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An increase in liver PPARγ2 is an initial event to induce fatty liver in response to a diet high in butter: PPARγ2 knockdown improves fatty liver induced by high-saturated fat☆,☆☆

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Abstract

The effects of a diet rich in saturated fat on fatty liver formation and the related mechanisms that induce fatty liver were examined. C57BL/6J mice were fed butter or safflower oil as a high-fat (HF) diet (40% fat calories) for 2, 4, 10, or 17 weeks. Although both HF diets induced similar levels of obesity, HF butter-fed mice showed a two to threefold increase in liver triacylglycerol (TG) concentration compared to HF safflower oil-fed mice at 4 or 10 weeks without hyperinsulinemia. At 4 weeks, increases in peroxisome proliferator-activated receptor γ2 (PPARγ2), CD36, and adipose differentiation-related protein (ADRP) mRNAs were observed in HF butter-fed mice; at 10 weeks, an increase in sterol regulatory element-binding protein-1c (SREBP-1c) was observed; at 17 weeks, these increases were attenuated. At 4 weeks, a single injection of adenoviral vector-based short hairpin interfering RNA against PPARγ2 in HF butter-fed mice reduced PPARγ protein and mRNA of its target genes (CD36 and ADRP) by 43%, 43%, and 39%, respectively, with a reduction in liver TG concentration by 38% in 5 days. PPARγ2 knockdown also reduced mRNAs in lipogenic genes (fatty-acid-synthase, stearoyl-CoA desaturase 1, acetyl-CoA carboxylase 1) without alteration of SREBP-1c mRNA. PPARγ2 knockdown reduced mRNAs in genes related to inflammation (CD68, interleukin-1β, tumor necrosis factor-α, and monocyte chemoattractant protein-1). In conclusion, saturated fatty acid-rich oil induced fatty liver in mice, and this was triggered initially by an increase in PPARγ2 protein in the liver, which led to increased expression of lipogenic genes. Inactivation of PPARγ2 may improve fatty liver induced by HF saturated fat.

Keywords: Steatosis; PPARγ; SREBP-1c

1. Introduction

The prevalence of obesity in Western societies has increased dramatically, due in part to a high-fat (HF) diet. The consequences of obesity include the emerging epidemics of hepatic steatosis and nonalcoholic fatty liver disease (NAFLD) [1]. Obesity can induce NAFLD, but a large intake in dietary fat may induce fatty liver, independent of obesity. In obese women, the amount of hepatic fat appears to be related to the amount of fat in the diet rather than to endogenous fat deposits [2]. In humans, fatty liver in obese subjects was improved after a 2-week low-fat (LF) diet (16% of total energy from fat) but worsened after a HF diet (56% of total energy from fat), despite provision of an isocaloric diet [3]. In addition to the total amount of fat, dietary fat subtypes may also affect the degree of fatty liver formation. Patients with fatty liver have been shown to consume significantly more saturated fat per day than control subjects matched for body mass index [4]. Saturated fatty acids are thought to be associated with lipogenesis and an increased risk for lipid-induced disorders, including fatty liver [5]. However, it is not clear whether the mechanism that mediates fatty liver in a HF diet (which might be mediated by hyperinsulinemia) is the same as the mechanism that mediates the condition under high saturated fatty acids.

Peroxisome proliferator-activated receptor (PPAR) γ and sterol regulatory element-binding protein (SREBP)-1c, transcription factors by which lipogenesis is stimulated, may contribute to fatty liver formation in response to increased intake of fat or saturated fatty acids. Liver PPARγ expression was increased in several animal models of fatty liver, and liver-specific knockout of PPARγ could prevent fatty liver [6,7]. Adenovirus delivery of PPARγ in hepatocytes results in fatty liver [8], and PPARγ interfering RNA reduces the liver triacylglycerol (TG) concentration [9]. Increased expression of liver fatty acid translocase/CD36, a target gene of PPARγ, leads to fatty liver [10]. An increase in the expression of adipose differentiation-related

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protein (ADRP), a target gene of PPARγ, increased triglyceride accumulation in cytosolic droplets in primary rat hepatocytes [11]. PPARγ exists in 2 forms, PPARγ1 and PPARγ2, and both forms are expressed in hepatocytes. These 2 isoforms are generated by alternative promoter usage and mRNA splicing; PPARγ2 possesses an additional 30 amino acids at its N-terminus [12]. Both forms may contribute to formation of fatty liver. However, activation of SREBP-1c increases expression of lipogenic genes such as acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1), which lead to fatty liver [13]. Peroxisome proliferator-activated receptor γ coactivator (PGC)-1β is induced in response to dietary saturated fatty acids, whereupon it coactivates SREBP-1c to up-regulate de novo lipogenesis [14]. Therefore, the increased expression of SREBP-1c or PGC-1β in response to dietary high-saturated fatty acids might activate SREBP-1c and lead to fatty liver.

In mice, studies comparing HF diets with LF diets during fatty liver formation revealed that a diet high in fat promotes fatty liver, although the authors did not consider fat subtypes [15–17]. The increases in PPARγ protein and the mRNAs of its target genes, CD36 and ADRP, were observed in liver of mice fed a HF diet, whereas SREBP-1 and the mRNAs of its target genes, FAS and acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT), were not altered [16,17]. However, activation of SREBP-1c may also contribute to fatty liver in response to dietary saturated fatty acids. It was shown that intracellular oleate generated by SCD1, a target gene of SREBP-1c, is directly involved in the induction of lipogenesis previously attributed to saturated fat [18]. A diet high in saturated fatty acids may increase lipogenesis relative to a polyunsaturated fatty acid (PUFA)-rich diet because it contains less PUFA, the end product of which (arachidonic acid or docosahexaenoic acid) inhibits SREBP-1c activity [19].

To discover the role of PPARγ and lipogenic genes in fatty liver induced by a diet high in saturated fatty acids, C57BL/6J mice were fed butter that contained 71% (wt:wt) saturated fatty acids in the total fat. To examine the effects of HF, two types of control diet were prepared: a LF safflower oil diet and a HF safflower oil diet. Mice were fed 3 different diets and were killed after 2, 4, 10, and 17 weeks. Changes in liver TG concentration and the mRNA levels of hepatic lipogenic genes were measured at each time point. With fatty liver development, increases in PPARγ2 and its target gene mRNAs (CD36 and ADRP) were observed in mice fed butter. Therefore, we decreased PPARγ2 mRNA by treatment with short-hairpin RNAs (shRNAs) in mice fed a HF butter oil diet and increased PPARγ2 mRNA by treatment with adenoviral vector-based PPARγ2 cDNA in mice fed a normal laboratory diet, and from this the role of PPARγ2 in development of fatty liver in response to butter was elucidated.

2. Materials and methods

2.1. Animals

Six-week-old male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and fed a normal laboratory diet (CE2; Clea, Tokyo, Japan) for 1 week to stabilize metabolic conditions. Mice were exposed to a 12-h light/12-h dark cycle and maintained at a constant temperature of 22°C. Four mice were housed per plastic cage, each of which was equipped with plastic partitions to separate individual mice. Mice were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All animal procedures were reviewed and approved by the National Institute of Health and Nutrition.

2.2. Dietary experiments

At 7 weeks of age, mice were assigned to 1 of 3 groups. Two groups were fed a HF diet (40% fat calories) containing either safflower oil (high-oleic type) or butter. As a control, mice in the third group were fed a LF diet (10% fat calories) containing safflower oil, in which essential fatty acids are included. The detailed compositions of each diet and macronutrients in the experimental diets are listed in Table 1. Fatty acid compositions of dietary oils were measured by gas-liquid chromatography. The percentages of saturated fatty acid in the HF butter and HF safflower oil diets were 29.8% and 3.5% fat calories, respectively (Table 1). Safflower oil was purchased from Benibana (Tokyo, Japan); butter from Snow Brand Milk (Hokkaido, Japan); casein, sucrose, α-cornstarch, vitamin mixture (AIN-93), mineral mixture (AIN-93), and cellulose powder were from Oriental Yeast (Tokyo, Japan), and L-cystine was from Wako Pure Chemical Industries (Osaka, Japan). The methods of diet preparation were the same as those of our previous studies [20]. Food consumption was measured daily; the mean food intake per day was estimated by subtracting the food weight of each day from the initial food weight of the previous day.

