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Dietary β-conglycinin prevents fatty liver induced by a high-fat diet by a decrease in peroxisome proliferator-activated receptor γ2 protein☆,☆☆

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Abstract

Diets high in sucrose/fructose or fat can result in hepatic steatosis (fatty liver). Mice fed a high-fat diet, especially that of saturated-fat-rich oil, develop fatty liver with an increase in peroxisome proliferator-activated receptor (PPAR) γ2 protein in liver. The fatty liver induced by a high-fat diet is improved by knockdown of liver PPARγ2. In this study, we investigated whether β-conglycinin (a major protein of soy protein) could reduce PPARγ2 protein and prevent high-fat-diet-induced fatty liver in ddY mice. Mice were fed a high-starch diet (70 energy% [en%] starch) plus 20% (wt/wt) sucrose in their drinking water or a high-safflower-oil diet (60 en%) or a high-butter diet (60 en%) for 11 weeks, by which fatty liver is developed. As a control, mice were fed a high-starch diet with drinking water. Either β-conglycinin or casein (control) was given as dietary protein. β-Conglycinin supplementation completely prevented fatty liver induced by each type of diet, along with a reduction in adipose tissue weight. β-Conglycinin decreased sterol regulatory element-binding protein (SREBP)-1c and carbohydrate response element-binding protein (ChREBP) messenger RNAs (mRNAs) in sucrose-supplemented mice, whereas it decreased PPARγ2 mRNA (and its target genes CD36 and FSP27), but did not decrease SREBP-1c and ChREBP mRNAs, in mice fed a high-fat diet. β-Conglycinin decreased PPARγ2 protein and liver triglyceride (TG) concentration in a dose-dependent manner in mice fed a high-butter diet; a significant decrease in liver TG concentration was observed at a concentration of 15 en%. In conclusion, β-conglycinin effectively prevents fatty liver induced by a high-fat diet through a decrease in liver PPARγ2 protein.

Keywords: Soy protein; Steatosis; Sterol regulatory element-binding protein; Peroxisome proliferator-activated receptor gamma; Butter

1. Introduction

The prevalence of obesity in Western societies, which is associated with an increased consumption of fat or carbohydrate, has increased dramatically. Among the consequences of obesity are the emerging epidemics of hepatic steatosis and nonalcoholic fatty liver disease (NAFLD) [1]. Nonalcoholic steatohepatitis (NASH), an advanced form in NAFLD, is characterized by macrovesicular steatosis and parenchymal inflammation [2]. The prevalence of NAFLD ranges from 10% to 24% of the general population, whereas NASH affects about 3% of the lean population and almost half of morbidly obese people [3].

The accumulated hepatic lipids in patients with NAFLD include plasma nonesterified fatty acids (NEFAs) from adipose tissue, fatty acids produced in the liver via *de novo* lipogenesis (DNL) and dietary fatty acids, which enter the liver via spillover of NEFA derived from the lipolysis of chylomicron and via hepatic uptake of chylomicron remnants. Analysis of multiple stable isotopes in patients with NAFLD has revealed that of the triglyceride (TG) in the liver, 59% is derived from NEFAs, 26% is from DNL and 15% is from dietary fatty acids [4]. Roughly one fourth of fatty acids in the liver is produced from *de novo* 2-carbon precursors derived from glucose, fructose and amino acids. Dietary fat also contributes significantly to liver TG storage pools. Transcription factors, including sterol regulatory element-binding protein (SREBP)-1c, carbohydrate response element-binding protein (ChREBP), peroxisome proliferator-activated receptor (PPAR)α and PPARγ, that regulate liver TG concentrations contribute to fatty liver induced by dietary manipulations [5–8].

The C57BL/6J inbred mouse strain has been used for studies of obesity and diabetes because of its susceptibility to these diseases in response to a high-fat (HF) diet [9,10]. C57BL/6J mice also develop fatty liver in response to an HF diet [11–13]. However, these mice are resistant to sucrose/fructose-induced fatty liver because they possess adenine −468 bp from the putative 5′ end of the SREBP-1c gene [14]. The ddY mice possess guanine −468 bp in the SREBP-1c promoter and develop hepatic steatosis when fed either sucrose supplementation or

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Table 1
Dietary composition of experimental diets

β-Conglycinin (20 en%)	St		Suc		Safflower oil		Butter	
	−	+	−	+	−	+	−	+
	g/100 g							
Safflower oil	3.9	3.9	3.9	3.9	32.6	32.6		
Butter							32.6	32.6
Casein	18.0		18.0		25.5		25.5	
β-Conglycinin		18.9		18.9		26.8		26.8
α-Starch	68.3	67.4	68.3	67.4	27.7	26.4	27.7	26.4
Vitamin mix (AIN-93)	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5
Mineral mix (AIN-93)	3.5	3.5	3.5	3.5	5.1	5.1	5.1	5.1
Cellulose powder	5.0	5.0	5.0	5.0	7.3	7.3	7.3	7.3
L-Cystine	0.3	0.3	0.3	0.3	0.4	0.4	0.4	0.4

St, high-starch diet; Suc, high-starch diet with 20% (wt/wt) sucrose drink. β-Conglycinin contains approximately 5% carbohydrate per protein as glucose.

an HF diet. In our previous study, fish oil supplementation could prevent sucrose-induced fatty liver but did not prevent HF-diet-induced fatty liver in ddY mice [15]. This was due to the difference in mechanisms of dietary-induced fatty liver formation. Fish oil decreases liver SREBP-1c activity, which counteracts the increase of SREBP-1c messenger RNA (mRNA) in response to sucrose overconsumption. Fish oil does not decrease liver PPARγ mRNA that is increased in response to HF diets.

In the present study, we sought to find a dietary compound to prevent HF-diet-induced fatty liver. Soy protein could prevent fat accumulation [16]. We therefore investigated β-conglycinin, a major component of soy protein, to determine if this protein might effectively prevent HF-diet-induced fatty liver and the methods by which it might do so.

2. Materials and methods

2.1. Animals

Six-week-old male ddY mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) 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 the room was 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 National Institutes of Health's (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 (No. 0606).

2.2. Diet

At 7 weeks of age, ddY mice were assigned to one of several groups (n=5–9 in each group). To examine the effects of β-conglycinin on fatty liver induced by sucrose, high-safflower-oil, and high-butter diets, four groups were created: control mice were fed a high-starch diet, sucrose-supplemented mice were fed a high-starch diet plus 20% sucrose (wt/wt) in their drinking water, high-safflower-oil-fed mice were given 60 energy% (en%) safflower oil and high-butter-fed mice were given 60 en% butter. To examine the effects of β-conglycinin, all casein in the diet of each group was replaced by β-conglycinin. Detailed compositions of the experimental diets are listed in Table 1. To examine the dose-dependency of β-conglycinin in mice fed a high-butter-oil diet, 0, 5, 10, 15 and 20 en% of β-conglycinin were given in replacement of casein. Duration of the dietary manipulations was 11 weeks in all experiments. Fatty acid compositions of dietary oils were measured by gas–liquid chromatography. Safflower oil (high-oleic type) contained 45% (wt/wt) oleic acid (18:1n-9) and 46% linoleic acid (18:2n-6), and butter (salt-free type) contained 71% saturated fatty acid including 12% myristic acid (14:0), 33% palmitic acid (16:0) and stearic acid 11% (18:0). Diet preparations were similar to those of our previous studies [15]. Butter was purchased from Snow Brand Milk Corp. (Hokkaido, Japan). Safflower oil and β-conglycinin were kindly provided by Benibana Food (Tokyo, Japan) and Fuji Oil Co. (Osaka, Japan), respectively. β-Conglycinin was prepared by treatment of soybean protein extract with phytase [17]. Consumption of food was measured daily, and intake of sucrose water was measured weekly. Food intake per day was estimated by subtracting the food weight of that day from the initial food weight of the previous day. Average energy intakes during total experimental periods in each group of mice were calculated with these data.

