III. 研究成果の刊行に関する一覧表

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雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hashimoto Y, Yamada K, Tsushima H, Miyazawa D, Mori M, Nishio K, Ohkubo T, Hibino H, Ohara N, Okuyama H	Three dissimilar high fat diets differentially regulate lipid and glucose metabolism in obesity-resistant Slc:Wistar/ST rats	Lipids	48	803-815	2013

IV. 研究成果の刊行物・別刷

Lipids (2013) 48:803–815
DOI 10.1007/s11745-013-3805-3

ORIGINAL ARTICLE

Three Dissimilar High Fat Diets Differentially Regulate Lipid and Glucose Metabolism in Obesity-Resistant Slc:Wistar/ST Rats

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Received: 25 December 2012 / Accepted: 15 May 2013 / Published online: 27 June 2013

Abstract Epidemiologic and ecologic studies suggest that dietary fat plays an important role in the development of obesity. Certain Wistar rat strains do not become obese when fed high-fat diets unlike others. In a preliminary study, we confirmed that Slc:Wistar/ST rats did not become obese when fed high-fat diets. The mechanisms governing the response of hepatic lipid-metabolizing enzymes to large quantities of dietary lipids consumed by obesity-resistant animals are unknown. The aim of the present study is to examine how obesity-resistant animals metabolize various types of high-fat diets and why they do not become obese. For this purpose, male Slc:Wistar/ST rats were fed a control low-fat diet (LS) or a high-fat diet containing fish oil (HF), soybean oil (HS), or lard (HL) for 4 weeks. We observed their phenotypes and determined lipid profiles in plasma and liver as well as mRNA expression levels in liver of genes related to lipid and glucose metabolism using DNA microarray and quantitative reverse transcriptase polymerase chain analyses. The body weights of all dietary groups were similar due to isocaloric intakes, whereas the weight of white adipose tissues in the LS group was significantly lower. The HF diet lowered plasma lipid levels by accelerated lipolysis in the peroxisomes and suppressed levels of very-low-density lipoprotein (VLDL) secretion. The HS diet promoted hepatic lipid accumulation by suppressed lipolysis in the peroxisomes and normal levels of VLDL secretion. The lipid profiles of rats fed the LS or HL diet were similar. The HL diet accelerated lipid and glucose metabolism.

Keywords High-fat diet · Soybean oil · Fish oil · Lard · Plasma lipids · Hepatic lipids · Gene expression

Abbreviations

DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
LNA	Linoleic acid (18:2n-6)
MUFA	Monounsaturated fatty acid(s)
NEFA	Non-esterified fatty acid
PUFA	Polyunsaturated fatty acid(s)
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFA	Saturated fatty acid(s)
TAG	Triacylglycerol(s)

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Introduction

The incidence of obesity, metabolic syndrome, and diabetes mellitus is increasing globally to epidemic levels [1]. To address this issue, a more detailed understanding of these disease processes is urgently required [1]. Excessive intake of lipids is considered an etiological factor for the development of obesity [2]. In contrast, dietary fats and oils are important sources of energy, essential fatty acids, and fat-soluble vitamins. Moreover, they influence the profiles of lipids in plasma and liver [3–7]. Increased plasma cholesterol levels have been proposed to accelerate the development of atherosclerosis and increase the risk of coronary heart disease (CHD) [8], although other evidence indicates the opposite [9]. Dietary vegetable oils enriched in n-6 polyunsaturated fatty acid (n-6 PUFA) were proposed to induce lower serum cholesterol levels [10, 11]. However, this effect is transient, and excessive intake of n-6 PUFA increased the incidence of CHD [9]. In contrast, a diet containing fish oils rich in n-3 PUFA, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), delays the development of atherosclerosis [12] and reduces the incidence of CHD [3, 13–15]. The atherosclerosis-related diseases involved in inflammation and are caused by overexpression of cyclooxygenase (COX, EC 1.14.99.1) [16]. Different types of dietary fatty acids have been implicated in atherosclerosis and CHD, possibly through different mechanisms. To prevent and overcome these diseases, it is important to understand the different effects of various types of dietary high fats on lipid and glucose metabolism.

High-fat diets induce obesity and metabolic disorders in rodents that resemble the human metabolic syndrome [17]. Wistar rats are susceptible to obesity when fed high-fat diets [17]. Buettner et al. [7] reported that high-fat diets containing 42 energy % (en%) lard, olive oil, or coconut fat induced obesity in Wistar rats, although it was emphasized in a review [17] that the phenotype induced by high-fat diets varied distinctly among different studies. In fact, Piche et al. [18] did not observe a significant difference in the food intake and body weight of Wistar rats fed a diet supplemented with 10 % (approximately 22 en%) lard, corn oil, soybean oil, canola oil, or cod liver oil. Rand et al. [19] also fed Wistar rats with diets supplemented with 50 en% of sunflower oil or palm oil and found no significant difference in the body weights compared with feeding a low-fat laboratory chow. Substrains of Wistar rats kept in different laboratories may exhibit different obesity-related phenotypes. In a preliminary experiment, we confirmed that Slc:Wistar/ST rats did not become obese under our conditions when fed high-fat diets. Therefore, this strain was used in the present study as a model resistant to obesity.

Genetically obese animals have been the focus of research on obesity and metabolic syndrome [17], and animals that do not develop obesity have served mainly as controls. Little is known about how obesity-resistant animals metabolize various types of high-fat diets. A detailed comparison of the differential effects of various high-fat diets on lipid parameters influenced by lipid and glucose metabolism has yet to be investigated in obesity-resistant animals. Therefore, we examined the effects of various high-fat diets on plasma and hepatic lipid parameters and lipid metabolism using Slc:Wistar/ST rats that do not develop obesity. For this purpose, we maintained male rats on 3 different high-fat diets (HF, fish oil; HS, soybean oil; HL, lard) for 4 weeks and determined their body and tissue weights, total food consumption, oil composition, and energy metabolism, and lipid profiles of plasma and liver. The data were compared with those of rats fed a low-fat diet (LS, soybean oil supplemented), and those of obesity-susceptible animals.