Table 1
Dietary composition

	LF	HF	
	Safflower oil	Safflower oil	Butter
Components		g (% of total energy)	
Safflower oil	40 (9.7)	190 (41.5)	–
Butter	–	–	190 (41.5)
Casein	200 (20.3)	290 (26.3)	290 (26.3)
Sucrose	110 (11.7)	63 (5.4)	63 (5.4)
α-Cornstarch	552 (58.3)	315 (26.8)	315 (26.8)
Vitamin mixture (AIN-93)	10	14	14
Mineral mixture (AIN-93)	35	51	51
Cellulose powder	50	73	73
l-Cystine	3	4	4
Fatty acids		% of total energy	
ΣSFA	0.8	3.5	29.8
C4:0	n.d.	n.d.	1.8
C6:0	n.d.	n.d.	1.2
C8:0	n.d.	n.d.	0.6
C10:0	n.d.	n.d.	1.4
C12:0	n.d.	n.d.	1.5
C14:0	n.d.	n.d.	5.1
C16:0	0.5	2.3	13.5
C18:0	0.2	0.9	4.5
C20:0	0.1	0.2	0.1
ΣMUFA	4.4	18.8	10.0
C16:1	0.0	0.1	0.6
C18:1	4.4	18.7	9.0
C20:1	0.0	0.1	0.1
ΣPUFA	4.5	19.1	1.1
C18:2 (n-6)	4.4	19.0	0.8
C18:3 (n-3)	0.0	0.1	0.2

n.d., not detectable; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Initially, a time-course study was conducted (n=16 for each group). Mice were killed by cervical dislocation around noon after a 3 h fast at 2, 4, 10, and 17 weeks following initiation of the experimental diets; livers were isolated for analysis. Serum analysis was conducted at 8 weeks. In the second experiment, the effect on liver TG of the knockdown of PPARγ2 mRNA by an injection of adenoviral vector-based short hairpin interfering RNA was examined (I=16 for each diet group). Mice were fed LF safflower oil, HF safflower oil, or HF butter for 4 weeks. Recombinant adenovirus expressing shPPARγ2 (Ad-shPPARγ2 or Ad-Negative control) was injected intravenously in a single dose (2×10^9 plaque-forming units in 200 μl) into a non-fasted mouse in each group under anesthesia (diethyl ether). Mice were killed at 1 day or 5 days after the injection. Similar to the time-course study, mice were fasted 3 h before sacrifice.

2.3. Adenoviral shRNA constructs

A 19-nt sequence starting from nucleotide 63 of PPARγ2 was synthesized as a complementary antiparallel oligonucleotide with a loop sequence (tagtgctcctggttg) and *BamHI*- and *HindIII*-compatible ends. The nucleotide sequences for the shRNA against PPARγ2 were as follows: 5′gatccgCTATGAGCACTTCACAAGAtagtgctcctggttgTGTGTGAAGTGCTCATAGctttttta (forward) and 5′agcttaaaaaagCTATGAGCACTTCACAAGAcaaccaggagcactaTCTTGTGAAGTGCTCATAGcg (reverse). The forward and reverse oligonucleotides were annealed and ligated into a pBAsi-hU6 Neo DNA vector (Takara Bio, Tokyo, Japan) containing the human U6 promoter. Recombinant adenovirus (Ad) was generated using an Adenovirus Expression Vector Kit (Dual Version, Takara Bio). An *EcoRV* restriction fragment of recombinant pBAsi-hU6-shPPARγ2 was inserted into the *SwaI* site of a cosmid vector pAxcwit2 (Takara Bio) containing E1- and E3-deficient Ad type 5 sequences. Ad-shPPARγ2 was produced in human embryonic kidney cells (HEK293) and purified by an Adeno-X Purification Kit (Clontech, Mountain View, CA, USA). The shRNA negative control adenovirus (Takara Bio) was used as a control (Ad-Negative control).

2.4. Overexpression of PPARγ2 in liver by a single injection of adenoviral vector-based PPARγ2 cDNA

The full-length mouse *PPARγ* (Gen Bank accession no. BC021798) coding sequence was amplified by polymerase chain reaction (PCR). The primers used for amplifying

PPARγ2 cDNA were tailed with either an *XbaI* site (forward) or a *KpnI* site (reverse). PCR products were subcloned into the *XbaI*/*KpnI* site of the pShuttle vector provided in the Adeno-X Expression System (BD Biosciences, Palo Alto, CA). All sequences of PPARγ2 were verified by DNA sequencing. An *I-CeuI*/*PI-SceI* restriction fragment from pShuttle containing the cytomegalovirus-IE promoter/enhancer 5′ to the DGAT cDNAs insert and the polyadenylation signal was ligated into the adenoviral DNA backbone that was also restricted with *I-CeuI* and *PI-SecI*. Following amplification and purification of recombinant viral DNA from bacteria, recombinant viral DNA was further amplified by transfecting *PacI*-linearized recombinant viral DNA into HEK293 cells via the use of Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA). The adenoviruses containing PPARγ2 cDNA (Ad-PPARγ2) were purified for injection into mice with an Adeno-X Purification Kit (BD Biosciences). Adeno-X EGFP (Ad-GFP, BD Biosciences) was used as a control.

For administration to mice, each recombinant adenovirus (Ad- PPARγ2 or Ad-GFP) was injected intravenously in a single dose (2×10^9 pfu in 200 μl) into a non-fasted animal at 8 weeks of age.

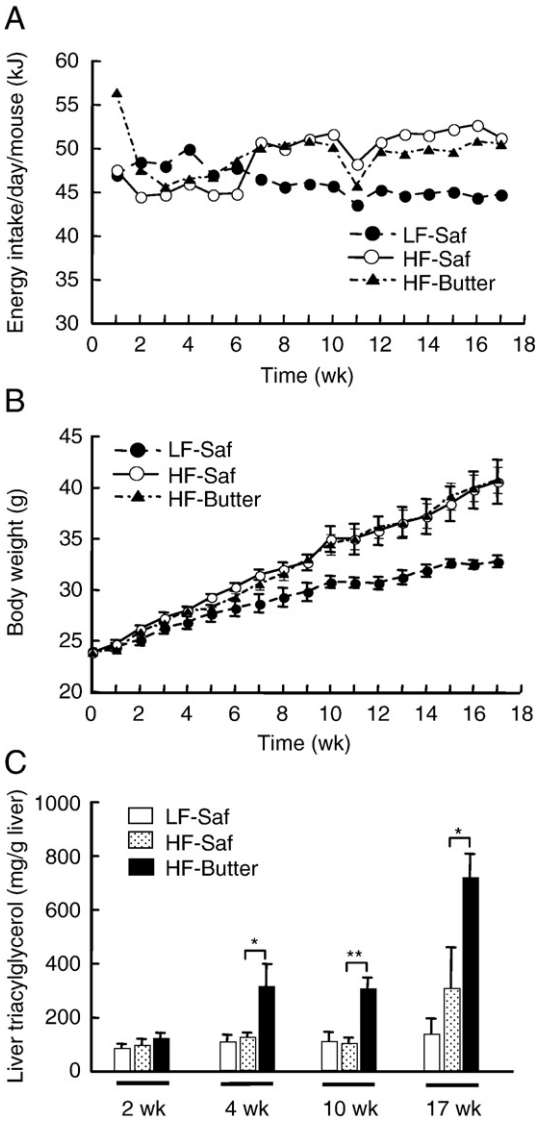

Fig. 1. Energy intake, body weight, and liver triacylglycerol changes during the experimental period in 3 groups of mice (n=16 each). Mice were given one of the three study diets shown in Table 1: LF safflower oil (LF-Saf), HF safflower oil (HF-Saf), or HF butter (HF-Butter). Four mice were killed at 2, 4, 10, and 17 weeks. Therefore, n decreased over time. (A) Energy intake. Food intake was measured daily during the study and was averaged weekly. Daily energy intake was calculated from the nutrient composition of the diet. The data represent the mean daily energy intake. At 17 weeks, the energy intake in mice fed a HF diet was significantly greater than that in mice fed a LF diet (P<.05). (B) Body weight. Body weight was measured weekly. At 17 weeks, the body weight of mice fed a HF diet was significantly greater than that in mice fed a LF diet (P<.05). (C) Hepatic triacylglycerol concentrations. *P<.05, **P<.01 vs HF-Saf. Data are means±SEM, n=4.