2.3. Quantitative reverse transcriptase polymerase chain reaction

Mice were killed, and livers were isolated for RNA preparation in the morning from 3-h fasted animals to avoid acute effects of food intake. RNA was extracted with TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA isolated from liver was reverse transcribed with ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) with random hexamers. The resulting complementary DNA was polymerase chain reaction (PCR) amplified in the 96-well format with SYBR Green PCR Master Mix and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression levels of test genes were normalized to those of an endogenous control, acidic ribosomal phosphoprotein P0 (36B4). The primers used for quantitative

Table 2
Body and tissue weights and total energy intake of mice after 11 weeks on experimental diets

	β-Conglycinin	St	Suc	Safflower oil	Butter	Two-way ANOVA P value		
						Diet	β-Conglycinin	Diet×β-conglycinin
n	−	8	8	8	8			
	+	8	8	8	8			
Weight (g)								
BW at start	−	28.3±0.8	28.3±0.8	28.4±1.2	28.6±0.9			
	+	28.4±1.4	28.4±1.4	28.6±1.4	28.5±1.4	.998	.946	.999
BW	−	45.3±1.9^{a,b}	53.4±1.0^{d}	53.7±2.1^{d,e}	59.8±2.2^{f}			
	+	36.6±1.7^{c}	41.0±1.2^{a,c}	47.7±2.2^{b,e}	46.1±1.7^{b}	<.001	<.001	.162
Liver	−	1.66±0.18^{a,b}	1.98±0.07^{b,c}	2.11±0.08^{b}	2.56±0.25^{d}			
	+	1.54±0.06^{a}	1.62±0.08^{a}	1.83±0.06^{a,b,c}	1.97±0.08^{b,c}	<.001	<.001	.308
Epididymal WAT	−	1.51±0.27^{a,b}	2.33±0.17^{b,d}	2.77±0.24^{d}	2.67±0.17^{d}			
	+	0.74±0.11^{c}	1.23±0.08^{a,c}	2.15±0.31^{b,d}	1.80±0.29^{a,b}	<.001	<.001	.716
Retroperitoneal WAT	−	0.38±0.07^{a,b}	0.52±0.07^{a,d}	0.60±0.07^{d}	0.58±0.07^{a,d}			
	+	0.16±0.03^{c}	0.27±0.03^{b,c}	0.51±0.05^{a,b}	0.41±0.07^{a,b}	<.001	<.001	.588
Mesenteric WAT	−	0.69±0.11^{a}	0.96±0.10^{c,d}	0.99±0.12^{c,e}	1.14±0.10^{de}			
	+	0.30±0.05^{b}	0.48±0.05^{a}	0.77±0.13^{a,c}	0.67±0.10^{a}	<.001	<.001	.553
Subcutaneous WAT	−	0.81±0.18^{a}	1.25±0.14^{c,d}	1.35±0.15^{c}	1.91±0.23^{e}			
	+	0.34±0.07^{b}	0.48±0.04^{a,b}	1.08±0.13^{a,c}	0.90±0.14^{a,d}	<.001	<.001	.085
Total energy intake (MJ/mouse)	−	5.51±0.03^{a}	6.73±0.12^{b}	7.64±0.09^{c}	7.59±0.13^{d}			
	+	5.52±0.03^{a}	6.93±0.11^{b}	7.45±0.08^{c}	7.71±0.11^{d}	<.001	.616	.184

Values are mean±S.E.M. Means without a common letter^{a,b,c,d,e} differ (P<.05). BW, body weight; St, high-starch diet; Suc, high-starch diet with 20% (wt/wt) sucrose drink; WAT, white adipose tissue.

reverse transcriptase PCR are listed in our previous report [15]. The other primers were as follows: aP2 5′, TTCGATGAAATCACCGCAGA and 3′, AGGGCCCCGCCATCT. FSP27 5′, CTGGAGGAAGATGGCACAATCGTG and 3′, CAGCCAATAAAGTCCTGAGGGTTCA.

2.4. Liver lipid analysis and hepatic histology

Liver lipids were measured by enzymatic colorimetry as described previously [15]. Frozen sections of formalin-fixed mouse liver were stained with oil red O with the use of standard techniques.

2.5. Serum chemistries

Serum was obtained at two points, after 24 h of fasting and 3 h after refeeding (postprandial). Serum glucose was measured on an Ascensia autoanalyzer (Bayer Medical, Ltd., Tokyo, Japan). Serum TG and NEFA levels were assayed by enzymatic colorimetry with TG E and NEFA C test kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum insulin was determined with a mouse insulin enzyme-linked immunosorbent assay kit (Morinaga, Kanagawa, Japan). In a time-course study, TG was measured at five points: after 24 h of fasting and at 1, 2, 3 and 5 h after refeeding.

2.6. 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 (7.5% gel) was electrophoretically transferred onto Clear Blot Membrane-P (ATTO, Tokyo, Japan) and immunoblotted with specific primary antibodies: PPARγ2 (PA1-824, 1:500 dilution, Thermo Scientific, Rockford, IL, USA) or SREBP-1 (IgG-2A4, a supernatant of hybridoma cell line CRL 2121, 1:1000 dilution, American Tissue Culture Collection, Manassas, VA, USA). Peroxidase-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology, Inc., 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 with NIH Image software (NIH, Bethesda, MD, USA).

2.7. Measurement of oxygen consumption and carbon dioxide production

Open-circuit indirect calorimetry was performed with an O_2/CO_2 metabolism measuring system for small animals (MK-5000RQ; Muromachi Kikai, Tokyo, Japan). The system monitored VO_2 and VCO_2 at 3-min intervals and calculated the respiratory quotient (RQ) ratio (VCO_2/VO_2). Spontaneous motor activity was measured using the Supermex infrared sensor (Muromachi Kikai). Measurements were performed for the dark (from 19:00 to 07:00) or light (from 07:00 to 16:30) period under *ad libitum* feeding conditions. The energy production rate was calculated with the formula used by Ferrannini [18] where the rate of energy production (kcal/min)=3.91 VO_2+1.10 VCO_2−3.34*N*, where *N* is the rate of urinary nitrogen excretion used to estimate protein oxidation. However, considering that only a small portion of resting and exercise energy expenditure arises from protein oxidation [19], the contributions of protein oxidation were neglected.

2.8. Statistical analysis

Values are shown as mean±S.E.M. One-way analysis of variance (ANOVA) was used for comparison among multiple groups. Two-way ANOVA was used to examine the two main effects of diet (starch, sucrose, safflower oil and butter), β-conglycinin supplementation and their interaction. Statistical significance of the interaction of diet and β-conglycinin indicated that the effects of β-conglycinin on the four diet types were statistically different. When differences were significant with respect to main or interaction effects, each group was compared with the others by Fisher's protected least significant difference test (StatView 5.0; Abacus Concepts, Inc., Berkeley, CA, USA). Postprandial TG concentration was plotted with respect to time and compared by two-way repeated-measures ANOVA (StatView 5.0). Statistical significance was set at $P<.05$.

