effects on growth parameters of intake of an HFD beyond one generation in mice. Changes in body weight (A), white adipose tissue weight (B), liver weight (C) and kidney weight (D) are shown. Values are means±S.E., $n = 10$. [a,b] Means in male or female groups with different superscripts are significantly different at $P<.05$.

and female groups (Fig. 2C, D). These results suggested that intake of an HFD beyond one generation promotes lipid accumulation in the white adipose tissue of female mice.

3.2. Lipid and glucidic metabolism parameters

Since the increase in white adipose tissue weight in female mice may have influenced lipid metabolism, we examined the effects of the HFD on lipid and glucidic metabolism parameters in serum and liver of female mice (Table 2). The serum TG levels in the F1 and F2 groups were 130% and 150% of that in the P group, respectively, with a significant increase in the F2 group; the serum TC levels were 113% and 127%, respectively, with a significant increase in the F2 group; the serum PL levels were 123% and 127%, respectively, with a significant increase in the F1 and F2 groups; and the serum FFA levels were 130% and 151%, respectively, with a significant increase in the F1 and F2 groups. These results indicate that intake of an HFD beyond one generation affected lipid metabolism and increased the serum lipid levels in female mice.

The serum glucose levels in the F1 and F2 groups were 131% and 134% of that in the P group, respectively, with a significant increase in both groups. The serum insulin level in the F1 and F2 groups were 156% and 490% of that in the P group, respectively, with a significant increase in F2 group. No significant differences in serum adiponectin levels were found among the three groups. These results indicate that intake of an HFD beyond one generation affected glucidic metabolism and increased the glucose and insulin levels in the serum of female mice.

The liver TG levels in the F1 and F2 groups were 134% and 158% of that in the P group, respectively, with a significant increase in the F2 group. The respective liver PL levels were 95% and 91%, with a significant decrease in the F2 group. No significant differences in liver TC levels were found among the three groups. These results indicate that intake of an HFD beyond one generation affected lipid metabolism and increased the TG level in the liver of female mice.

Next, we examined the effects of the HFD on the activity of lipid metabolic enzymes in the liver of female mice (Table 2). The activities of three enzymes associated with fatty acid synthesis, FAS, G6PDH, and ME, in the F1 and F2 groups were 120% and 134%, 121% and 161%, and 141% and 143% of those in the P group, respectively, with a significant increases in the activities of FAS and G6PDH in the F2 group. The activities of two enzymes associated with fatty acid β-oxidation, mitochondrial CPT and peroxisomal ACO, in the F1 and F2 groups were 106% and 114% and 107% and 114% of those in the P group, respectively. The CPT activity showed a significant increase in the F2 group. These results indicate that intake of an HFD beyond one generation increased the activities of enzymes associated with fatty acid metabolism in the liver of female mice and induced activation of lipid metabolism.

Table 2
Effects of the continuous HFD intake that exceeded the generation on lipid and glucidic parameters in serum and liver of female mice

	P	F1	F2
Serum			
TG (mg/ml)	127±12[a]	165±10[ab]	190±15[b]
TC (mg/ml)	110±11[a]	124±7[ab]	139±4[b]
PL (mg/ml)	209±19[a]	257±8[b]	264±6[b]
FFA (mEq/ml)	0.83±0.06[a]	1.08±0.04[b]	1.26±0.04[b]
Glu (mg/ml)	226±7[a]	296±19[b]	302±16[b]
Insulin (ng/ml)	2.0±0.7[a]	3.1±0.2[a]	9.8±2.0[b]
Adiponectin (μg/ml)	13.4±2.2	12.1±1.9	11.0±2.2
Liver			
TG (mg/g)	57.1±4.4[a]	76.6±6.1[ab]	90.1±5.7[b]
TC (mg/g)	7.7±0.6	6.9±0.3	6.8±0.6
PL (mg/g)	31.1±0.6[a]	29.7±0.9[ab]	28.5±0.1[b]
Enzymatic activity in the liver			
FAS (nmol/min/mg protein)	65.1±3.4[a]	78.3±5.2[ab]	87.4±6.7[b]
G6PDH (nmol/min/mg protein)	32.2±1.4[a]	38.8±3.8[ab]	51.8±5.7[b]
ME (nmol/min/mg protein)	253±12	356±52	361±42
CPT (nmol/min/mg protein)	1.96±0.06[a]	2.07±0.04[ab]	2.24±0.04[b]
ACO (nmol/min/mg protein)	1.57±0.09	1.68±0.09	1.80±0.10

Values are means±S.E., $n=10$.
[a,b] Means in a row with different superscripts are significantly different at $P<.05$.