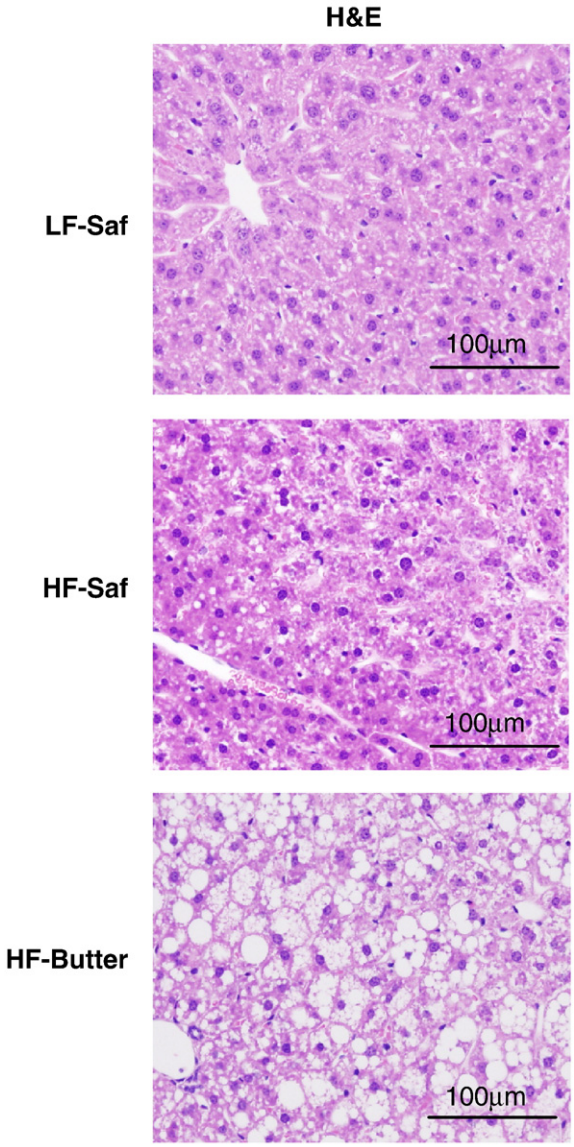

Fig. 2. Hematoxylin-and-eosin (H&E) staining of liver sections from representative mice from each group at 17 weeks. H&E staining showed microvesicular and macrovesicular fatty change within hepatocytes in HF butter-fed mice. LF-Saf, LF safflower oil; HF-Saf, HF safflower oil; HF-Butter, HF butter.

Table 2
Serum analysis after 8 weeks on study diets

		LF	HF		ANOVA
		Safflower oil	Safflower oil	Butter	*P*
Glucose, mmol/L	0 h[a]	5.05±0.17	5.55±0.17	5.33±0.17	.278
	3 h[b]	9.83±0.28	8.66±0.22	8.60±0.56	.981
NEFA, mEq/L	0 h	1.12±0.05	1.08±0.06	1.04±0.07	.700
	3 h	0.49±0.06	0.55±0.05	0.64±0.06	.205
Serum TG, μmol/L	0 h	891±68	983±91	963±94	.883
	3 h	833±78	724±70	700±72	.813
Insulin, pmol/L	0 h	38±5	34±5	38±3	.763
	3 h	598±98	327±29	288±26	.328
Leptin, nmol/L	0 h	0.11±0.03	0.15±0.04	0.14±0.04	.795
	3 h	1.50±0.19	0.89±0.12	1.39±0.24	.069

Values are means±SEM, n=8.
[a] 0 h, serum obtained from 24-h fasted mice.
[b] 3 h, serum obtained from animals 3 h after food.

2.5. Liver lipid analysis

Liver lipids were extracted quantitatively with an ice-cold mixture of chloroform and methanol (2:1, v:v) by the method of Folch et al. [21]. Total TG concentration in the liver homogenates was measured by enzymatic colorimetric methods with a TG E test (Wako Pure Chemical Industries).

2.6. Hepatic histology

Mouse livers were fixed in 4% neutral-buffered formalin, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin. Frozen sections of formalin-fixed liver were stained with Oil red O with the use of standard techniques.

2.7. Serum chemistry

Serum TG, nonesterified fatty acid (NEFA), and alanine aminotransferase (ALT) were assayed enzymatically with colorimetric kits TG E, NEFA-C, and Transaminase CII tests (Wako Pure Chemicals Industries), respectively. Serum insulin and leptin were assayed with a mouse insulin ELISA kit and a mouse leptin ELISA kit (Morinaga, Kanagawa, Japan), respectively. Serum glucose was measured on an Ascensia autoanalyzer (Bayer Medical, Tokyo, Japan).

2.8. Quantitative reverse transcriptase-PCR

Methods of RNA preparation and quantitative reverse transcriptase-PCR (RT-PCR) were described previously [20]. Primers used for quantitative RT-PCR are listed in Supplemental Table 1.

2.9. Western blot analysis

Nuclear protein from liver was extracted with a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Protein (100 μg) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% gel) was electrophoretically transferred onto Clear Blot Membrane-P (ATTO,