3. Results

3.1. β-Conglycinin reduces liver TG concentration in sucrose-supplemented, high-safflower-oil and high-butter diets

To examine the effect of β-conglycinin on fatty liver induced by different type of diets, β-conglycinin (20 en%) was given to control (high-starch), sucrose-supplemented, high-safflower-oil-fed and high-butter-fed mice. In the absence of β-conglycinin, as reported in our previous study [15], all three groups of mice showed larger body weights and liver TG concentrations after 11 weeks of feeding than did control mice (Table 2, Fig. 1A). Among dietary groups, mice fed a high-butter diet showed the most profound liver TG accumulation. β-Conglycinin reduced liver TG concentration in all four groups of mice at 11 weeks; however, the reduction in liver TG in control mice did not reach significance (Fig. 1A). Oil red O staining confirmed hepatic TG accumulation in sucrose-supplemented, high-safflower-oil-fed and high-butter-fed mice and also confirmed that β-conglycinin decreased dietary-induced hepatic TG accumulation in

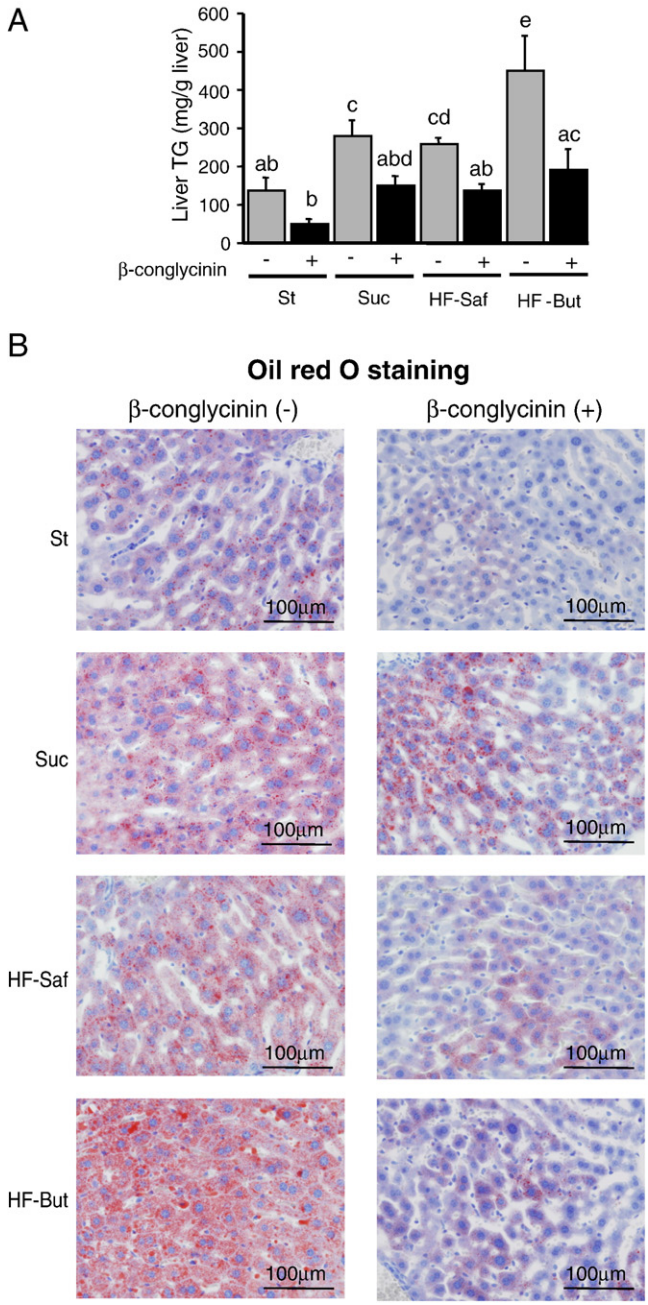

Fig. 1. β-Conglycinin prevents sucrose (Suc)-, high-safflower-oil (HF-Saf)- and high-butter (HF-But)-induced TG accumulation. The experimental diet including 20 en% casein (control) or β-conglycinin was given to mice for 11 weeks. Hepatic lipids were extracted, and TG concentrations were measured (A). Oil red O staining of liver sections from representative mice (B). Values are mean±S.E.M. (n=5–9). Means without a common letter differ ($P<.05$). Gray columns show liver TG concentrations in mice fed a casein diet, and black columns show concentrations in mice fed β-conglycinin. Two-way ANOVA P values are significant in regard to effects of diet and interaction. St, high-starch diet.

these three groups (Fig. 1B). Mean energy intakes during the experimental periods did not differ in the presence of β-conglycinin (Table 2). Two-way ANOVA analysis revealed that β-conglycinin also significantly reduced body weight and liver and white adipose tissue (WAT) weights in several body locations (Table 2).

3.2. β-Conglycinin prevents increases in SREBP-1c and ChREBP mRNAs in a sucrose-supplemented diet but prevents increase in PPARγ2 mRNA in high-safflower-oil and high-butter diets

The mechanisms underlying the prevention of fatty liver in mice supplemented with β-conglycinin were elucidated by hepatic gene expression. Sucrose supplementation increased the mRNAs of transcription factors SREBP-1c and ChREBP (although this increase did not reach significance), whereas HF diets increased PPARγ2 mRNA, relative to the control diet (Table 3). As observed in our previous study [15], the increase in PPARγ2 mRNA was much larger in mice fed a high-butter diet than in mice fed a high-safflower-oil diet. Target genes of SREPB-1c, such as fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1) and acetyl-CoA carboxylase 1 (ACC1) mRNAs, and target genes of ChREBP, such as liver-type pyruvate kinase (LPK), ACC1 and FAS mRNAs, were increased in sucrose-supplemented mice. A target gene of PPARγ2, fatty acid translocase (CD36) mRNA, was increased in high-butter-fed mice. There were no significant differences in mRNAs of PPARα and their target genes, medium-chain acyl-CoA dehydrogenase (MCAD) and acyl-CoA oxidase (ACO) mRNAs, between dietary groups; however, mRNA in carnitine palmitoyltransferase (CPT)1 was significantly lower in high-butter-fed mice than in control mice.

β-Conglycinin significantly prevented increases in mRNAs of SREBP-1c and ChREBP and lipogenic genes, such as FAS, SCD1, ACC1 and LPK, in sucrose-supplemented mice. β-Conglycinin decreased mRNAs of SREBP-1c, ChREBP, FAS, SCD1, ACC1 and LPK in control (high-starch) mice, although the decreases were not significant. In both groups of HF-diet-fed mice, β-conglycinin significantly prevented increases in mRNAs of PPARγ2 but did not affect SREBP-1c and ChREBP mRNAs. β-Conglycinin also decreased mRNA of CD36 (a target gene of PPARγ2) in high-butter-fed mice. β-Conglycinin affected target genes of PPARα differently between dietary groups. β-Conglycinin significantly decreased mRNAs of MCAD (and also decreased mRNAs of PPARα, ACO and CPT1, but not significantly) in sucrose-supplemented mice; however, β-conglycinin increased mRNA of MCAD in high-safflower-oil-fed mice. Alterations in PPARα and its target genes did not contribute to the prevention of fatty liver by β-conglycinin supplementation because a mild increase in MCAD mRNA by β-conglycinin was observed only in mice fed the high-safflower-oil diet. These data indicated that decreases in SREBP-1c and ChREBP mRNAs in sucrose-supplemented mice and prevention of an increase in PPARγ2 mRNA in high-safflower-oil-fed and high-butter-fed mice, which was observed in β-conglycinin supplementation, may contribute to the anti-fatty liver effects of β-conglycinin.