To understand the molecular basis of the effects of these high-fat diets (HF, HS and HL), we took advantage of microarray technology, which is a useful tool for high-throughput quantitative analysis of gene expression [20]. This method has evolved rapidly during the past decade with dramatic improvements in probe selection, sensitivity, image acquisition, and data analysis. The liver plays central roles in lipid, glucose, and cholesterol metabolism. The close connection between lipid and glucose metabolism maintains energy homeostasis [21]. Therefore, the expression levels of 326 genes associated with lipid and glucose metabolism were analyzed using DNA microarrays. Moreover, the mRNA expression levels of 19 genes involved in inflammatory responses as well as lipid metabolism were examined using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. To complement gene transcription analysis, we determined the expression levels and identities of hepatic proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS). The mechanisms by which various fatty acids differentially alter the lipid profiles in the liver and plasma of male Wistar rats were revealed based on the data obtained from these techniques.

Slc:Wistar/ST rats did not become obese when fed high-fat diets, because their caloric intakes were similar and independent of the type and amount of dietary fatty acids. However, the weights of visceral white adipose tissues (WAT) were significantly greater in the high-fat dietary groups than in the low-fat dietary group. The HF diet lowered plasma lipid levels by stimulated peroxisomal β-oxidation and suppressed very-low-density lipoprotein (VLDL) secretion. The HS diet induced hepatic lipid accumulation via suppression of peroxisomal β-oxidation

and normal levels of VLDL secretion. The hepatic and plasma lipid profiles of rats fed the HL and LS diets were similar. The HL diet did not induce hepatic lipid accumulation. Lipid and glucose metabolism were accelerated in rats fed the HL diet. This study, to our knowledge, is the first to show the differential effects of 3 high-fat diets on lipid and glucose metabolism in Slc:Wistar/ST rats.

Materials and Methods

Animals and Diets

Male Slc:Wistar/ST rats (specific-pathogen-free) at 4 weeks-of-age were purchased from Japan SLC, Inc., Hamamatsu, Japan and were housed two per cage with a 12-h light–dark cycle at 23 °C under controlled environmental conditions. The experiments were carried out according to the Guidelines for Animal Care and Use, and the protocols for the experiments were approved by the Institutional Animal Care and Use Committee of Kinjo Gakuin University College of Pharmacy. We prepared high-fat (20 % w/w) diets by mixing fish oil, soybean oil, or lard with normal (conventional) low-fat powdered diet (F-1 fish meal-free containing 4.4 % w/w lipid by weight of which 1.7 % w/w was soybean oil) (Funabashi Farm Co., Ltd.; Chiba, Japan). The final oil content of high-fat diets was 23.5 % (w/w). Total energy content and fat energy were 468.0 kcal/100 g and 45.2 en% and 360.0 kcal/100 g and 11.0 en%, respectively, for the high-fat diets and normal low-fat diet (LS). After feeding the LS diet for 1 week, rats were divided randomly into 4 groups, 6 animals/group, and maintained for 4 weeks with free access to the LS, HF, HS, HL diets and water. The powdered diet was provided in a container with a dome-shaped cover (Roden Café: Oriental Yeast Co., Ltd.; Tokyo, Japan) to prevent the diet from dropping out of the container and to prevent contamination with wood chips entering into the container. The remaining diet was weighed, discarded, and replenished 3 times a week. Body weight was measured weekly.

Determination of Fatty Acid Compositions of Diets

The lipids in the experimental diets were extracted with chloroform/methanol according to the method of Bligh and Dyer [22], and the fatty acid compositions were determined by gas chromatography as described elsewhere [23].

Determination of Lipid and Protein Profiles in Plasma and Liver

Rats were fasted overnight and anesthetized with sevoflurane, and then euthanized by cardiac puncture. Blood was collected in a tube containing EDTA. The plasma was separated by centrifugation at 3,000 rpm for 15 min at 4 °C, and stored at −80 °C. The livers and hearts were weighed, frozen in dry ice-acetone (−78 °C), and then stored at −80 °C. The epididymal white adipose tissue (WAT), perirenal WAT, and brown adipose tissue (BAT) were isolated and weighed. The total visceral WAT is defined as the weight of epididymal fat tissues and perirenal fat tissues. Liver samples for mRNA analysis were transferred into the RNAlater® solution (Ambion Cat # AM7020) to prevent RNA degradation during storage. Glucose, total cholesterol (t-Cho), triacylglycerol (TAG), HDL-cholesterol (HDL-C), non-esterified fatty acid (NEFA), and total protein (TP) present in plasma were measured using a clinical auto-analyzer (TMS-1024: Tokyo Boeki Medical System Ltd.; Tokyo, Japan). Approximately 100 mg of the liver was homogenized in phosphate buffered saline (PBS), and the homogenate was then divided into two portions to measure protein and lipid concentrations. The lipids were extracted from the homogenate 3 times with chloroform/methanol according to the method of Bligh and Dyer [22] as mentioned above and were extracted from the organic phase dried under N_2 gas, and solubilized with isopropanol containing 10 % Triton X-100. The levels of hepatic t-Cho, NEFA, phospholipid (PL), and TAG were measured using Wako assay kits as follows: L-type Wako CHO M, NEFA C-test Wako, L-type Wako phospholipid, and L-type Wako TG M. All assay kits and a Multi-Calibrator Lipid standard were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. The protein concentration of the liver was determined by the method of Hartree [24] using bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO) as a standard.

DNA Microarray Analysis

RNA was extracted from liver samples using an RNeasy Protect Mini Kit (Qiagen, Valencia, CA) combined with DNase I according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Two micrograms of RNA from 6 samples of each group were pooled and the expression levels of 326 genes (http://www.kurabo.co.jp) associated with lipid and glucose metabolism were analyzed using a GeneSQUARE (Kurabo Industrial Ltd.; Osaka, Japan) multi-sample DNA microarray system. Sample processing and data acquisition were carried out by Kurabo Industrial Ltd. Gene expression levels of the HF, HS, or HL groups were normalized to those of the LS group and the values of experimental groups were expressed as fold changes, which are ratios to those of the LS group. Significant differences were judged from fold changes of ≥ 2.0 or ≤ 0.5.