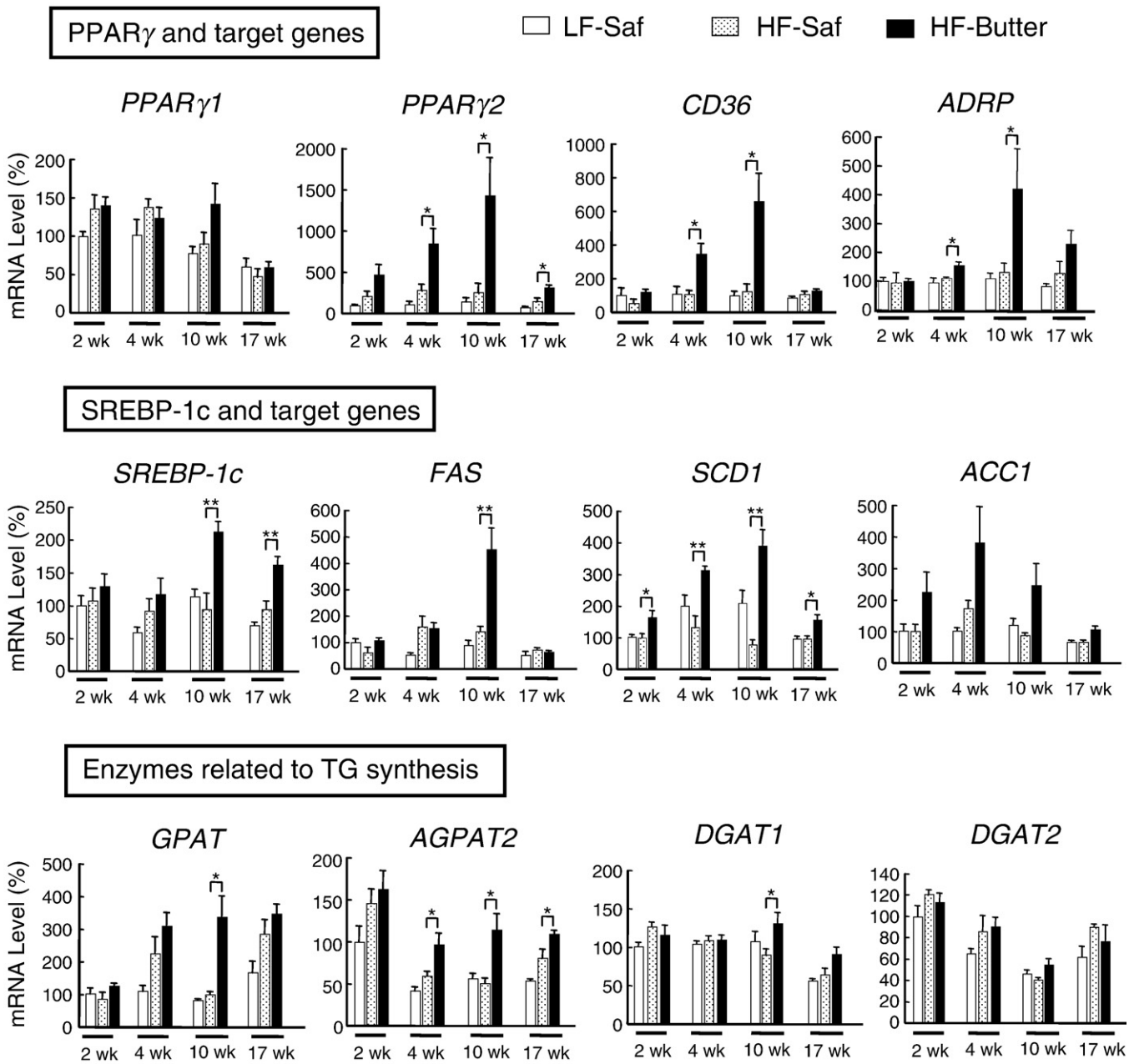

Fig. 3. Hepatic gene expression of transcription factors related to lipogenesis, their target genes, and enzymes related to lipid synthesis in mice fed LF safflower oil (LF-Saf), HF safflower oil (HF-Saf), or HF butter (HF butter) for 2, 4, 10, and 17 weeks. Quantitative RT-PCR analysis was performed with specific primers. The graphs show the percent mRNA levels relative to those of LF-Saf-fed mice at 2 weeks. Values are means±SEM, $n=4$. *$P<.05$, **$P<.01$ vs HF-Saf. No letter indicates that the 1- or 2-way ANOVA P value was not significant, and thus post-hoc tests were not performed.

Tokyo, Japan) and immunoblotted with specific primary antibodies: PPARγ2 (PA1-824, 1:500 dilution, Thermo Scientific, Rockford, IL, USA). Peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:8000 dilution) was used as the secondary antibody. Bands were visualized with an enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK) and quantified using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

2.10. Statistical analysis

Data are presented as means±S.E.M. Comparisons of data from multiple groups were made by two-way analysis of variance (ANOVA) (StatView 5.0; Abacus Concepts, Berkeley, CA, USA). When differences were significant, each group was compared with the others by Fisher's protected least significant difference test. As an example, two-way ANOVA was used to examine the effects of a HF diet (safflower oil and butter), feeding period (2, 4, 10, 17 weeks), and their interaction. Comparisons of data between two groups were made by unpaired Student's *t* test. Statistical significance was defined as $P<.05$. ANOVA *P* values are shown when appropriate.

3. Results

3.1. Energy intake, body weights, and liver TG concentrations

Mice were fed a HF diet (40% fat calories) containing safflower oil or butter. As a control, mice were fed an LF diet (10% fat calories) containing safflower oil. Average energy intake for the 3 groups over the 17 weeks is shown in Fig. 1A. Energy intake in each of the HF groups was slightly greater than that in the LF group, but there was no significant difference in energy intake between the HF groups. As the HF diets were slightly hypercaloric and contained the same amount of other nutrients, statistical comparisons were limited to the effects of the two HF diets rather than including comparisons to the LF diet. Changes in body weight are shown in Fig. 1B. Each group of mice fed a HF diet showed similar increases in body weight compared to those of LF diet-fed mice. However, there was a marked difference in liver TG levels between the HF groups; HF butter-fed mice showed greater increases in liver TG concentrations than did HF safflower oil-fed mice after 4 weeks of feeding (Fig. 1C). Compared with the LF diet, liver TG concentrations in mice fed a HF safflower oil diet began to increase after 17 weeks of feeding. A similar change in the whole-liver TG level was observed (data not shown). Hematoxylin and eosin staining showed microvesicular and macrovesicular fatty change within hepatocytes in HF butter-fed mice at 17 weeks of feeding (Fig. 2).

3.2. Blood metabolite and hormone concentrations

Hepatic fat accumulation may be a result of peripheral insulin resistance and increased insulin concentrations [22]. Increased plasma concentrations of glucose and NEFA may also promote hepatic steatosis [23–25]. Leptin deficiency leads to fatty liver [26]. At 8 weeks, blood glucose, NEFA, TG, insulin, and leptin concentrations were measured at two points: fasting (24-h fasting, 0 h) and postprandial (3 h after food presentation after 24-h fasting) (Table 2). There were no significant differences in fasting

Table 3
Effect of 1 day of Ad-shPPARγ2-mediated knockdown on energy intake, serum analysis, and mRNAs related to lipid synthesis and inflammation in the liver after 4 weeks of feeding

		LF	HF		Two-way ANOVA *P* value		
	shPPARγ2	Safflower oil	Safflower oil	Butter	Dietary fat	shPPARγ2	Dietary fat×shPPARγ2
Energy intake (kJ/day/mouse)	−	63±1	106±7	115±5			
	+	63±5	98±8	112±1	0.078	0.348	0.685
Serum analysis							
Glucose (mmol/L)	−	7.88±0.50	9.05±0.33	8.22±0.67			
	+	7.83±0.28	7.77±0.22	7.55±0.50	0.273	0.056	0.580
Insulin (pmol/L)	−	210±43	294±50	325±48			
	+	165±22	243±59	339±46	0.237	0.728	0.525
TG (μmol/L)	−	645±32	495±28[a]	443±54[a,b]			
	+	352±45	315±26[b]	375±62[a,b]	0.936	0.018	0.239
ALT (nkat/L)	−	403±55	410±25	343±90			
	+	300±98	448±62	325±90	0.208	0.905	0.696
PPARγ and its target genes (mRNA)							
PPARγ1	−	100±5	119±5	129±9			
	+	109±8	135±9	129±6	0.725	0.318	0.300
PPARγ2	−	100±7	158±16[a]	270±30[b]			
	+	50±14	107±11[a]	116±32[a]	0.026	0.001	0.051
CD36	−	100±6	106±19	139±19			
	+	92±22	98±12	94±8	0.351	0.105	0.236
ADRP	−	100±2	111±20	120±4			
	+	79±5	114±15	110±9	0.699	0.693	0.536
SREBP-1c and its target genes (mRNA)							
SREBP-1c	−	100±13	82±15	87±4			
	+	76±15	81±14	81±6	0.839	0.753	0.806
FAS	−	100±10	94±13[a]	81±7[a,b]			
	+	61±7	67±12[a,b]	54±6[b]	0.223	0.021	0.952
SCD1	−	100±5	65±13[a]	71±9[a]			
	+	56±4	30±7[b]	52±9[a,b]	0.172	0.017	0.444
ACC1	−	100±9	106±8	117±6			
	+	61±2	83±25	113±4	0.156	0.340	0.496
Genes related to inflammation (mRNA)							
CD68	−	100±26	105±9[a]	102±4[a]			
	+	64±3	74±5[b]	71±9[b]	0.647	0.001	0.979
MCP-1	−	100±21	126±16	128±16			
	+	108±9	123±16	115±9	0.853	0.609	0.745
TNF-α	−	100±13	114±12	108±20			
	+	104±15	116±3	128±13	0.842	0.424	0.542
IL-1β	−	100±5	109±15[a]	113±18[a]			
	+	96±8	89±11[a,b]	61±3[b]	0.647	0.001	0.979

Mice fed LF safflower oil, HF safflower oil, or HF butter for 4 weeks were injected with Ad-shPPARγ2 (+) or an Ad-Negative control (−) and were killed 1 day later. The percentages of mRNA levels relative to those of a LF safflower oil diet without shPPARγ2 injection are shown. Values are means±S.E.M. ($n=4$). Means without a common letter differ ($P<.05$).

and postprandial blood glucose, NEFA, TG, or leptin concentrations between the HF groups, suggesting that alterations in substrate entry into (glucose, NEFA, postprandial TG) and exit from [fasting TG corresponds to very low-density lipoprotein (VLDL)-TG] the liver, or lipolysis in adipose tissues were not major causes of fatty liver in mice fed HF butter.