3.3. Postprandial TG concentrations are increased in mice fed safflower oil or butter, and β-conglycinin partially prevents postprandial TG (3 h after refeeding) increases in mice fed safflower oil

Increased plasma concentrations of glucose, postprandial TG, NEFA and insulin may promote hepatic steatosis [20]. To examine substrate

Table 3
Hepatic gene expression profile related to liver TG of mice after 11 weeks on experimental diets

	β-Conglycinin	St	Suc	Safflower oil	Butter	Two-way ANOVA *P* value		
						Diet	β-Conglycinin	Diet×β-conglycinin
n	−	8	8	8	8			
	+	8	8	8	8			
SREBP-1c and its target genes								
SREBP-1c	−	100±23^{a,b}	133±28^{b}	92±18^{a,b}	106±19^{a,b}			
	+	53±7^{a}	72±14^{a}	97±13^{a,b}	73±16^{a}	.662	.026	.432
FAS	−	100±17^{a}	293±58^{c}	84±23^{a,b}	72±15^{a,b}			
	+	42±10^{a,b}	56±11^{a,b}	40±8^{a,b}	23±2^{b}	<.001	<.001	<.001
SCD1	−	100±24^{a}	268±57^{c}	25±5^{b}	46±5^{a,b}			
	+	54±10^{a,b}	28±3^{b}	12±5^{b}	17±2^{b}	<.001	<.001	<.001
ACC1	−	100±10^{a}	242±39^{c}	70±11^{a,b}	46±5^{b}			
	+	55±8^{a,b}	70±12^{a,b}	38±5^{b}	28±1^{b}	<.001	<.001	<.001
ChREBP and its target gene								
ChREBP	−	100±21^{a,b}	140±27^{b}	58±8^{c}	60±4^{a,c}			
	+	66±12^{ac}	60±10^{ac}	82±12^{ac}	86±8^{a,c}	.179	.124	.001
LPK	−	100±36^{a,b}	295±37^{c}	48±12^{a,b}	52±12^{a,b}			
	+	60±16^{a,b}	104±15^{a}	49±8^{a,b}	39±6^{b}	<.001	<.001	<.001
PPARγ and its target gene								
PPARγ1	−	100±41	238±80	218±66	184±51			
	+	187±39	254±67	105±24	107±39	.189	.569	.250
PPARγ2	−	100±30^{a,b}	190±63^{a,b}	293±101^{a,c}	411±136^{c}			
	+	140±31^{a,b}	162±64^{a,b}	60±24^{b}	103±21^{a,b}	.288	.011	.053
CD36	−	100±16^{a}	158±24^{a,b,c}	168±28^{a,c}	198±66^{c}			
	+	135±34^{a,b,c}	70±11^{b}	119±16^{a,b,c}	94±12^{a,b}	.649	.027	.132
PPARα and its target genes								
PPARα	−	100±12	85±15	88±8	89±8			
	+	84±15	68±9	103±6	99±7	.255	.773	.311
MCAD	−	100±12^{a,b}	109±11^{a}	116±12^{a}	114±11^{a}			
	+	118±20^{a}	61±10^{b}	185±30^{c}	106±14^{a,b}	.002	.517	.006
ACO	−	100±13^{a,b}	93±10^{a,b}	126±15^{b}	99±10^{a,b}			
	+	90±20^{a,b}	65±7^{a}	130±10^{b}	112±15^{b}	.004	.559	.433
CPT1	−	100±17^{a,b}	89±13^{a,c}	100±10^{a,d}	57±14^{cd}			
	+	93±28^{a,c}	48±5^{c}	105±8^{a}	55±12^{b,c}	.007	.288	.436

Values are mean±S.E.M.. Means without a common letter^{a,b,c,d} differ (P<.05). St, high-starch diet; WAT, white adipose tissue.

and hormonal contributions to liver TG concentration, serum glucose, NEFA, TG and insulin concentrations at two points, after 24 h of fasting (fasting) and 3 h after refeeding (postprandial) at day 1 or day 56 (8 weeks) after the initiation of feeding experiments, were measured. The day-1 measurement aimed to examine the acute effects of diet and β-conglycinin, whereas the day-56 measurement examined both the acute and chronic effects of diet and β-conglycinin.

In the absence of β-conglycinin, as expected, control (high-starch) and sucrose-supplemented mice showed a higher postprandial glucose concentration than did HF-diet-fed mice at days 1 and 56 (Fig. 2). At day 56, fasting glucose concentrations were slightly higher in HF-diet fed mice than in control and sucrose-supplemented mice. Blood NEFA concentrations were markedly suppressed after refeeding in control and sucrose-supplemented mice, whereas there were no significant differences between fasting and postprandial NEFA concentrations in HF-diet-fed mice. Increased postprandial NEFA concentrations in HF-diet-fed mice might be due to spillover of the increased lipolysis of chylomicrons. At day 56, fasting NEFA concentrations were higher in sucrose-supplemented mice than in HF-diet-fed mice, suggesting increased lipolysis in very low density lipoprotein (VLDL)–TG. At day 1, as expected, postprandial TG concentrations were markedly increased in HF-diet-fed mice, whereas there were no marked differences between fasting and postprandial TG concentrations in control and sucrose-supplemented mice. At day 56, fasting TG (VLDL) concentrations were higher in sucrose-supplemented mice than in control and HF-diet-fed mice. At day 1, postprandial insulin concentrations were lower in HF-diet-fed mice than in sucrose-supplemented and control mice, as reflected by a decrease in glucose concentration. These data suggest that in sucrose-supplemented mice, increased *de novo* TG synthesis in the liver may lead to a fasting TG (VLDL) concentration, whereas in HF-diet-fed mice, increased entry of dietary fat or its metabolite (NEFA) into the liver might be a cause of fatty liver, as indicated in human studies [4].

There were no significant effects of β-conglycinin on glucose and insulin concentrations in each diet group at day 1; however, β-conglycinin lowered the fasting glucose concentration at day 56