Determination of mRNA Levels Using qRT-PCR

RNA was extracted from the liver as described above and was used as template to synthesize cDNA in 20 μl of RT-reaction mixtures using a High Capacity RNA-to-cDNA kit (Life Technologies, Carlsbad, CA). To quantify the expression level of genes, the mRNA levels of various genes (Table 1) in the liver were determined using a 7900HT Fast Real-time PCR system (Life Technologies). Primers (TaqMan® gene expression assays) for genes associated with lipid metabolism are listed in Table 1, which were obtained from Life Technologies. There was no cross-reactivity with liver genomic DNA except the genes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) and acetyl-CoA acetyltransferase 2 (ACAT2, EC 2.3.1.9) probes. Therefore, DNase I was used in the RNA extraction step as described above. Ten microliters of a two-fold concentrated TaqMan Gene Expression Master Mix (Life Technologies), 1 μl (0.4 μM) of each primer, and the cDNA template were mixed in a final volume of 20 μl. The Hot-Start AmpliTaq Gold DNA polymerase was activated at 50 °C for 2 min and then at 95 °C for 10 min followed by a two-step PCR for 40 cycles (15 s at 95 °C, 1 min at 60 °C). Amplicon sequence specific methods are based on the use of oligonucleotide probes labeled with a donor fluorophore and an acceptor dye (quencher). Data acquisition and analysis were performed using the TaqMan data worksheet and software according to the manufacturer's instructions (Life Technologies), and the ΔΔCt method [25, 26], respectively. Briefly, the cycle threshold (Ct) values for each reaction were automatically calculated by the ABI Prism sequence detection software (version 2.3) by determining the PCR cycle number. Gene expression levels were normalized to those of the reference gene *Gapdh* and those of the experimental groups (HF, HS, or HL group) were normalized to those of the dietary LS group (defined as 1.00).

SDS-PAGE

The liver was homogenized in PBS, and the protein concentration was determined as described above. Samples containing 15 μg protein were analyzed using SDS-PAGE with a 5–20 % (linear gradient) polyacrylamide slab gel (Anateck K.K. Tokyo, Japan) according to the method of Laemmli [27]. The separated proteins were visualized by staining the gels with 0.1 % Coomassie Blue. The Bio-Rad Precision Plus Protein™ standard was used to determine relative mobility.

Table 1 TaqMan® gene expression assays

Target gene	ABI ID no.	GenBank ID	Gene name	Protein function
Apob	Rn01499054_m1	NM_019287.2	Apolipoprotein B	VLDL secretion
Mttp	Rn01522970_m1	NM_001107727.1	Microsomal triglyceride transfer protein	VLDL assembly
Acat1	Rn00567139_m1	NM_017075.1	Acetyl-Coenzyme A acetyltransferase 1	
Acat2	Rn01526241_g1	NM_001006995.1	Acetyl-Coenzyme A acetyltransferase 2	
Acox1	Rn00569216_m1	NM_017340.2	Acyl-Coenzyme A oxidase 1	Fatty acid β-oxidation
Cpt1a	Rn00580702_m1	NM_031559.2	Carnitine palmitoyltransferase 1a	Fatty acid β-oxidation
Srebf1	Rn01495769_m1	XM_213329.4	Sterol regulatory element binding transcription factor 1	Fatty acid biosynthesis
Fasn	Rn01463550_m1	NM_017332.1	Fatty acid synthase	Lipogenesis marker
Ppara	Rn00566193_m1	NM_013196.1	Peroxisome proliferator activated receptor alpha	Lipid metabolism
Pparg	Rn00440945_m1	NM_013124.2	Peroxisome proliferator activated receptor gamma	Lipid metabolism
Ucp2	Rn01754856_m1	NM_019354.2	Uncoupling protein 2	Proton carrier
Ldlr	Rn00598438_m1	NM_175762.2	Low-density lipoprotein receptor	Lipoprotein uptake
Hmgcr	Rn00565598_m1	NM_013134.2	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Cholesterol biosynthesis
Cat	Rn00560930_m1	NM_012520.1	Catalase	
Tlr4	Rn00569848_m1	NM_019178.1	Toll-like receptor 4	LPS receptor
Nfkb1	Rn01399583_m1	XM_342346.4	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	
Ptgs1 (COX-1)	Rn00566881_m1	NM_017043.3	Prostaglandin-endoperoxide synthase 1	Prostaglandin synthesis
Ptgs2 (COX-2)	Rn01483828_m1	NM_017232.3	Prostaglandin-endoperoxide synthase 2	Prostaglandin synthesis
Gapdh	Rn99999916_s1	NM_017008.3	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping

Protein Identification Using MALDI-TOF/MS

The protein bands were cut out of the gel, extracted using trypsin digestion, and analyzed using MALDI-TOF/MS at AB SCIEX (Framingham, MA) by the method of Bonk and Humeny [28].

Statistical Analysis

Data are presented as means ± standard deviations (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using the SPSS statistical software package (SPSS 11.0J). A p value <0.05 was considered statistically significant.

Results

Fatty Acid Compositions of Diets

The fatty acid compositions of the diets are shown in Table 2. Their salient features are as follows: HF diet, 30 % of n-3 PUFA; the HS diet, 51 % of n-6 PUFA; and the HL diet, 36 % of saturated fatty acids (SFA) and 47 % of monounsaturated fatty acids (MUFA). In the LS and HS diets, the percentages of total SFA plus MUFA, n-6 PUFA, and n-3 PUFA were similar.

Total Food and Energy Intake

As shown in Table 3A, the total food intake of the LS group was significantly higher than that of the other 3 dietary groups ($p < 0.01$). However, total energy intake was not significantly different among the 4 dietary groups. The total oil intake was the lowest in the LS group.

Body and Tissue Weights

Body and tissue wet weights are shown in Table 3B. Neither body nor heart weights were significantly different between groups. In contrast, the liver weights of the HF group were significantly higher compared with those of the HS, HL, or LS groups. Moreover, the order of the weight of WAT including epididymal fat and perirenal fat, was HS > HL > HF ≫ LS groups, and the WAT weight of the LS group was significantly lower than that of the HF ($p < 0.05$), HS ($p < 0.01$), or HL ($p < 0.01$) group. The BAT weight of the HF group was the highest among all groups.

Lipid and Protein Levels in Plasma and Liver

As shown in Table 4A, the plasma levels of t-Cho and HDL-C in the HF group were significantly lower than those in the other 3 groups. The t-Cho and NEFA levels in the livers of the HS group were the highest ($p < 0.01$) (Table 4B). Moreover, the hepatic TAG level in the HS group was the highest among all groups and significantly higher compared with that of the HF group ($p < 0.01$). This result has important implications for understanding the regulation of hepatic lipolysis and lipogenesis by dietary fatty acids. Plasma and hepatic lipid profiles of the LS and HL groups were similar (Table 4), although there were major differences in the oil contents and fatty acid compositions between the LS and HL diets (Table 2). These results suggest that different sources of high-fat diets influenced lipid metabolism differently and modulated plasma and hepatic lipid profiles. Moreover, the plasma levels of glucose were not significantly different among 4 groups (Table 4A).