However, at 17 weeks, blood glucose and insulin concentrations after 3 h of fasting were significantly increased in each of the HF groups compared to the concentration in the LF group, indicating that mild diabetes had developed in mice fed a HF diet, irrespective of the type of fat. Serum glucose concentrations were 6.55±0.50, 11.27±0.17, and 10.55±0.39 mmol/L in LF safflower-, HF safflower oil- and HF butter-fed mice, respectively (n=4), and insulin concentrations were 356±121, 592±103, and 484±86 pmol/L, in LF safflower oil-, HF safflower oil-, and HF butter-fed mice, respectively (n=4).

3.3. Alterations in lipogenic enzyme mRNAs

The increased TG accumulation in the livers of mice fed HF butter was likely due to alterations in lipid metabolism in the liver. To elucidate this mechanism, gene expression profiles related to lipogenesis were measured at 2, 4, 10 and 17 weeks (Fig. 3). Gene expression profiles in the liver differed during the feeding period.

PPARγ1 levels were not altered but PPARγ2 expression was increased in HF butter-fed mice at 4, 10 and 17 weeks, compared to HF safflower oil-fed mice. In parallel with increased PPARγ2 mRNA, CD36 and ADRP mRNAs were increased in HF butter-fed mice at 4 and 10 weeks. These increases in PPARγ2, CD36 and ADRP mRNAs were attenuated at 17 week.

Compared to HF safflower oil-fed mice, HF butter-fed mice showed 1.5 to 2-fold increases in SREPB-1c mRNA levels at 10 and 17 weeks. FAS mRNA expression at 10 week and SCD1 mRNA expression at 2, 4, 10 and 17 weeks were increased in HF butter-fed mice. However, these increased expressions were attenuated at 17 weeks. ACC1 mRNA expression was increased in HF butter fed mice, but the changes were not significant. The coactivator PGC-1β binds to SREBP-1c and activates its transcriptional activity [14]. However, expression of PGC-1β mRNA was not altered among the groups (data not shown).

TG synthesis requires several enzymes. GPAT is responsible for the first esterification step of glycerol-3-phosphate to monoacylglycerol [27], and 1-acylglycerol 3-phosphate acyltransferase (AGPAT) is responsible for the second esterification step of monoacylglycerol to diacylglycerol [28]. Diacylglycerol acyltransferase (DGAT) is responsible for the final esterification step of diacylglycerol to triacylglycerol [29,30]. GPAT expression is reported to be regulated by SREBP-1c [31], but it is not clear whether expression of AGPAT and DGAT is also regulated by SREBPs. Compared to HF safflower oil-fed mice, GPAT expression was greater in HF butter-fed mice at 10 weeks. AGPAT2 expression was also greater in HF butter-fed mice at 4, 10 and 17 weeks. DGAT1 and DGAT2 expression was not altered markedly at any time point.

Table 4
Effect of 5 days of Ad-shPPARγ2-mediated knockdown on energy intake, serum analysis, and mRNAs related to lipid synthesis and inflammation in the liver after 4 weeks of feeding

		LF	HF		Two-way ANOVA P value		
	shPPARγ2	Safflower oil	Safflower oil	Butter	Dietary fat	shPPARγ2	Dietary fat×shPPARγ2
Energy intake (kJ/5days/mouse)	−	305±3	397±15	506±65			
	+	307±4	392±28	541±35	0.067	0.973	0.884
Serum analysis							
Glucose (mmol/L)	−	9.21±0.22	8.88±0.33	8.77±0.39			
	+	8.72±0.22	8.55±0.83	8.83±0.17	0.849	0.767	0.742
Insulin (pmol/L)	−	136±29	170±14^{a}	241±24^{a,c}			
	+	129±17	269±38^{b,c}	339±24^{b}	0.056	0.001	0.622
TG (μmol/L)	−	574±111	483±70^{a,b}	663±138^{b}			
	+	483±53	304±128^{a}	344±59^{a,b}	0.311	0.034	0.519
ALT (nkat/L)	−	302±77	450±100	385±53			
	+	277±48	417±43	358±30	0.351	0.646	0.955
PPARγ and its target genes (mRNA)							
PPARγ1	−	100±10	109±7^{a,b}	132±10^{a}			
	+	86±9	90±6^{b}	106±9^{a,b}	0.042	0.020	0.693
PPARγ2	−	100±13	161±10^{a}	259±37^{b}			
	+	46±10	110±22^{a}	137±22^{a}	0.025	0.004	0.171
CD36	−	100±12	149±17	168±20			
	+	69±8	132±20	95±30	0.709	0.067	0.239
ADRP	−	100±13	122±6^{a}	135±3^{a}			
	+	88±14	120±20^{a}	82±5^{b}	0.264	0.026	0.038
SREBP-1c and its target genes (mRNA)							
SREBP-1c	−	100±33	94±9	120±10			
	+	86±16	124±18	155±21	0.082	0.054	0.881
FAS	−	100±25	123±8^{a}	124±6^{a}			
	+	81±15	109±29^{a}	58±6^{b}	0.125	0.022	0.112
SCD1	−	100±13	97±3^{a,b}	113±8^{a}			
	+	94±10	53±8^{c}	77±16^{b,c}	0.075	0.002	0.692
ACC1	−	100±16	125±9^{a}	119±9^{a}			
	+	93±10	95±14^{a,b}	64±10^{b}	0.121	0.002	0.291
Genes related to inflammation (mRNA)							
CD68	−	100±8	104±4^{a}	122±8^{a}			
	+	43±8	77±12^{b}	52±3^{c}	0.641	<0.001	0.017
MCP-1	−	100±8	132±12^{a}	140±30^{a}			
	+	15±2	40±9^{b}	27±7^{b}	0.865	<0.001	0.550
TNF-α	−	100±13	117±14^{a}	110±21^{a}			
	+	24±4	35±10^{b}	18±2^{b}	0.420	<0.001	0.727
IL-1β	−	100±14	130±17^{a}	118±4^{a,b}			
	+	83±11	97±17^{a,b}	88±12^{b}	0.458	0.039	0.924

Mice fed LF safflower oil, HF safflower oil, or HF butter for 4 weeks were injected with Ad-shPPARγ2 (+) or an Ad-Negative control (-) and were killed 5 days later. The percentages of mRNA levels relative to those of a LF safflower oil diet without shPPARγ2 injection are shown. Values are means±S.E.M. (n=4). Means without a common letter differ (P<.05).

We examined mRNA levels of PPARα and its target genes including acyl-CoA oxidase and medium-chain acyl-CoA dehydrogenase and carnitine palmitoyltransferase1 [32]. Expression levels of these genes were not altered in the HF groups (data not shown). The expression level of carbohydrate responsive element-binding protein mRNA was not altered between HF groups either (data not shown).