3.3. Liver and white adipose tissue

Lipid accumulation in the liver and white adipose tissue of mice fed an HFD was confirmed. These tissues were stained with hematoxylin and eosin and observed by optical microscopy (Fig. 3). The results showed that lipid accumulation in the liver of the F2 group was promoted compared with that in the P group (Fig. 3A). The size of adipocytes in the white adipose tissue of the F2 group was larger than those in the P group (Fig. 3, B and C). Hence, hypertrophy of adipocytes was induced in the F2 group. These results indicated that lipid accumulation was promoted in hepatocytes and adipocytes of the F2 group compared with the P group.

3.4. Expression of mRNA for lipid and glucidic metabolism-related genes in the liver

The detailed mechanism of changes in lipid metabolism in mice fed a continuous HFD was examined. Lipid accumulation-related changes in mRNA expression of 334 genes related to lipid and glucidic metabolism were examined using DNA microarray analysis (Table 3). Genes with a change in mRNA expression of over twofold were identified as follows. Increased mRNA expression with intake of an HFD beyond one generation was found for Acly and Cdkn1a/p21 in the liver and decreased mRNA expression was found for Cfi, Cyp7a1 and Igfbp2. These results were confirmed. The mRNA levels of selected genes were measured by qRT-PCR (Table 3). The mRNA levels for Acly in the F1 and F2 groups were 118% and 183% of that in the P group, respectively. The respective mRNA levels for Cdkn1a/p21 were 271% and 615%, respectively, with a significant increase in the F1 and F2 groups. The mRNA levels for Cfi, Cyp7a1 and Igfbp2 in the F1 and F2 groups were 84% and 85%, 54% and 16%, and 45% and 42% of those in the P group, respectively. Cyp7a1 showed a significant decrease in the F2 group.

These results were probed in detail. Lipid accumulation-related changes in mRNA expression for Acly and Cdkn1a/p21-related genes were examined (Table 4). First, the mRNA levels for Acly-related genes associated with fatty acid synthesis and fatty acid catabolism system were measured. The mRNA levels for Fas, which is associated with fatty acid synthesis, in the F1 and F2 groups were 159% and 299% of that in the P group, respectively, with a significant increase in F2 group. The mRNA levels for G6pdx, which is associated with fatty acid synthesis, in the F1 and F2 groups were 181% and 187% of that in the P group, respectively, with a significant increase in F1 and F2 groups. No significant differences in mRNA levels for Srebf1 and Me1 (fatty acid synthesis) and PPARα, Cpt1a, Acox1 and Acox2 (fatty acid catabolism) were found among the three groups. Next, the mRNA levels of Cdkn1a/p21-related genes involved in cell-cycle arrest were measured. The mRNA level for Gadd45γ in the F2 group was 230% of that in the P group. No significant differences in mRNA levels for p53, which regulates Cdkn1a/p21 and Gadd45γ, were found among the three groups. These results suggested that lipid accumulation in the liver of mice fed an HFD beyond one generation was induced by function of Fas, G6pdx and Cdkn1a.

3.5. Expression of mRNA for lipid metabolism-related genes in the white adipose tissue

The detailed mechanism of the changes in white adipocytes in mice fed an HFD was examined. The mRNA levels of genes associated with lipid accumulation in white adipose tissue were examined using