Hepatic Gene Expression Profiling by DNA Microarray Analysis

To investigate whether gene expression in the liver was influenced by dietary fatty acids, gene expression levels were analyzed by DNA microarray analysis on 326 genes associated with lipid and glucose metabolism. Gene expression levels of the experimental groups (HF, HS, or HL group) were normalized to those of the dietary LS group. Significant differences were judged from fold changes of ≥2.0 or ≤0.5. The results of eight genes related to glucose and lipid metabolism that satisfied these criteria are shown in Table 5. There were prominent differences in HF group compared to other high-fat dietary groups. Down-regulated mRNA level of pyruvate kinase (PKLR, EC 2.7.1.40), which is an irreversible enzyme for glycolysis, and up-regulated mRNA level of pyruvate dehydrogenase kinase isozyme 4 (PDK4, EC 2.7.11.2), which inhibits production of acetyl-CoA from pyruvic acid, suggesting glucose catabolism in the HF group was suppressed. Moreover, the expression level of solute carrier family 2 (Glut 2, *Slc2a2*) mRNA was down-regulated. In the HS and HL groups, the expression level of cytochrome P450 7a1 (CYP7A1, EC 1.14.13.17), which promotes catabolism of sterols, was strongly up-regulated. Moreover, genes encoding acyl-CoA oxidase 1 (ACOX1, EC 1.3.3.6) and enoyl-CoA, hydratase/3-hydroxyacyl-CoA dehydrogenase (EHHADH, EC 4.2.1.17/EC 1.1.1.35) in the HF group were expressed at 3.20- and 6.25-fold higher levels, respectively, than those in the HS and HL groups (Table 5B). *Acox1* and *Ehhadh* encode enzymes that catalyze peroxisomal β-oxidation. Up-regulation of glucokinase mRNA (GCK, EC 2.7.1.2), which functions in glycogen synthesis, and down-regulation of *PDK4* mRNA were detected in the HS and HL groups (Table 5A).

Table 2 Fatty acid composition of experimental diets

Fatty acids	Diet			
	LS	HF	HS	HL
	% (wt/wt) of total fatty acids			
14:0	0.35 ± 0.02	2.26 ± 0.18	0.14 ± 0.03	1.09 ± 0.07
14:1	Nd	0.07 ± 0.01	0.01 ± 0.01	0.06 ± 0.01
15:0	0.08 ± 0.00	0.62 ± 0.02	0.03 ± 0.00	0.07 ± 0.00
15:1	0.10 ± 0.07	0.04 ± 0.02	Nd	Nd
16:0 DMA	Nd	Nd	Nd	0.01 ± 0.05
16:0	15.32 ± 0.32	19.56 ± 0.43	11.30 ± 0.07	23.56 ± 0.12
16:1	0.48 ± 0.01	3.46 ± 0.08	0.10 ± 0.00	1.77 ± 0.05
17:1	0.14 ± 0.00	0.70 ± 0.01	0.05 ± 0.00	0.27 ± 0.00
18:0 DMA	Nd	0.03 ± 0.01	0.66 ± 0.47	Nd
18:0	5.81 ± 0.03	5.36 ± 0.18	3.41 ± 0.44	10.32 ± 1.58
18:1 DMA	Nd	Nd	Nd	2.20 ± 1.55
18:1 n-9	18.20 ± 0.12	19.20 ± 0.49	26.08 ± 0.01	40.24 ± 0.05
18:1 n-7	1.10 ± 0.01	2.22 ± 0.06	1.11 ± 0.10	1.98 ± 0.05
18:2 n-6	50.35 ± 0.20	10.13 ± 0.21	50.28 ± 0.00	15.51 ± 0.15
18:3 n-6	0.14 ± 0.00	0.17 ± 0.00	0.29 ± 0.02	0.03 ± 0.01
18:3 n-3	4.09 ± 0.02	4.09 ± 2.02	5.20 ± 0.00	0.97 ± 0.02
20:0	0.27 ± 0.00	0.48 ± 0.07	0.32 ± 0.06	0.20 ± 0.00
20:1	0.51 ± 0.00	1.63 ± 0.07	0.33 ± 0.01	0.68 ± 0.01
20:2 n-6	Nd	0.23 ± 0.01	0.02 ± 0.01	0.31 ± 0.00
20:3 n-6	0.05 ± 0.04	0.10 ± 0.00	0.02 ± 0.04	0.05 ± 0.00
20:4 n-6	0.14 ± 0.10	1.53 ± 0.05	0.08 ± 0.00	0.17 ± 0.06
20:3 n-3	Nd	0.18 ± 0.09	0.01 ± 0.00	0.02 ± 0.02
20:5 n-3	0.83 ± 0.01	5.82 ± 0.13	0.03 ± 0.01	0.05 ± 0.02
22:0	0.21 ± 0.09	0.16 ± 0.01	0.30 ± 0.00	0.28 ± 0.17
22:1	0.06 ± 0.02	0.87 ± 0.05	Nd	0.02 ± 0.02
22:2	Nd	Nd	0.01 ± 0.01	Nd
22:4 n-6	Nd	0.19 ± 0.01	0.05 ± 0.13	0.10 ± 0.02
22:5 n-6	Nd	0.95 ± 0.03	0.04 ± 0.00	0.01 ± 0.01
22:5 n-3	1.52 ± 0.56	0.66 ± 0.13	Nd	0.01 ± 0.01
22:6 n-3	Nd	18.68 ± 0.58	0.02 ± 0.00	0.02 ± 0.01
24:0	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.00	Nd
24:1	0.12 ± 0.00	0.47 ± 0.03	Nd	Nd
Sum SFA	22.17	28.60	16.29	35.54
Sum MUFA	20.71	28.67	27.68	47.21
Sum n-6 PUFA	50.68	13.31	50.77	16.18
Sum n-3 PUFA	6.44	29.42	5.25	1.07
n-6/n-3	7.87 ± 0.23	0.45 ± 0.04	9.67 ± 2.04	15.17 ± 1.40
P/S	2.58	1.49	3.44	0.49
P/(S + M)	1.33	0.75	1.27	0.21

Values (%) are expressed as means ± SD (3 time points were measured). The positions of the double bonds numbered from the methyl terminus are designated as n-9, n-6, and n-3

Nd not detected, *DMA* dimethylacetal derivatives, *SFA* saturated fatty acid(s), *MUFA* monounsaturated fatty acid(s), *LS*, *HF*, *HS*, or *HL* denote a control low-fat diet, a high-fat diet containing fish oil, soybean oil, or lard, respectively

Hepatic Gene Expression Profiling by qRT-PCR

Nineteen genes involved in inflammation response genes as well as lipid metabolism-related genes were selected and their mRNA expression levels were measured by qRT-PCR. The results are shown in Table 6 and are illustrated in Fig. 1. No significant differences were detected between the groups in the expression levels of genes encoding microsomal triglyceride transfer protein (MTP), ACAT2, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA R, EC 1.1.1.34), and prostaglandin-endoperoxide synthase 1 (PTGS1, COX-1) (Table 6). In contrast, the expression levels of *Acat1* and *Acox1* mRNAs were the most abundant ($p < 0.01$) in the livers of the HF group.