3.4. Decrease in liver TG concentration in Ad-shPPARγ2-injected mice

Because increases in PPARγ and its activation (estimated by increased expression of its target gene) in HF butter-fed mice were observed earlier than was an increase in SREBP-1c mRNA, the role of increased PPARγ2 mRNA expression in fatty liver formation was investigated. We deactivated PPARγ2 by adenovirus-mediated RNA interference in which shRNAs were used to inhibit PPARγ2 gene expression in vivo and then examined phenotypic changes in each group of mice. Mice fed LF safflower oil, HF safflower oil, and HF butter for 4 weeks received a single injection of adenovirus (Ad-shPPARγ2 or Ad-negative control) and were killed 1 or 5 days later. Energy intake, body weight, liver weight and epididymal white adipose tissue weight did not differ between Ad-shPPARγ2- and Ad-shNegative control-injected mice (only data related to energy intake is shown in Tables 3 and 4). Ad-shPPARγ2 injection did not affect serum glucose and insulin concentrations but did decrease serum TG concentrations (ANOVA $P<.05$). However, there were no differences in the mRNAs of MTP and apoB, which regulate VLDL secretion, in each group of mice (unpublished observation, T. Yamazaki and O. Ezaki). An increased export of lipids from the liver, by which fatty liver formation is prevented, might not occur. Serum ALT concentration, a marker of liver injury, did not differ between the groups, suggesting that knockdown of PPARγ2 did not cause cell damage.

One day after Ad-shPPARγ2 injection, the liver PPARγ2 mRNA level in mice fed HF butter injected with Ad-shPPARγ2 was 57% lower than in mice injected with an Ad-shNegative control (Table 3). The PPARγ2 mRNA level in mice fed LF safflower oil or HF safflower oil, was also decreased by Ad-shPPARγ2 injection but it was not significant. The lack of a significant decrease in PPARγ2 mRNA in mice fed LF safflower oil or HF safflower oil by Ad-shPPARγ2 injection might be due to lower expressions of PPARγ2 mRNA in these mice. Five days after injection, decreases in the PPARγ2 mRNA level in mice injected with Ad-shPPARγ2 were maintained in mice fed HF butter (by 47%, Table 4). PPARγ1 mRNA levels were not altered after Ad-shPPARγ2 injection.

One day after injection, the liver TG concentration was decreased by 25% in mice fed LF safflower oil, 27% in mice fed HF safflower oil and 23% in mice fed HF butter, although they did not reach significance (Fig. 4). Five days after injection, the decrease in liver TG concentration was augmented in mice fed LF safflower oil (41% decrease), HF safflower oil (37% decrease), and HF butter (38% decrease). The decrease in liver TG in mice fed HF butter was statistically significant. Oil red O staining confirmed hepatic TG accumulation in mice fed HF butter and also confirmed that knockdown of PPARγ2 decreased HF butter induced hepatic TG accumulation (Fig. 5).

Deactivation of PPARγ2 affected expression of other genes. In HF butter-fed mice, ADRP mRNAs were decreased at 5 days after Ad-shPPARγ2 injection, but a decrease in CD36 mRNA did not reach significance (Table 4). A reduction in FAS, SCD1, and ACC1 mRNAs was observed at 5 days after injection without decreasing SREBP-1c mRNA, whereas these reductions did not reach significance at 1 day after injection. Interestingly, knockdown of PPARγ2 reduced mRNAs of CD68, a maker of macrophage and major inflammatory cytokines [monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α)] at 5 days after injection (Table 4). Reductions in CD68 and interleukin-1β (IL-1β) mRNAs were observed at 1 day after injection (Table 3). There were no significant differences in these mRNAs between the HF-diet groups.

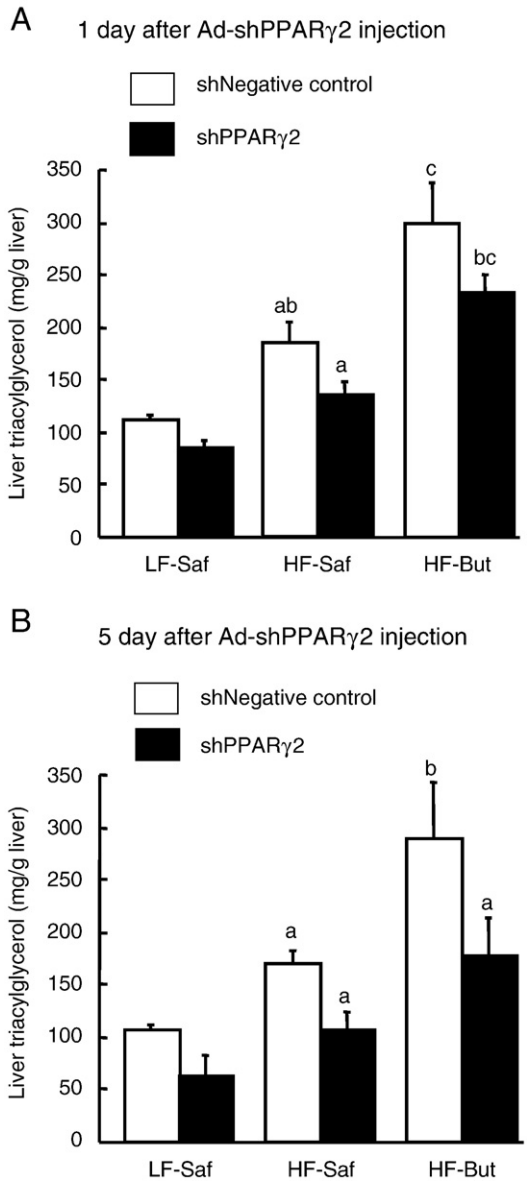

Fig. 4. Effect of PPARγ2 RNAi-mediated knockdown for 1 or 5 days on liver triacylglycerol concentration. Mice fed diets of LF safflower (LF-Saf), HF safflower oil (HF-Saf) and HF butter (HF-But) for 4 weeks were injected with Ad-shPPARγ2 or an Ad-Negative control and were killed 1 or 5 days later. The graphs show the percent triacylglycerol levels relative to those of Ad-Negative control-injected LF-fed mice. Values are means±SEM, $n=4$. Means without a common letter differ ($P<.05$). Two-way ANOVA P values are significant in effects of diet and interaction.

3.5. Overexpression of PPARγ2 in mouse liver increases liver TG with an increase in SREBP-1c mRNA

To examine whether PPARγ2 affects expression of lipogenic genes in vivo, we injected 2×10^9 pfu of recombinant virus containing either PPARγ2 or the control GFP cDNA into mice. This dose has been found to cause expression of the foreign gene in a majority of hepatocytes [33]. Five days after injection of Ad-PPARγ2 into mice, PPARγ2 mRNA increased by 85-fold (Table 5). This increase of PPARγ2 mRNA was sixfold larger than that in HF butter-fed mice at 10 weeks. The