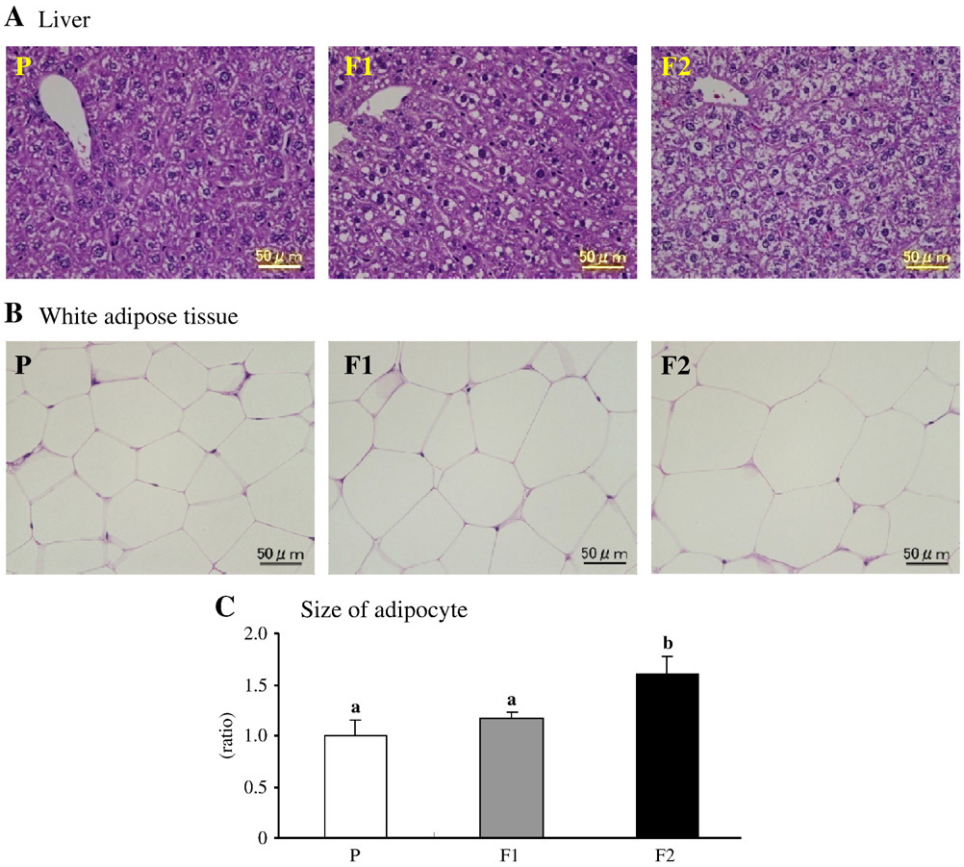


Fig. 3. Effects on liver and adipose tissue of intake of an HFD beyond one generation in mice. Changes in hematoxylin–eosin staining of liver (A) and white adipose tissue (B) and size of adipocytes (C) are shown. Values are means±S.E., $n = 10$. [a,b] Means in a row with different superscripts are significantly different at $P<.05$.

Table 3
Liver mRNA expression of lipid and glucidic metabolism-related genes that were gradually increased or decreased by the continuous HFD intake that exceeded the generation measured using DNA microarray and qRT-PCR assay

DNA microarray	F1 *vs.* P	F2 *vs.* P		Gene function
Acly	1.11	2.17		Fatty acid biosynthesis
Cdkn1a/p21	1.58	3.04		Cell-cycle arrest
Cfi	0.89	0.45		Immune/defense response
Cyp7a1	0.44	0.12		Cholesterol catabolism
Igfbp2	0.68	0.46		Insulin signal
qRT-PCR	P	F1	F2	Gene function
Acly	1.00±0.15	1.18±0.19	1.83±0.36	Fatty acid biosynthesis
Cdkn1a/p21	1.00±0.11[a]	2.71±0.26[b]	6.15±0.73[c]	Cell-cycle arrest
Cfi	1.00±0.04	0.84±0.16	0.85±0.24	Immune/defense response
Cyp7a1	1.00±0.27[a]	0.54±0.17[ab]	0.16±0.02[b]	Cholesterol catabolism
Igfbp2	1.00±0.28	0.45±0.15	0.42±0.14	Insulin signal

Values are means±S.E., $n=10$.
[a–c] Means in a row with different superscripts are significantly different at $P<.05$.

qRT-PCR (Table 4). The mRNA levels for Cdkn1a/p21 in the F1 and F2 groups were 157% and 310% of that in the P group, respectively, with a significant increase in the F2 group. This result was similar to that found in liver. The mRNA levels for p53 in the F1 and F2 groups were 162% and 195% of that in the P group, respectively, with a significant increase in the F2 group. The mRNA levels for Gadd45γ in the F1 and F2 groups were 180% and 253% of that in the P group, respectively, with a significant increase in the F2 group. No significant differences in mRNA levels for PPARγ (cellular differentiation and proliferation); and Fas, G6pdx and Me1 (fatty acid synthesis) were found among the three groups. These results suggest that adipocyte hypertrophy and lipid accumulation in white adipose tissue in mice fed an HFD beyond one generation is induced by function of Cdkn1a, p53 and Gadd45γ.