Table 3 A. Total intakes of food, oil, and energy consumption by 2 rats kept in a cage over the entire 4-week period. B. Body and organ weights after feeding rats with the LS, HF, HS, or HL diet

	LS	HF	HS	HL
A. Food intake (2 rats/cage)				
Food (g)	$1033.3 \pm 21.0^{a,b,c}$	802.2 ± 11.3^{a}	814.6 ± 44.6^{b}	830.3 ± 31.8^{c}
Oil (g)	$45.5 \pm 0.9^{a,b,c}$	188.5 ± 2.6^{a}	191.4 ± 10.4^{b}	195.1 ± 7.5^{c}
Energy (kcal)	3720 ± 76	3754 ± 53	3812 ± 209	3886 ± 149
B. Tissue wet wt. (g)				
Body	292 ± 53	312 ± 13	313 ± 11	315 ± 16
Liver	9.61 ± 2.17^{d}	$11.20 \pm 0.54^{a,b,d}$	8.63 ± 0.43^{a}	8.87 ± 0.25^{b}
Heart	1.18 ± 0.12	1.17 ± 0.10	1.16 ± 0.09	1.10 ± 0.13
WAT	$5.66 \pm 0.64^{b,c,d}$	$7.92 \pm 1.23^{d,e}$	$10.39 \pm 1.81^{c,e}$	9.99 ± 2.66^{b}
BAT	$0.22 \pm 0.12^{a,b}$	$0.49 \pm 0.05^{a,d}$	0.43 ± 0.08^{b}	0.34 ± 0.13^{d}

A. Values represent means ± SD, ($n = 3$). Values indicated by the same letter are significantly different. a, b, c: $p < 0.01$. B. The weights of WAT and BAT represent those of epididymal plus perirenal adipose or brown adipose tissues, respectively. Values are means ± SD, $n = 6$ for each group. Values indicated by the same letter are significantly different. a, b, c: $p < 0.01$; d, e: $p < 0.05$

Table 4 Plasma (A) and liver (B) lipid profiles

	LS	HF	HS	HL
A. Plasma				
t-Cho (mg/dL)	62.7 ± 4.8^{b}	$35.3 \pm 6.5^{a,b,d}$	52.7 ± 12.1^{d}	64.7 ± 11.0^{a}
TAG (mg/dL)	42.3 ± 18.6	22.7 ± 9.7	39.6 ± 23.6	45.3 ± 13.1
HDL-C (mg/dL)	51.8 ± 4.8^{c}	$27.6 \pm 4.4^{a,b,c}$	48.5 ± 5.8^{a}	52.4 ± 7.4^{b}
NEFA (UEq/L)	753.0 ± 151.7	642.7 ± 89.5	718.0 ± 96.8	719.7 ± 96.1
TP (g/dL)	5.3 ± 0.27^{e}	$5.7 \pm 0.11^{a,d,e}$	5.2 ± 0.28^{a}	5.3 ± 0.16^{d}
Glucose (mg/dL)	151.3 ± 25.0	128.4 ± 9.6	125.7 ± 18.6	132.7 ± 10.9
B. Liver				
t-Cho (mg/g)	3.26 ± 0.42^{c}	3.00 ± 0.26^{a}	$5.93 \pm 0.79^{a,b,c}$	3.44 ± 0.37^{b}
TAG (mg/g)	28.1 ± 6.9	20.1 ± 4.81^{a}	41.2 ± 13.5^{a}	30.0 ± 8.9
PL (mg/g)	$16.4 \pm 2.2^{b,c}$	$21.7 \pm 1.66^{a,b}$	20.4 ± 1.36^{c}	18.0 ± 1.9^{a}
NEFA (mg/g)	0.80 ± 0.10^{c}	0.50 ± 0.07^{a}	$2.00 \pm 0.84^{a,b,c}$	0.97 ± 0.39^{b}
TP (mg/g)	270 ± 6	274 ± 25	268 ± 3	254 ± 26

TP total protein, *t-Cho* total cholesterol, *TAG* triacylglycerol, *NEFA* non-esterified fatty acid, *HDL-C* HDL-cholesterol, *PL* phospholipid(s)

Values represent means ± SD, $n = 6$ rats per diet group. Values indicated by the same letter are significantly different. a, b, c: $p < 0.01$; d, e: $p < 0.05$

The expression level of *Hmgcr* mRNA in the HF group was not up-regulated. This indicates that because ACAT1 catalyzes the conversion of two acetyl-CoA molecules into acetoacetyl-CoA, the increase in acetyl-CoA produced from accelerated peroxisomal β-oxidation is likely to be converted into ketone bodies instead of cholesterol. In contrast, the expression level of carnitine palmitoyl transferase (CPT1A, EC 2.3.1.21) mRNA in the HF group was down-regulated suggesting that β-oxidation in mitochondria was suppressed. Moreover, the levels of genes encoding SREBP-1 and fatty acid synthase (FASN, EC 2.3.1.85) were suppressed by 0.5- and 0.8-fold, respectively, compared with those of LS group. SREBP-1, encoded by *Srebf1*, is a transcription factor that regulates various lipogenic genes including *Fasn*.

In the HS group, lipids were accumulated in the liver (Table 4B), and the expression level of *Acox1* mRNA was down-regulated (Tables 5B, 6). However, the level of *Fasn* mRNA was similar to that of the LS group (Table 6). Furthermore, the expression levels of apolipoprotein B-100 (*Apob*) and *Mttp* mRNAs were similar to those of the LS group but lower than those of the HL group (Table 6).