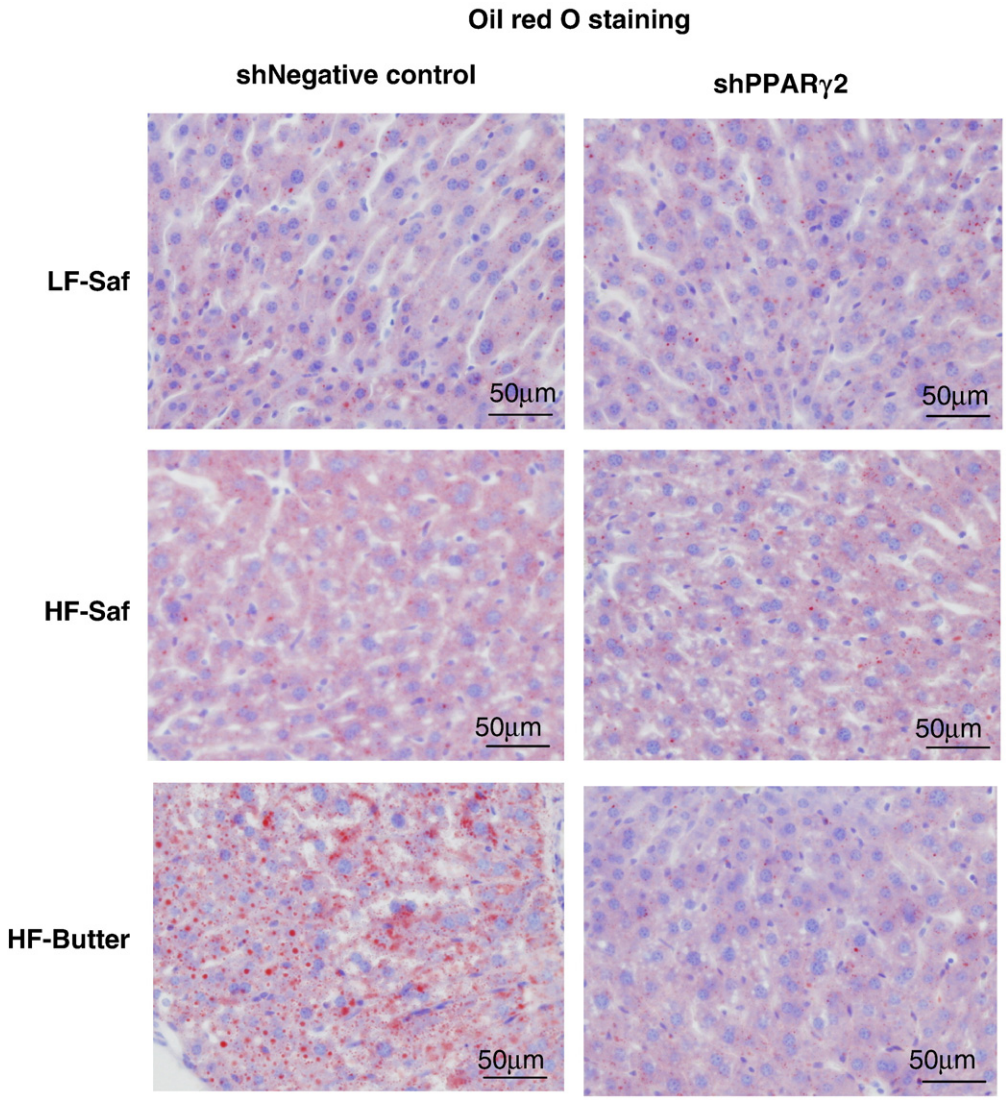

Fig. 5. Oil red O staining of liver sections from representative mice. Mice fed diets of LF safflower oil (LF-Saf), HF safflower oil (HF-Saf), or HF butter (HF-But) for 4 weeks were injected with Ad-shPPARγ2 or an Ad-Negative control and were killed 5 days later. Ad-shPPARγ2 markedly decreased hepatic triacylglycerol accumulation in HF butter-fed mice.

increased PPARγ2 protein leads to an increase in expression of the mRNAs of the PPARγ2 target genes, CD36 and ADRP, by 17- and 1.7-fold, respectively, and also an increase in expression of SREBP-1c and its target genes, FAS, SCD1 and ACC1, by 1.9-, 2.9-, 2.5- and 2.6-fold, respectively. As expected, liver TG concentration in Ad-PPARγ2-injected mice increased by 3.7-fold without increasing energy intake. Overexpressing PPARγ2 increased mRNAs of MCP-1 by 1.7-fold but did not affect levels of CD68, IL-1β, and TNF-α.

3.6. Western blot of PPARγ2

To examine whether altered expression of PPARγ2 mRNA affected its protein levels, we conducted Western blot analysis on the knockdown and overexpression experiments of PPARγ2 with an antibody that detects only PPARγ2 (but not PPARγ1). PPARγ2 protein (MW, 57 kDa) levels in mice fed a HF diet were greater than those in mice fed a LF diet (Fig. 6A and B). As expected, the increase in PPARγ2 protein levels in HF butter-fed mice (4.7±0.5-fold increase at 5 days after injection, n=4, relative to LF diet-fed mice) was larger than those in HF safflower oil-fed mice (2.7±0.1-fold increase at 5 days after injection, n=4, relative to LF diet-fed mice). Knockdown by

Table 5
Effect of Ad-PPARγ2 mRNAs 5 days after injection and the relation to lipid synthesis and inflammation and triacylglycerol in the liver

	Ad-GFP (control)	Ad-PPARγ2	P value
Liver triacylglycerol (mg/g liver)	46±8	172±36	.003
Energy intake (kJ/5 days/mouse)	277±2	270±4	.136
PPARγ and its target genes (mRNA)			
PPARγ1	100±8	97±11	.851
PPARγ2	100±33	8530±1580	<.001
CD36	100±12	1730±710	.029
ADRP	100±9	173±21	.007
SREBP-1c and its target genes (mRNA)			
SREBP-1c	100±8	190±28	.007
FAS	100±8	286±42	<.001
SCD1	100±21	254±25	<.001
ACC1	100±5	262±59	.013
Genes related to inflammation (mRNA)			
CD68	100±15	123±13	.270
MCP-1	100±15	166±22	.035
TNF-α	100±13	188±41	.083
IL-1β	100±16	74±17	.307

Mice fed a normal laboratory diet at 8 weeks of age were injected with Ad-PPARγ2 or an Ad-GFP (control) and were killed 5 days later. The percentages of mRNA levels relative to those of mice with Ad-GFP injection are shown. Values are means±S.E.M. (n=7 in Ad-GFP, n=6 in Ad-PPARγ2).