4. Discussion

Several reports have shown changes in lipid metabolism in children of mothers with a high-energy diet [4,7–9], but it is unclear if these changes also occur in the third generation. In this study, we examined the influence of intake of an HFD beyond one generation on lipid metabolism in normal mice (Fig. 1). Lipid accumulation in the liver and white adipose tissue of third-generation female mice was promoted by function of Cdkn1a. This is the first evidence to show that intake of an HFD beyond one generation influences lipid metabolism in the third generation. The changes in lipid metabolism in the third-generation female mice were also markedly different to those in the second-generation mice, which is a particularly interesting result.

Table 4
Liver and adipose tissue mRNA expression of lipid metabolism-related genes that were gradually increased or decreased by the continuous HFD intake that exceeded the generation measured using qRT-PCR assay

	P	F1	F2	Gene function
Liver				
Srebf1	1.00±0.18	1.26±0.08	1.44±0.31	Fatty acid biosynthesis
Fas	1.00±0.14[a]	1.59±0.14[ab]	2.99±0.27[b]	
G6pdx	1.00±0.14[a]	1.81±0.09[b]	1.87±0.21[b]	
Me1	1.00±0.31	1.59±0.08	1.98±0.30	
PPARα	1.00±0.10	1.20±0.17	1.38±0.18	Fatty acid β-oxidation
Cpt1a	1.00±0.19	1.23±0.12	1.54±0.30	
Acox1	1.00±0.17	1.22±0.18	1.68±0.36	
Acox2	1.00±0.19	1.07±0.12	1.41±0.23	
p53	1.00±0.20	1.18±0.13	1.28±0.11	Cell-cycle arrest
Gadd45γ	1.00±0.28	1.81±0.16	2.30±0.55	
Adipose tissue				
Cdkn1a/p21	1.00±0.17[a]	1.57±0.22[ab]	3.10±0.21[b]	Cell-cycle regulation
p53	1.00±0.16[a]	1.62±0.18[ab]	1.95±0.24[b]	
Gadd45γ	1.00±0.12[a]	1.80±0.16[ab]	2.53±0.13[b]	
PPARγ	1.00±0.21	0.82±0.04	0.72±0.15	Lipid metabolism
Fas	1.00±0.32	1.09±0.11	1.51±0.08	Fatty acid biosynthesis
G6pdx	1.00±0.16	1.26±0.31	1.33±0.28	
Me1	1.00±0.26	1.11±0.01	1.44±0.22	

Values are means±S.E., $n=10$.
[a,b] Means in a row with different superscripts are significantly different at $P<.05$.

Body weight gain and white adipose tissue weight increased in second- and third-generation female mice (F1 and F2 groups, respectively) compared with the first generation (P group) with intake of an HFD in all generations (Fig. 2). The body weight gain in the F1 and F2 groups may have originated from an increase of white adipose tissue because liver and kidney weights were unchanged. This suggests that the female mice were susceptible to obesity with intake of an HFD. The changes in female mice were larger than those in male mice, so we analyzed the changes in females in detail. The serum TG, TC and PL levels and liver TG accumulation all increased in the F2 group (Table 2 and Fig. 3). Secretion of VLDL, which contains abundant TG, increases when lipids accumulate in the liver [18]. Therefore, our results suggest that the serum lipid levels were increased by lipid accumulation in the liver and that intake of an HFD beyond one generation promoted lipid accumulation in liver and caused fatty liver [nonalcoholic fatty liver disease (NAFLD)] (Figs. 3 and 4).