In the HL group, the expression levels of *Apob*, *Srebf1*, *Fasn*, low-density lipoprotein receptor (*Ldlr*), and peroxisomal proliferator-activated receptor gamma (*Pparg*) mRNAs were significantly higher compared with any of the

Table 5 DNA microarray analysis of expression levels of genes involved in glucose and lipid metabolism-related genes in the liver

Target gene	GenBank ID	Gene name	Protein function	HF	HS	HL
A. Glucose metabolism related						
Gck	NM_012565.1	Glucokinase	Glycogen synthesis	Nd	2.0	2.5
Slc2a2 (Glut2)	NM_012879.2	Solute carrier family 2 (facilitated glucose transporter) member 2	Glucose transporter	0.4	1.0	1.2
Pklr	NM_012624.3	Pyruvate kinase, liver and RBC	Glucose catabolism	0.5	1.3	1.7
PDK4	NM_053551.1	Pyruvate dehydrogenase kinase, Isozyme 4	Inhibition of pyruvate dehydrogenase	2.9	0.5	0.5
B. Lipid metabolism related						
Srebf1	XM_213329.4	Sterol regulatory element binding transcription factor 1	Fatty acid biosynthesis	0.5	2.6	3.1
Cyp7a1	NM_012942.1	Cytochrome P450, family 7, Subfamily a, polypeptide 1	Sterol catabolism	1.5	2.7	1.7
Acox1	NM_0173402	Acyl-CoA oxidase, palmitoyl	Fatty acid β-oxidation	1.6	0.5	0.5
Ehhadh	NM_133606.1	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	Fatty acid β-oxidation	2.5	0.4	0.4

Data are expressed relative to those of the LS group. The data show the differences in gene expression

Significant differences were judged from fold changes of ≥2.0 or ≤0.5

Nd not determined

Table 6 Expression levels of mRNA of hepatic genes involved in lipid metabolism and inflammation

Target gene	LS	HF	HS	HL
Apob	1.00 ± 0.07^{c}	0.67 ± 0.09^{a}	0.99 ± 0.16^{b}	$1.79 \pm 0.64^{a,b,c}$
Mttp	1.00 ± 0.11	1.03 ± 0.17	1.09 ± 0.21	1.41 ± 0.45
Acat1	1.00 ± 0.11^{c}	$1.76 \pm 0.13^{a,b,c}$	1.22 ± 0.20^{a}	1.08 ± 0.23^{b}
Acat2	1.00 ± 0.24	0.92 ± 0.57	0.75 ± 0.22	1.06 ± 0.50
Acox1	$1.00 \pm 0.13^{c,e}$	$1.47 \pm 0.25^{a,b,c}$	$0.72 \pm 0.10^{a,e}$	0.84 ± 0.16^{b}
Cpt1a	1.00 ± 0.15	0.79 ± 0.13^{a}	0.95 ± 0.10	1.10 ± 0.16^{a}
Srebf1	$1.00 \pm 0.36^{c,e}$	$0.54 \pm 0.21^{a,d}$	$2.15 \pm 0.28^{b,d,e}$	$3.57 \pm 1.14^{a,b,c}$
Fasn	1.00 ± 0.34^{f}	0.76 ± 0.46^{a}	1.08 ± 0.42^{e}	$2.01 \pm 0.74^{a,e,f}$
Ppara	1.00 ± 0.33	0.72 ± 0.15^{e}	1.18 ± 0.57	1.59 ± 0.76^{e}
Pparg	1.00 ± 0.19^{b}	1.96 ± 0.41^{c}	1.60 ± 0.38^{a}	$2.98 \pm 1.03^{a,b,e}$
Ucp2	1.00 ± 0.21^{f}	0.97 ± 0.19^{e}	1.06 ± 0.21	$1.36 \pm 0.21^{e,f}$
Ldlr	1.00 ± 0.18^{c}	1.04 ± 0.22^{a}	1.02 ± 0.26^{b}	$1.84 \pm 0.26^{a,b,c}$
Hmgcr	1.00 ± 0.31	1.15 ± 0.24	0.92 ± 0.33	0.90 ± 0.38
Cat	1.00 ± 0.09^{f}	0.91 ± 0.08^{a}	0.98 ± 0.15^{e}	$1.34 \pm 0.32^{a,e,f}$
Tlr4	1.00 ± 0.41	0.79 ± 0.31	1.33 ± 0.40^{e}	0.75 ± 0.19^{e}
Nfkb1	1.00 ± 0.13	0.68 ± 0.28^{a}	1.01 ± 0.10	1.25 ± 0.28^{a}
Ptgs1 (COX-1)	1.00 ± 0.60	0.40 ± 0.43	0.60 ± 0.73	0.40 ± 0.60
Ptgs2 (COX-2)	1.00 ± 0.30^{c}	0.72 ± 0.24^{a}	0.95 ± 0.19^{b}	$4.06 \pm 2.76^{a,b,c}$

Data are expressed relative to those of the LS group

Values represent means ± SD ($n = 6$). Values indicated by the same letter are significantly different. a, b, c, d: $p < 0.01$; e, f: $p < 0.05$

3 other groups. The expression level of uncoupling protein 2 (*Ucp2*) mRNA was significantly higher compared with those of the HF and LS groups ($p < 0.05$). However, the expression levels of mRNA associated with β-oxidation were not up-regulated. The mRNA levels of *Srebf1* and *Acox1* were consistent with the results of DNA microarray analysis (Tables 5B, 6).

The mRNA expression levels for genes related to inflammation were also examined (Table 6). The expression level of *Ptgs2* (COX-2) mRNA in the HL group was the highest ($p < 0.01$) among all groups, while the *Ptgs1* (COX-1) mRNA levels were similar (Table 6).

SDS-PAGE Analysis and Protein Identification

Hepatic proteins (15 μg/lane) were analyzed using SDS-PAGE (Fig. 2). The staining intensity of a 72-kDa band in the HF preparation was approximately 3-times stronger than