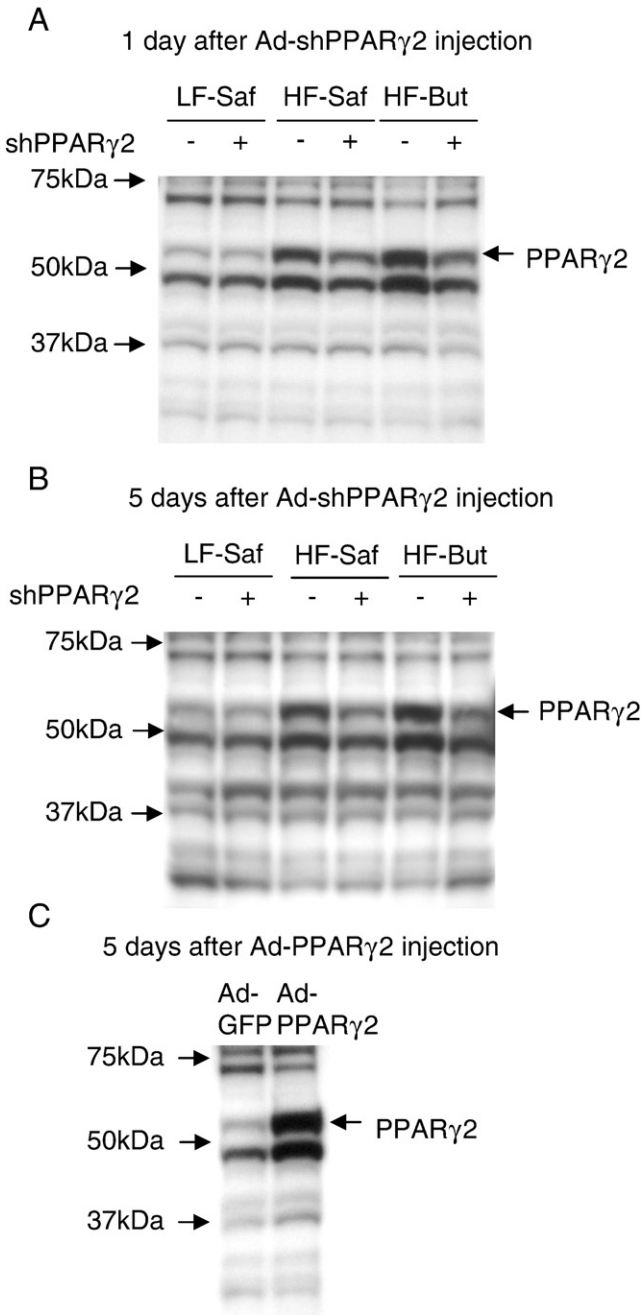

Fig. 6. Western blot analyses of PPARγ2. Mice fed LF safflower oil (LF-Saf), HF safflower oil (HF-Saf), or HF butter (HF-But) for 4 weeks were injected with Ad-shPPARγ2 or an Ad-Negative control and were killed 1 (A) and 5 (B) days later. Mice fed the standard mouse diet (CE2) at 8 weeks of age were injected with Ad-PPARγ2 or an Ad-GFP and killed 5 days later (C). Nuclear protein from liver was extracted and separated by SDS-PAGE (7.5% gel). Typical results of Western blot analyses of PPARγ are shown. Nonspecific bands are shown as markers of protein loading.

Ad-shPPARγ2 reduced PPARγ protein levels by 43%. Ad-PPARγ2-injected mice increased protein levels by 13-fold (Fig. 6C).

4. Discussion

Relative to a low saturated fatty acid oil (safflower oil) diet, we found in this study that an increased intake of saturated fatty acid-rich oil (butter) induces fatty liver in mice. Increased intake of safflower oil as well as butter induced obesity, suggesting that the development of fatty liver rather than obesity is more dependent on specific types of dietary fatty acids. Analysis of lipogenic gene expression showed that increased expressions of lipogenic genes (CD36, ADRP, FAS, SCD1, and GPAT), which are mediated by activation of PPARγ2 and SREBP-1c, contributed to fatty liver development in HF butter-fed mice. Increases in PPARγ2 and SREBP-1c mRNA expression were time dependent, with an initial increase in PPARγ2 observed, then an increase in SREBP-1 mRNA occurred, which was followed by attenuation of both mRNAs. The mechanism of this attenuation is not known at present, but some adaptive mechanisms to prevent further TG accumulation might occur at later stage in liver. At 8 weeks of feeding, serum insulin concentrations under fasting and feeding conditions were not altered in HF-fed mice, suggesting that high butter oil-induced fatty liver at early stages was not mediated by hyperinsulinemia.

Increases in PPARγ2 protein (or its mRNA) and expression of its target genes (CD36 and ADRP) were observed prior to the increase in SREBP-1c mRNA in mice fed a HF butter diet. Knockdown of PPARγ2 significantly decreased the liver TG content in mice fed a HF butter diet, without decreasing SREBP-1c mRNA expression. In mice fed HF-margarine, another diet that contains a large amount of saturated fatty acids (50% of total diet), fatty liver formation and an increase in mRNA expression of PPARγ2 and its target genes was observed (unpublished observation, T. Yamazaki, O. Ezaki). In addition, in mice fed HF-margarine, knockdown of PPARγ2 decreased the liver TG content by about 25%. These data suggested that an increase in PPARγ2 protein is an initial event that leads to fatty liver in mice fed saturated fat-rich oils.

Knockdown of PPARγ2 protein reduced not only the expression of its target genes (CD36 and ADRP) but also lipogenic genes (FAS, SCD1, ACC1) with no alterations of SREBP-1c mRNA. Similar changes have been reported in animal models of fatty liver [6,7,34]. Liver-specific PPARγ knockout mouse on a *ob*/*ob* background showed reduced liver TG contents with reductions in FAS, SCD1, and ACC1 mRNAs and no alteration of SREBP-1c mRNA [7]. In another mouse model of fatty liver, treatment of apoB/BATless mice for 4 weeks with injections of PPARγ antisense oligonucleotide (twice a week) resulted in reduction of PPARγ2 protein, liver TG, and mRNA levels of FAS and ACC1 without a reduction of SREBP-1c mRNA [34]. It remains unclear whether PPARγ directly or indirectly regulates the transcription of these genes. However, it was shown that SCD1 is regulated by PPARα and has the peroxisome proliferator response element-like (PPRE-like) sequence in its promoter [35]. These data suggest that PPARγ might directly bind this PPRE-like motif and increase SCD1 expression. In contrast, overexpression of PPARγ2 in liver increased liver TG with increased expression of the mRNA of SREBP-1c. In agreement with these findings, a hepatic cell line stably expressing PPARγ2 showed increased mRNA expression of FAS and ACC with an increase in SREBP-1c mRNA and protein [36]. Therefore, it is also conceivable that PPARγ2 activation increased SREBP-1c mRNA directly or indirectly and that it stimulates lipogenesis under some metabolic conditions.

By contrast, thiazolidinediones (TZDs), which are PPARγ activators, can improve insulin resistance and hepatic steatosis in patients with nonalcoholic steatohepatitis [37]. Neither liver PPARγ nor muscle PPARγ was essential for the beneficial effects of rosiglitazone to occur, suggesting that TZDs normally act via adipose tissues [6]. TZDs increase expression of adiponectin in the adipose tissues and cause a mild increase in its serum concentration [38], which alleviates alcoholic and nonalcoholic fatty liver disease [39]. Recently, it was reported that adiponectin suppresses hepatic SREBP-1c mRNA in an adipoR1/LKB1/AMPK dependent pathway [40]. Therefore, TZDs may improve fatty liver indirectly by increasing adiponectin concentrations through adipose tissues, which overcome PPARγ activation in the liver. The HF butter diet did not increase blood adiponectin concentrations at 10 week's feeding (1085±34, 839±67, 943±67

pmol/L in mice fed LF safflower oil, HF safflower oil, and HF butter, respectively, $n=4$). Therefore, diets high in saturated fat may fail to prevent fatty liver formation.

Interestingly, knockdown of PPARγ2 reduced mRNAs of CD68, a marker of macrophage and major inflammatory cytokines (MCP-1, IL-1β and TNF-α) in liver. In contrast, overexpression of PPARγ2 increased mRNAs of MCP-1 by 1.7-fold. It is not clear that alterations of inflammatory cytokine expression in liver were causes or results of alterations in liver TG concentration.

To elucidate the mechanism of increased PPARγ expression in mice fed a HF butter diet, the expression of several genes that regulate PPARγ expression was investigated. PPARγ expression is suppressed by the transcription factor hairy/enhancer of split 1 (HES-1), and HES-1 expression is increased by the cAMP response element-binding protein (CREB); this pathway is involved in the inhibition of the hepatic TG synthesis observed during fasting [9]. The PPARγ promoter contains the conserved binding site of the liver X receptor (LXR) and is transactivated by the expression of LXRα [41]. The activation of PXR up-regulates PPARγ and CD36 [42]. However, expression of HES-1, CREB, LXRα, and PXR mRNA was not altered in HF butter-fed mice (data not shown). Insulin increases the expression of PPARγ2 mRNA in human adipocytes and in mouse hepatocytes [43,44]. However, insulin concentrations did not differ in the HF diet-fed groups (Table 2). At present, the mechanisms for increased PPARγ2 mRNA expression in mice fed a diet high in saturated fatty acids are not clear.

In this study, total fat intake in the HF diet was 40% fat calories, similar to that in human diets. In humans, ADRP was strongly expressed in hepatocytes from patients with fatty liver when compared with normal liver samples [17], suggesting that PPARγ2 activation might be occurred in fatty liver in humans. A large intake of saturated fatty acid rich oils (butter or margarine) may induce hepatic steatosis in humans, possibly by activating PPARγ2. However, inactivation or decreasing of liver PPARγ2 protein may improve fatty liver under a HF saturated fat diet.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.04.009.

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