The synthesis and catabolism systems for lipid metabolic enzymes in the liver were both activated in the F2 group (Table 2), and the serum insulin level increased in this group. Since insulin promotes the fatty acid synthesis system [19], it is likely that insulin activated fatty acid synthesis in the liver and promoted lipid accumulation. An increase in serum FFA and lipid accumulation activate fatty acid β-oxidation in the liver [20] and such changes may have induced activation of the fatty acid β-oxidation system in the current study, since serum FFA, glucose and insulin were all increased by intake of an HFD beyond one generation (Table 2). Since an increase in serum FFA inhibits insulin-induced glucose metabolism [21], our results suggest that insulin function was suppressed by the increase in serum FFA, leading to increases in serum glucose and insulin, and development of insulin resistance. In addition, both white adipocyte hyperplasia and hypertrophy were observed in the F2 group (Figs. 2 and 3), and these changes can also increase the serum FFA level [22].

The mechanisms underlying these changes in female mice were revealed. The expression levels of lipid metabolism-related genes were measured by DNA microarray and qRT-PCR analysis. A significant increase in the expression level of Cdkn1a/p21 was found in both liver and white adipose tissue (Table 3 and 4). This gene arrests the cell cycle at G1 and inhibits DNA synthesis [23,24]. Cdkn1a/p21 is also highly up-regulated in adipocyte hypertrophy and fatty liver [25,26] and is crucial for maintaining adipocyte hypertrophy since obesity is improved by knockdown of this gene [27]. Therefore, our results suggest that lipid accumulation in the liver and white adipose tissue was promoted by Cdkn1a/p21. The expression level of p53, which regulates Cdkn1a/p21 [23], in the white adipose tissue was increased by the HFD. The expression levels of Cdkn1a/p21 and Gadd45γ, which are also regulated by p53, were increased (Table 4). p53 is activated by modifications such as phosphorylation and acetylation induced by various stimuli [28,29]. Therefore, our findings suggest that p53 was activated by a response to a stimulus produced by intake of an HFD in female mice and that Cdkn1a/p21 activity was induced by activated p53. Lipid accumulation in the liver and white adipose tissue was then promoted by Cdkn1a/p21, with resultant development of obesity, NAFLD and insulin resistance. We first thought that lipid accumulation in the liver and adipose tissue of female mice were caused chiefly by insulin function, since insulin activates fatty acid synthesis at the transcriptional level and promotes lipid accumulation in liver and white adipose tissue [19]. However, fatty acid synthesis at the transcriptional level was not greatly activated in white adipose tissue (Table 4). Additionally, insulin represses fatty acid β-oxidation at the

transcriptional level in the liver [30], but we did not find repression of the fatty acid β-oxidation system (Table 4). These results suggest that insulin was unable to exert an effect because of insulin resistance in the liver and white adipose tissue. Therefore, the main mechanism underlying lipid accumulation in liver and white adipose tissue is likely to be the effect of Cdkn1a/p21.

Cyp7a1 is a rate-limiting enzyme in cholesterol catabolism [31]. The expression level of Cyp7a1 was decreased greatly by intake of an HFD beyond one generation (Table 3), but there was no significant change in the hepatic cholesterol level (Table 2). These results suggest that homeostasis of cholesterol was strongly maintained. The decrease in liver PL by the high-energy diet (Table 2) may be due to a decrease in the ratio of the weight of the cell membrane to the total hepatocyte weight, since these cells were enlarged by lipid accumulation (Fig. 3).

There were no significant differences in body weight gain and white adipose tissue weight in male mice (Fig. 2). It has been reported that female mice are more vulnerable to the maternal diet [7,10], and our results suggest that female mice were more sensitive to the HFD in this study. Obesity and increased body weight and white adipose tissue weight of children are promoted by a maternal HFD during pregnancy and lactation [4,7–9]. In this study, we made the new discovery that intake of an HFD beyond one generation strongly affects the subsequent generation. These results are interesting, but we are unable to clarify the mechanism completely. In particular, the reasons for the increased expression of Cdkn1a/p21 are uncertain. In addition, the relation between p53 and Cdkn1a/p21 is not clear. Epigenetic effects are known to be involved in nutritional determination of body weight and body composition [32–34], and therefore, DNA methylation or histone acetylation may be involved. Further work will be necessary to examine the details of the mechanism.

Acknowledgment

This study was supported by KAKENHI (20780099) of JSPS, Japan. There are no conflicts of interest.

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