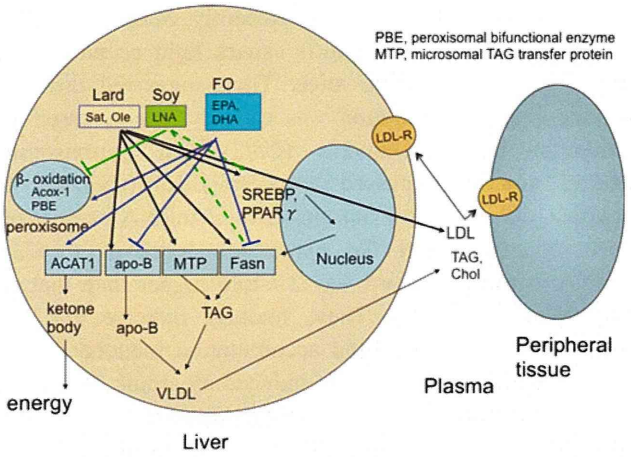

Fig. 1 Scheme summarizing the differential effects of dietary fatty acids on lipid metabolism in male Wistar rats. Data are based on the results of DNA microarray analysis (Table 5) and qRT-PCR analysis (Table 6). Major fatty acids were saturated (Sat) and monounsaturated (Ole) for lard, linoleic acid (LNA) for soybean oil (Soy), and EPA and DHA for fish oil (FO). The *arrow* indicates stimulation or up-regulation, and the *up tack* indicates inhibition or down-regulation

the corresponding bands (indicated by an arrow in Fig. 2) in the LS, HS, and HL preparations. Using MALDI-TOF/MS, the band was identified as EHHADH. In this search, protein scores >49 were judged as significant ($p < 0.05$), and in this case, the score was 233. This result agrees with the DNA microarray analysis (Table 5B). Moreover, the bands observed at approximately 150 kDa (indicated by an asterisk in Fig. 2) in the HF or HS group were weaker than the corresponding bands in the LS and HL groups. These bands were identified as carbamoyl-phosphate synthase (ammonia) mitochondrial precursor (EC 6.3.4.16). This enzyme participates in the synthesis of urea from proteins but not in lipid metabolism. The protein score was 457.

Discussion

In the present study, the caloric intakes of Slc:Wistar/ST rats were similar and independent of total food consumption or the type and amount of dietary fatty acids (Table 3A). The average body weights of high-fat groups were not significantly higher compared with the control LS group (<8 %, Table 3B). We confirmed that body weights of rats fed these high-fat diets even for 3 months were not significantly different (Yamada et al., unpublished observations). However, the weight of WAT increased depending on the intake of fats and oils; the WAT weight of the LS group was lower than that of the HF ($p < 0.05$), HS ($p < 0.01$), and HL ($p < 0.01$) groups (Table 3B). Farley et al. [29] reported that obesity-susceptible animals are hyperphagic; rats remaining lean on a high-fat diet consumed the same amount of calories as standard chow-fed controls. Consistent with these findings, the food intakes of the Slc:Wistar/ST rats in the present study were isocaloric (Table 3A), resulting in no significant differences in the body weights of these dietary groups.

The HF diet reduced plasma lipid levels (Table 4A) and the HS diet induced hepatic lipid accumulation (Table 4B). Plasma and liver lipid parameters of the HL group were similar to those of the LS group (Table 4). To investigate whether gene expressions in the liver were influenced by dietary fatty acids, we determined the levels of hepatic mRNAs encoding proteins involved in mediating lipid and glucose metabolism as well as those involved in inflammation. Dietary fish oil is known to decrease plasma TAG levels by suppressing VLDL secretion from the liver [30]. However, their effects on the expression of genes involved in the synthesis, assembly, and secretion of VLDL are unclear. In the HF group, plasma levels of t-Cho and

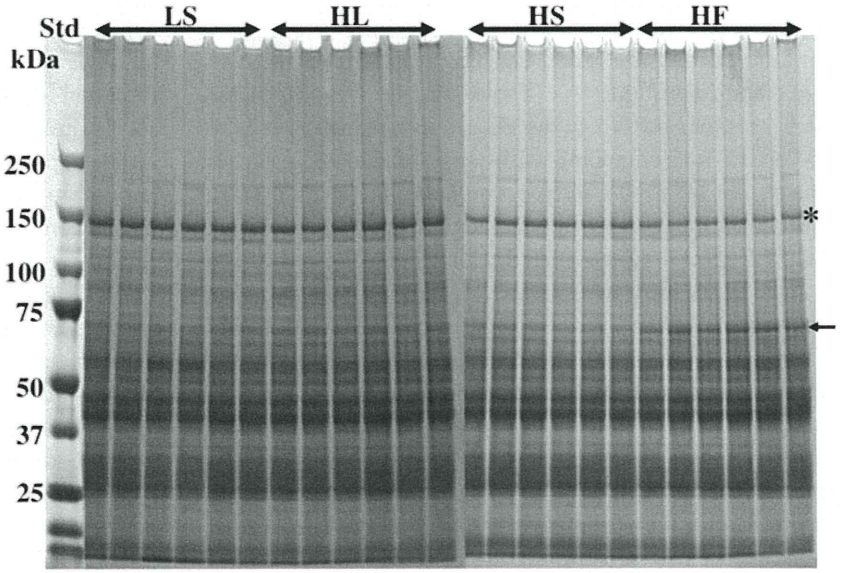

Fig. 2 SDS-PAGE analysis of liver proteins. The *arrow* and *asterisk* indicate EHHADH (EC 4.2.1.17/EC 1.1.1.35) and carbamoyl-phosphate synthase (ammonia) mitochondrial precursor (EC 6.3.4.16), respectively

HDL-C were significantly lower compared with those of the other 3 dietary groups, and the level of TAG was the lowest of all groups (Table 4A). In the HF group, the expression level of *Apob* mRNA was suppressed, but not that of *Mttp* (Table 6), suggesting that ApoB-100, but not MTP, regulates VLDL secretion. Moreover, n-3 PUFA (EPA and DHA) stimulated the degradation of ApoB-100 by post-ER presecretory proteolysis [31–34]. Therefore, VLDL secretion in the HF group was likely reduced by decreased ApoB-100 synthesis and stimulated posttranslational degradation.

Ikeda et al. [35] suggested that in rats, fish oil depresses TAG secretion from the liver. Hagve and Christophersen [36] reported that n-3 fatty acids were more readily converted to ketone bodies than n-6 fatty acids in cultured hepatocyte. Moreover, fish oil supplementation increased blood concentrations of ketone bodies in human subjects [37]. These findings are consistent with our observations that the HF group expressed the highest levels of *Acat1* mRNA (Table 6). In the HF group, glucose catabolism and uptake were suppressed through down-regulation of the levels of *Pklr* and *Slc2a2* (Glut 2) mRNAs, respectively (Table 5A). However, the plasma glucose level in the HF group was not increased (Table 4A). Suppression of glucose catabolism likely ensures preferential utilization of EPA and DHA by peroxisomal β-oxidation, because their melting points are too low to form stable oil droplets inside cells. Further, the expression level of *PDK4* mRNA was up-regulated (Table 5A).