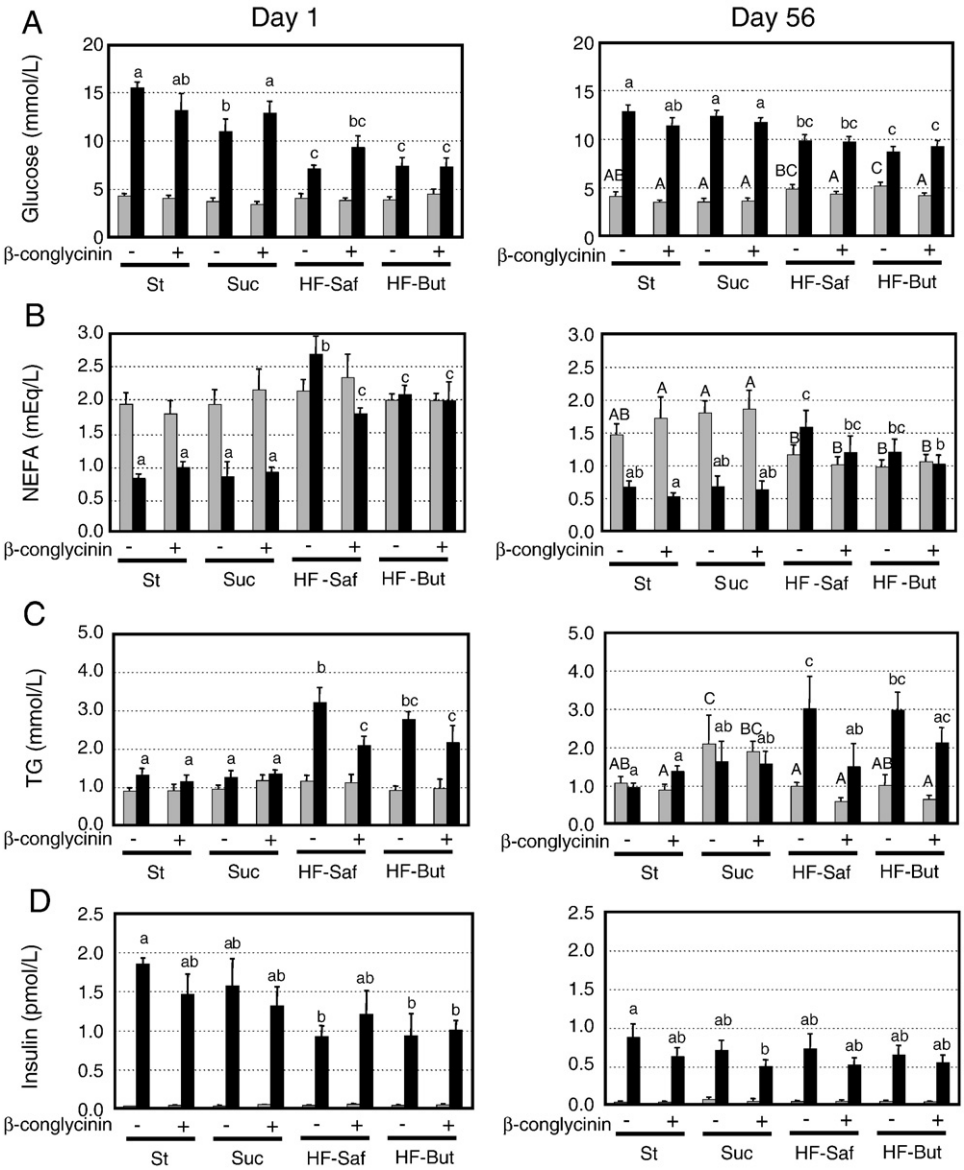

Fig. 2. Serum analysis of glucose (A), NEFA (B), TG (C) and insulin (D) in mice at day 1 (acute effects) and day 56 (chronic effects) after the initiation of experimental diets. Values are mean±S.E.M. (n=8). Means without a common letter differ (P<.05). Gray columns show concentrations in serum obtained from 24-h-fasted mice, and black columns show concentrations in serum obtained from animals 3 h after refeeding. St, high-starch diet; Suc, sucrose-supplemented diet; HF-Saf, high-safflower oil diet; HF-But, high-butter diet.

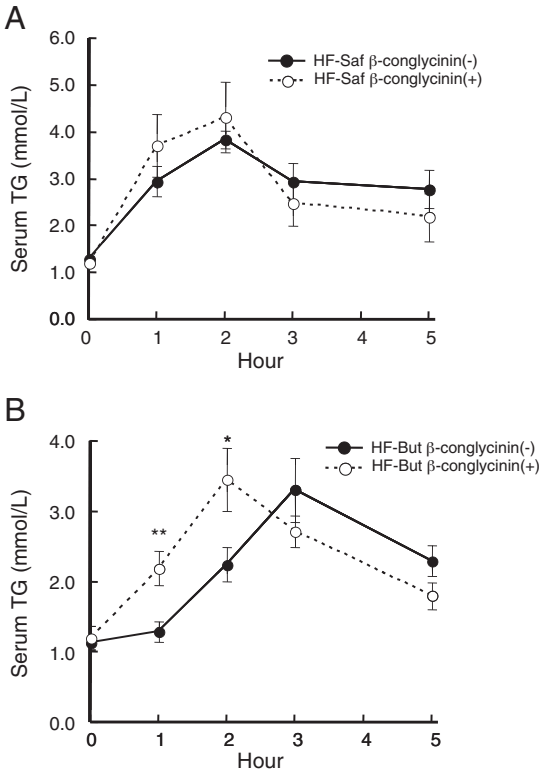

Fig. 3. Time course of postprandial TG concentrations after an HF diet with or without β-conglycinin at day 1. The TG concentration was measured at 0, 1, 2, 3 and 5 h after the initiation of a high-safflower-oil (A) or high-butter (B) diet in 24-h-fasted mice at day 1. Mice were fed with either casein or β-conglycinin. Values are mean±S.E.M. (n=10). Repeated ANOVA P values in panels A and B are 0.339 and 0.0002, respectively. *P<.05, **P<.01 vs. no β-conglycinin.

in mice fed an HF diet. β-Conglycinin significantly lowered postprandial TG concentrations in high-safflower-oil-fed mice at days 1 and 56; however, it did not alter these concentrations in the other diet groups.

3.4. β-Conglycinin accelerated appearance of postprandial TG in mice fed an HF diet

To examine in detail the effects of β-conglycinin (20 en%) on postprandial TG concentration in mice fed an HF diet, TG concentration was measured at 0 (24 h of fasting), 1, 2, 3 and 5 h after the initiation of high-safflower-oil or high-butter feeding at day 1. β-Conglycinin accelerated an initial increase in blood TG concentration (at 1 and 2 h) but decreased TG concentration later (at 3 and 5 h), although the differences were not significant in mice fed a high-safflower-oil diet (Fig. 3). Therefore, total increases in postprandial TG were not altered by the presence of β-conglycinin.

3.5. Dose-dependent effects of β-conglycinin on reduction of liver TG

To examine the dose-dependency of β-conglycinin-mediated reduction of liver TG in mice fed HF diets, mice were fed a high-butter diet with increasing amounts of β-conglycinin from 0 to 20 en% in 5-en% increments, replacing casein, for 11 weeks, and their phenotypes were examined. The reduction of weights in epididymal, retroperitoneal, mesenteric and subcutaneous WAT was dose-dependent, and a significant reduction was observed in epididymal fat at a β-conglycinin dose of 10 en% with no alterations of energy intake (Table 4). The reduction of liver TG concentration was also dose-dependent, and a significant reduction was observed at a β-conglycinin dose of 15 en% (Fig. 4A). Decreases in PPARγ2 mRNA and proteins were also dose-dependent (Fig. 4B, C). In mRNAs of several PPARγ2 target genes (CD36, adipose differentiation-related protein [ADRP], FSP27 and aP2), β-conglycinin decreased mRNAs of FSP27 and CD36 in a dose-dependent manner, whereas only a high concentration of β-conglycinin decreased mRNAs of ADRP and aP2 (Fig. 5). Overexpression of CD36, ADRP or FSP27 in the liver leads to increased liver TG concentration [21–23], suggesting that decreased expression of these PPARγ2 target genes is responsible for a reduction of liver TG by β-conglycinin. β-Conglycinin-induced decrease in PPARγ2 is also accompanied by a decrease in mRNA of lipogenic genes, FAS, SCD1, ACC1 and acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT), without a decrease in SREBP-1c mRNA and its mature protein (Fig. 6).

3.6. Energy production and physical activity levels in mice fed β-conglycinin

β-Conglycinin-supplemented mice showed a decrease in fat accumulation in liver and adipose tissues without significant changes in food intake and lipid absorption. To examine whether energy production and substrate utilization were altered in β-conglycinin supplementation, control (casein only) and 20 en% β-conglycinin-supplemented mice were fed *ad libitum* under a high-butter diet, and their oxygen consumption was measured and RQ ratio was calculated at 2 and 8 weeks after the initiation of the experimental diet (Table 5). To account for the effects of body weight on energy production, two different times were examined. At 2 weeks, there was no significant change in body weights, whereas at 8 weeks, body weights in mice fed β-conglycinin were significantly less than those in control mice.

Table 4
Dose-dependent effects of β-conglycinin on body and tissue weights and total energy intake of mice after 11 weeks on a high-butter diet

β-Conglycinin (en%)	0	5	10	15	20	ANOVA P value
n	8	8	8	8	8	
Weight (g)						
BW at start	35.9±0.3	35.9±0.4	35.9±0.5	35.9±0.3	35.9±0.3	.975
BW	66.6±3.0	66.0±2.2	64.6±2.8	63.4±0.8	56.4±2.6	.208
Liver	3.14±0.43	3.15±0.32	3.02±0.34	2.82±0.35	2.37±0.25	.484
Epididymal WAT	3.03±0.18[a]	2.95±0.18[a]	2.32±0.13[b]	2.27±0.18[b]	2.11±0.23[b]	.032
Retroperitoneal WAT	0.66±0.06	0.60±0.03	0.53±0.04	0.52±0.04	0.52±0.06	.051
Mesenteric WAT	1.54±0.14	1.46±0.14	1.26±0.11	1.19±0.11	0.98±0.17	.054
Subcutaneous WAT	1.89±0.17	1.88±0.18	1.81±0.27	1.79±0.13	1.35±0.22	.415
Gastrocnemius	0.51±0.01	0.49±0.01	0.49±0.01	0.48±0.01	0.46±0.02	.061
Quadriceps	0.53±0.02	0.56±0.04	0.56±0.02	0.58±0.02	0.58±0.02	.172
BAT[c]	0.34±0.05	0.34±0.03	0.34±0.04	0.31±0.03	0.26±0.03	.571
Total energy intake (MJ/mouse)	8.04±0.39	8.45±0.38	8.43±0.40	8.27±0.33	8.13±0.46	.685

Values are mean±S.E.M. Means without a common letter[a,b] differ (P<.05). BW, body weight; WAT, white adipose tissue; BAT, brown adipose tissue.

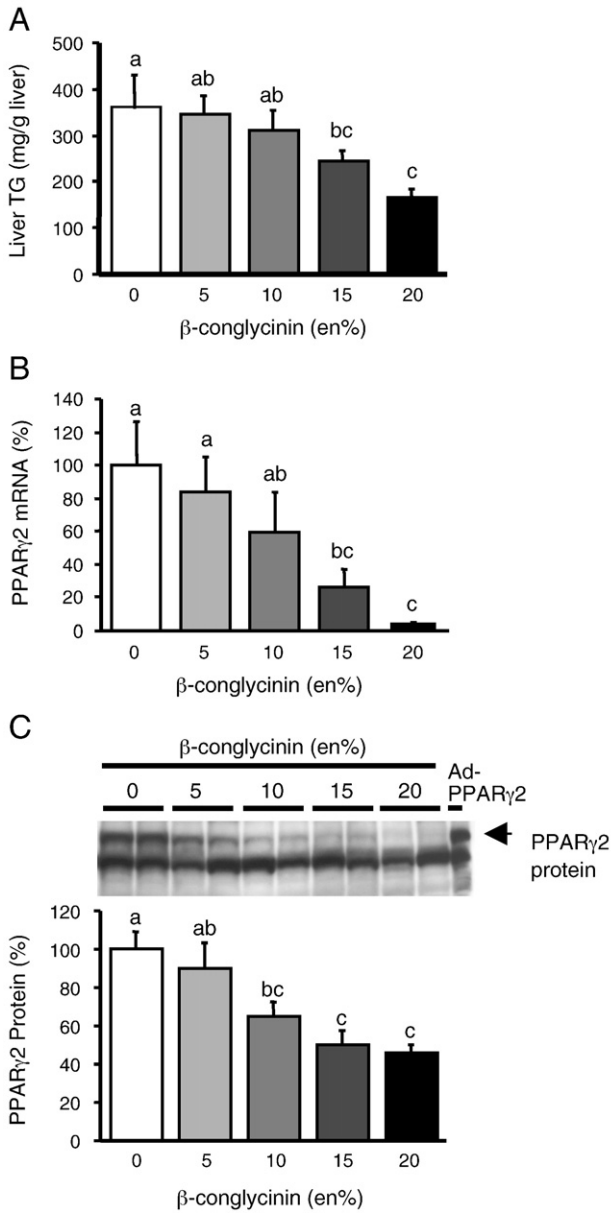

Fig. 4. Dose-dependent effects of β-conglycinin on TG concentration (A), PPARγ2 mRNA (B) and PPARγ2 protein (C) in liver. Mice were fed a high-butter diet with increasing amounts of β-conglycinin from 0 to 20 en% in 5-en% increments replacing casein for 11 weeks. Values are mean±S.E.M. (n=8). Means without a common letter differ (P<.05). One-way ANOVA P values with respect to β-conglycinin effect are significant for TG concentration, PPARγ2 mRNA and protein.

Oxygen consumption and RQ ratio for control and β-conglycinin-supplemented mice did not differ significantly during the dark cycle (feeding period) or light cycle (sleeping period) at either 2 or 8 weeks. Energy production did not differ significantly between the two groups when expressed on either a $kg^{0.75}$ of body weight or mouse. Daily activity levels also did not differ significantly between the two groups.

4. Discussion

In the present study, we found that β-conglycinin effectively prevented fatty liver induced by diets high in sucrose/fructose or fat. However, the mechanisms to prevent fatty liver differed according to the etiology of the fatty liver: β-Conglycinin prevented increases in SREBP-1c and ChREBP mRNAs in sucrose-supplemented mice (and in control mice), whereas it prevented an increase in PPARγ2 mRNA in mice fed an HF (high-safflower-oil or high-butter) diet. β-Conglycinin was unique in preventing the increase of PPARγ2 mRNA and its protein in HF diets. In our previous study, fish oil counteracted the increase in SREBP-1c mRNAs in sucrose-supplemented mice by deactivating SREBP-1c but failed to prevent the increase in PPARγ2 mRNA in an HF diet [9,15].

Soy protein shows antilipogenic and antiobesity effects when compared with casein [24,25]. Soy protein is composed of two main protein components, β-conglycinin (7S globulin) and glycinin (11S globulin). A β-conglycinin-rich diet, but not a casein-rich or glycinin-rich diet, resulted in significant decreases in serum TG, glucose and insulin levels with a decrease in body weights in normal mice (ICR) and genetically obese mice (KK-A^y) under energy-restricted conditions [26]. Digestibility of casein and soy protein isolate (given as 35% energy of the total diet) in rats was 94.1%±0.6% and 93.3%±0.4%, respectively [25], indicating that bioavailability of β-conglycinin was not impaired even if a large amount of β-conglycinin was given. In a human randomized, double-blind, placebo-controlled study, 12-week consumption of 5 g of soybean β-conglycinin per day significantly reduced serum TG concentrations from 2.65 to 2.29 mmol/L, whereas consumption of 5 g of casein did not [27]. In this study, a 20-week consumption of 5 g of soybean β-conglycinin per day significantly reduced computed-tomography-measured visceral fat area by 5.5±2.2 cm^2, whereas consumption of 5 g of casein increased this area by 4.2±1.6 cm^2 in obese subjects. Therefore, it is likely that soybean β-conglycinin might be the active component of soy protein that prevents hyperlipidemia and obesity.