As shown in Table 4B, the HS group accumulated the largest amount of lipids in the liver. The HS and LS diets contained by weight, 23.5 % of soybean oil and 4.4 % oils of which 1.7 % was contributed by the soybean oil, respectively. However, their fatty acid compositions were similar (Table 2). Total oil intakes by the HS and LS groups were 95.7 and 22.7 g, respectively. The mRNA expression levels of lipid metabolism-related genes were similar between the HS and LS groups, except for those of *Acox1* and *Srebf1* mRNAs (Tables 5B, 6). The expression levels of *Acox1* mRNA in the HS group were suppressed to 0.5- or 0.7-fold compared with those of the LS group as determined by DNA microarray analysis or qRT-PCR, respectively, suggesting that the hepatic lipid accumulation in the HS group was associated with decreased degradation of fatty acids in peroxisomes, but not in mitochondria (Table 6).

Lipogenesis was not significantly increased in the HS group, because the mRNA expression level of *Fasn* was not up-regulated (Table 6). Moreover, the level of VLDL secretion in the HS group was lower than that in the HL group, because the mRNA expression levels of *Apob* and *Mttp* were not up-regulated in the HS group. The normal levels of VLDL secretion contributed to hepatic lipid accumulation in the HS group. Recently, Seino et al. [21] reported that atopic dermatitis causes lipid accumulation in the liver of NC/Nga mice. They suggested that the hepatic lipid accumulation was caused by suppressed β-oxidation in mitochondria (*Cpt2*) and peroxisomes (*Acox1*) and by suppressed sterol catabolism (*Cyp7a1*). In contrast, our data show that only *Acox1* mRNA expression was suppressed and the expression level of *Cyp7a1* mRNA in the HS group was 2.7-fold higher than that of LS group (Table 5B). These findings indicate that the mechanism of hepatic lipid accumulation induced by the HS diet is different from that induced by atopic dermatitis in a mouse model. Feeding high-fat diets (42 en% of lard, olive oil, coconut fat) to obesity-susceptible male Wistar rats induced obesity and hepatic lipid accumulation [7]. This accumulation was caused by increased lipogenesis (*Fasn*), reduced lipolysis in mitochondria (*Cpt1a*), and normal levels of secretion of VLDL (*Apob*) [7]. Further, a large amount of linoleic acids (LNA, 18:2n-6) was present in the HS liver (HS, 27.3 %; HF, 10.2 %; HL, 15.5 %; and LS, 27.0 % of the total fatty acids). Hepatic lipid accumulation in the HS group was likely reversible, because no accumulation of collagen was detected (Fig. 2).

Total oil intakes by the HL and LS groups were 97.6 and 22.7 g, respectively. However, their hepatic and plasma lipid profiles were similar. In the HL group, large amounts of SFA and MUFA derived from lard were secreted from the liver as VLDL through up-regulation of *Apob* and *Mttp* mRNAs and were delivered to peripheral tissues or were partly transported back to the liver as LDL through up-regulated *Ldlr* mRNA (Table 6). The liver of the HL group continued synthesizing lipids from carbohydrates and proteins by up-regulating the expression of *Srebf1* and *Fasn* mRNAs (Tables 5B, 6) and by suppressing the expression of *PDK4* mRNA to stimulate acetyl-CoA production (Table 5A). Moreover, up-regulation of mRNAs for *Pklr* and *Gck* suggests that glucose catabolism and glycogen synthesis in the HL liver, respectively, were accelerated. Because of altered gene expression required to enable rapid mobilization of TAG to peripheral tissues in the HL group, plasma and hepatic cholesterol and TAG levels were maintained at levels similar to those in the LS group.

We found here that various types of dietary high fat produced different plasma and liver lipid profiles because of the metabolic properties of obesity-resistant Slc:Wistar/ST rats that differ from those of obesity-susceptible animals. The observed phenotypes are different those from reported for obesity-susceptible animals [3, 4, 7], which are hyperphagic and acquire hepatic lipid accumulation when consuming high-fat diets, except for a high-fish oil diet [7, 17]. In contrast, our results showed that the

obesity-resistant Slc:Wistar/ST rats are isocaloric and do not exhibit hepatic lipid accumulation even when consuming high-fat diets, except one that includes soybean oil.

Ptgs1 (COX-1) is constitutively expressed [38–42], and *Ptgs2* (COX-2) expression is induced by mitogens [43–48]. They each catalyze the conversion of arachidonic acid to prostaglandin H2, a rate-limiting step in the biosynthesis of prostaglandins and thromboxane [49]. COX-3, the third and most recently discovered COX isozyme, is encoded by *Ptgs1*, and is differentially spliced [50, 51]. Significantly higher expression levels of COX-2 ($p < 0.01$), but not COX-1 mRNA, were detected in the HL group (Table 6) compared with other groups. Dietary lard induced significantly increased expression of mRNA and protein levels of COX-2 compared with a dietary fish and soybean oil mixture in the Long-Evans Cinnamon rat [52].

The SFA moieties of lipopolysaccharide (LPS), but not unsaturated fatty acids, induce nuclear factor kappa light polypeptide gene enhancer in B-cells 1 (NF-κB) activation and expression of COX-2 that are mediated through a common signaling pathway triggered by toll-like receptor 4 (TRL4) [49, 53, 54]. Therefore, the large amounts of SFA present in the HL diet might induce the expression of COX-2 mRNA through TLR4 and the NF-κB signaling pathways. The HL diet might induce up-regulated COX-2 mRNA in the coronary artery as well, similar to liver, and cause coronary atherosclerosis or cardiovascular disease [16]. In contrast, the products generated by COX-2 might serve as ligands for differentiation [55] and proliferation of adipocytes [56] through up-regulation of *Pparg* mRNA [57–60]. Indeed, significant up-regulation of the mRNA level of *Pparg* in the HL group was observed (Table 6). Because TAGs in lard are adequate as depot fat and directly transported to peripheral tissues, the capacity to adipose tissue to accept TAGs must be concurrently increased.

Here, we have revealed the differential effects of high-fat diets containing fish oil, soybean oil, or lard on plasma and liver lipids, and discussed their possible mechanisms based on gene expressions in obesity-resistant SLC:Wistar/ST rats. However, information on metabolic rates of hepatic lipid synthesis, excretion form the liver, transport, and turnover of depot fats must be determined to better define the mechanisms involved.

Acknowledgments This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (070025). We thank Dr. Taeko Murakami and Dr. Haruo Nakagaki, Aichi-Gakuin University, for statistical analyses, and Mikie Sato and Dr. Fuminobu Yoshimura, Aichi-Gakuin University, for analyses using a TOF–MS/MS method. We are also grateful to Yui Imai and Eri Yamada, Kinjo Gakuin University College of Pharmacy, for experimental assistance and animal care.

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