Although the molecules in β-conglycinin by which it may exert its beneficial effects are not identified, recent studies suggest that the mechanisms might be due to active peptides in β-conglycinin. For example, soy hydrolysates from β-conglycinin inhibited lipid accumulation and induced adiponectin secretion in 3T3-L1 adipocytes [28], and short peptides derived from β-conglycinin suppressed the secretion of apoB in HepG2 cells [29]. Three peptides, KNPQLR, EITPEKNPQLR and RKQEEDEDEEQQRE, were found to inhibit FAS activity via direct binding of the FAS molecule [30]. Soybean beta 51-63 peptide stimulates cholecystokinin secretion in enteroendocrine STG-1 cells [31]. It is conceivable that some peptides in β-conglycinin decreased PPARγ2 protein in liver and prevented HF-diet-induced fatty liver.

The effects of β-conglycinin on PPARγ2 were specific to liver because β-conglycinin supplementation did not affect PPARγ2 mRNA expression in WAT (Yamazaki T., unpublished observation). In our recent study, mice fed an HF diet, especially a diet rich in saturated fat, showed increases in PPARγ2 protein and TG concentrations in liver [32]. Furthermore, adenovirus-mediated knockdown of PPARγ2 decreased PPARγ2 protein in liver and improved HF-diet-induced fatty liver [32]. Knockdown of PPARγ2 protein reduced the expression of not only its target genes (CD36 and ADRP) but also lipogenic genes (FAS, SCD1, ACC1) with no alterations of SREBP-1c mRNA [32]. In the present study, β-conglycinin also decreased mRNAs of lipogenic genes, such as FAS, SCD1, ACC1 and GPAT, in a dose-dependent manner with no decrease in SREBP-1 mRNA and its mature protein in mice fed an HF diet. Similar changes have been reported in animal models of fatty liver [8,33,34]. Liver-specific PPARγ knockout mice on an ob/ob background showed reduced liver TG content with reductions in FAS, SCD1 and ACC1 mRNAs and no alteration of SREBP-1c mRNA [33]. In another mouse model of fatty liver, treatment of apoB/BAT-less 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

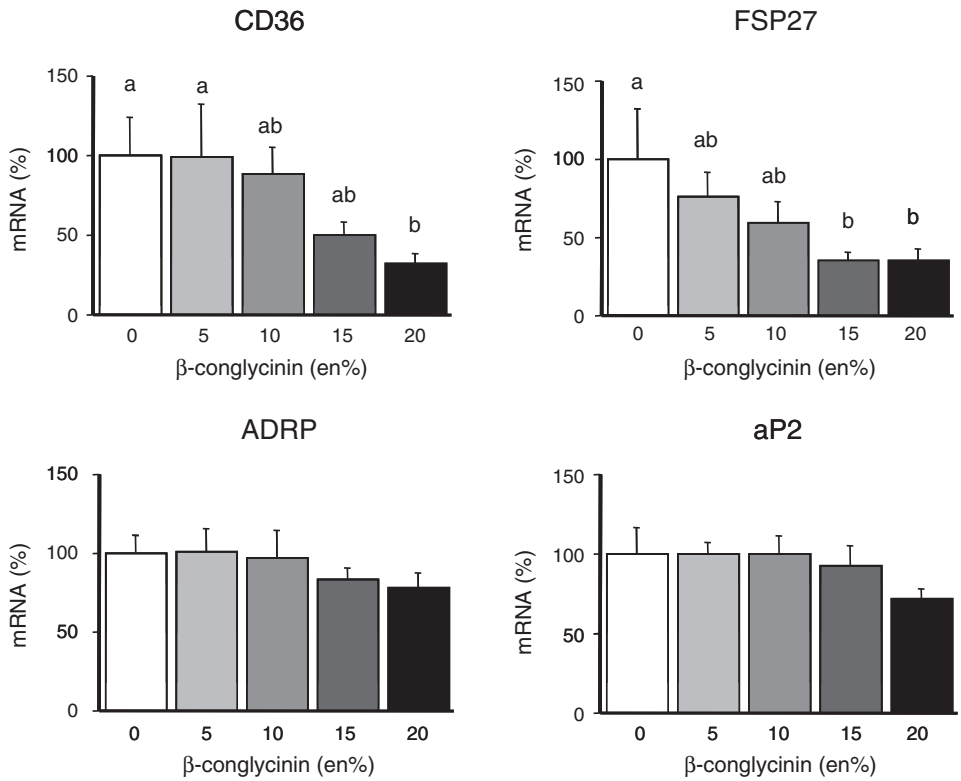

Fig. 5. Dose-dependent effects of β-conglycinin on mRNAs of target genes of PPARγ2, CD36, FSP27, ADRP and aP2. Mice were fed a high-butter diet with increasing amount of β-conglycinin from 0 to 20 en% in 5-en% increments replacing casein for 11 weeks. Values are mean±S.E.M. (n=8). Means without a common letter differ (P<.05). One-way ANOVA P values with respect to β-conglycinin effect are significant for CD36 and FSP27.

whether PPARγ2 directly or indirectly regulates the transcription of these genes. 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 PPARγ2 might directly bind this PPRE-like motif and increase SCD1 expression. It is conceivable that PPARγ2 directly and indirectly deactivates mature SREBP-1c protein by protein modifications of SREBP-1c, as observed in its phosphorylation [36]. β-Conglycinin effectively prevented sucrose-induced fatty liver with decreasing SREBP-1c mRNA. The mechanism of SREBP-1c mRNA reduction in response to β-conglycinin in sucrose-supplemented mice is unclear at present.

β-Conglycinin did not decrease the total amount of postprandial TG after an HF-diet feeding (Fig. 3), suggesting that inhibition of fat absorption in the intestine and a decrease in fat intake were not observed in mice supplemented with β-conglycinin. This further suggests that increased energy production might occur in mice supplemented with β-conglycinin. However, we did not detect any changes in energy production or physical activity levels. This might be due to the sensitivity of indirect calorimetry. The 20 en% of β-conglycinin reduced body weight by 10.2 g during the 11-week experimental period. If we assume this reduction was due to a reduction of body fat, and 1 g tissue fat is equivalent to 29.3 kJ (7 kcal), a total of 299 kJ (10.2×29.3) was consumed during the 11 weeks, which corresponds to 3.88 kJ (299/77) per day. By indirect calorimetry, ddY mouse fed β-conglycinin consumed, on average, 55.2±1.7 (n=8) and 62.8±2.5 kJ per day (n=6) at 2 and 8 weeks of feeding, respectively. A 6.6% (3.88/59) increase in total energy production might be difficult to detect by indirect calorimetry. Small increases in energy production might occur in some tissues by unknown mechanisms. Slight activation of PPARα (1.5-fold increase in mRNAs of PPARα, MCAD, CPT1) in skeletal muscle but not UCP1 expression in brown adipose tissue was observed in mice fed a β-conglycinin-supplemented diet (Yamazaki T., unpublished observation), suggesting that absorbed dietary fat might be taken preferentially into skeletal muscles rather than liver due to a reduction in liver PPARγ2. It is conceivable that a reduction in liver PPARγ2 protein may be due to certain hormonal changes (increased adiponectin concentration and decreased basal insulin concentration) caused by a reduction in adipose tissue, rather than to the direct effects of β-conglycinin or its metabolite to decrease liver PPARγ2 mRNA expression. Serum adiponectin concentrations at 11weeks of feeding were 683±30 and 556±41 nmol/L (n=8) with and without β-conglycinin, respectively (P<.05), and serum insulin concentrations at 11 weeks of feeding were 0.59±0.21 and 1.05± 0.21 pmol/L (n=8) with and without β-conglycinin, respectively (P<.05). In agreement with these findings, it was recently reported that β-conglycinin supplementation in rats increased adiponectin and insulin sensitivity [37].

The favorable effect of β-conglycinin in reducing liver TG and fat tissue weights was dose-dependent. A significant difference was observed at 10 en% for reducing epididymal WAT weight and at 15 en% for reducing liver TG. However, in humans, the estimated average intake of β-conglycinin was very low (less than 1 en%). Mean intake of beans (mostly soybeans) and their related foods in Japanese over 1 year of age was 56.0 g/day, and protein intake was 4.8 g [38]. Because about 20% of the soy protein consumed was β-conglycinin [39], about 1 g of β-conglycinin was consumed per day on average. If we assume that average energy intake in humans is 2000 kcal/day and energy from protein is 4 kcal/g, then 1 g of β-conglycinin corresponds to 0.2 en%. The US Food and Drug Administration has approved food labeling with the health claim that consumption of at least 25 g of soy protein per day is required to reduce total and low density lipoprotein cholesterol levels [40].

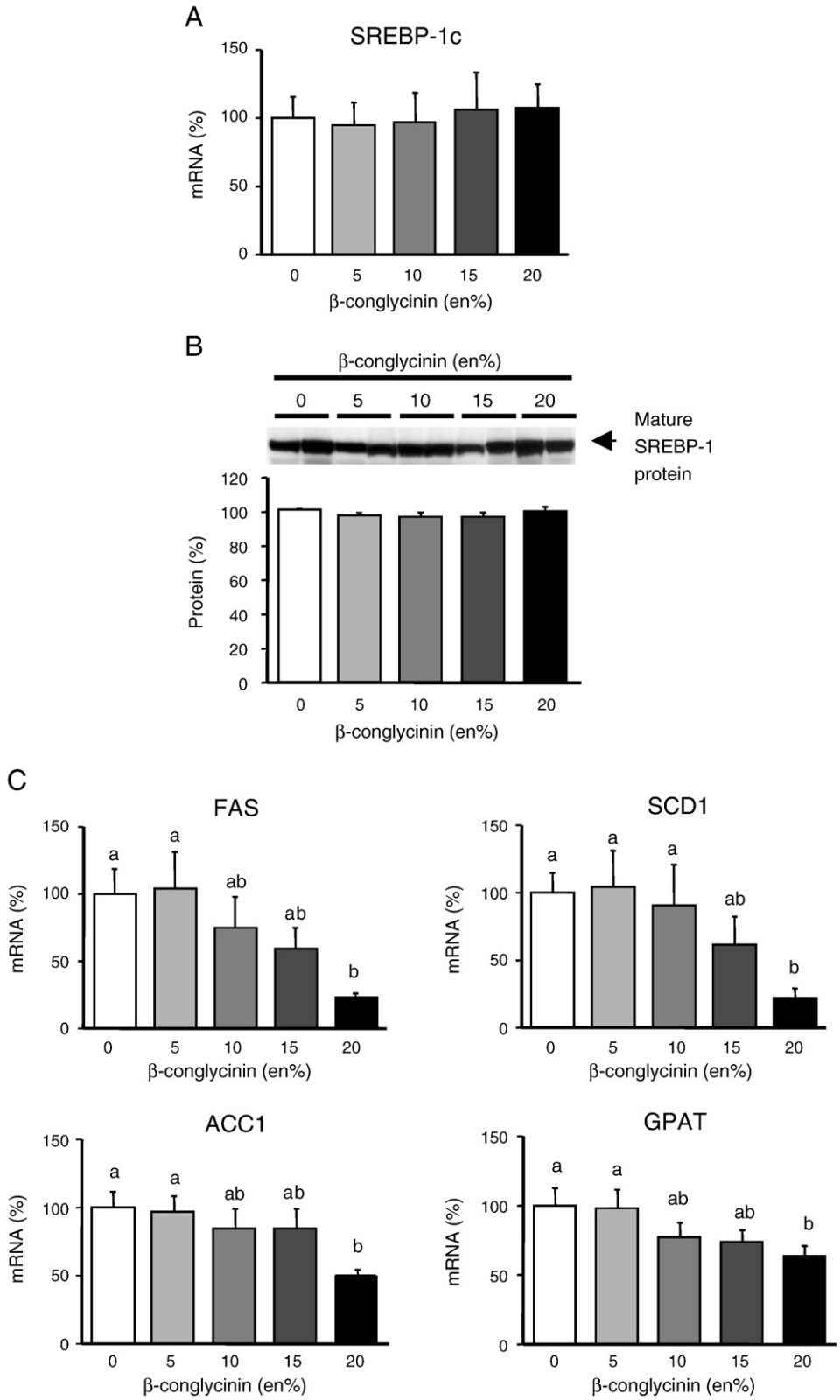

Fig. 6. Dose-dependent effects of β-conglycinin on SREBP-1c mRNA (A), mature SREBP-1 protein (B) and mRNAs of target genes of SREBP-1c, FAS, SCD1, ACC1 and GPAT (C). Mice were fed a high-butter diet with increasing amount of β-conglycinin from 0 to 20 en% in 5-en% increments replacing casein for 11 weeks. Values are mean±S.E.M. (n=8). Means without a common letter differ (P<.05). One-way ANOVA P values with respect to β-conglycinin effect are significant for FAS, SCD1, ACC1 and GPAT.

However, in humans, additional supplementation of 5 g of β-conglycinin effectively reduced intraabdominal obesity [27]. This amount corresponds to 1 en%, which is lower than the dose required to elicit a decrease in WAT weight in ddY mice, suggesting that humans might be more sensitive to β-conglycinin than are ddY mice.

Table 5
Oxygen consumption, carbon dioxide production, RQ ratio and spontaneous motor activity of mice after 2 and 8 weeks with and without β-conglycinin (20 en%) on a high-butter diet

	2 weeks		8 weeks	
β-Conglycinin	−	+	−	+
n	8	8	6	6
Body weight (g)	42.9±0.9	42.3±0.7	51.1±2.2	46.3±2.8
Dark				
VO_2 (ml/min/kg$^{0.75}$)	22.9±0.5	22.8±0.7	22.8±0.4	23.4±0.5
VCO_2 (ml/min/kg$^{0.75}$)	17.2±0.5	17.1±0.7	18.5±0.4	18.8±0.6
RQ	0.75±0.01	0.75±0.01	0.81±0.01	0.80±0.01
Activity (count/min)	175±12	176±13	144±18	161±9
Energy production (J/min/kg$^{0.75}$)	452±13	452±17	460±8	469±13
Energy production (J/min/mouse)	41.1±1.0	41.5±1.3	49.2±1.9	46.6±1.5
Light				
VO_2 (ml/min/kg$^{0.75}$)	19.0±0.4	18.5±0.8	19.5±0.5	20.3±0.4
VCO_2 (ml/min/kg$^{0.75}$)	14.2±0.4	13.7±0.7	15.6±0.4	16.1±0.4
RQ	0.74±0.01	0.73±0.01	0.80±0.01	0.79±0.01
Activity (count/min)	79±9	64±8	64±8	81±8
Energy production (J/min/kg$^{0.75}$)	377±8	364±17	389±8	410±8
Energy production (J/min/mouse)	35.1±0.8	32.2±1.5	42.0±1.9	40.8±1.8

To our knowledge, β-conglycinin is the first compound found that reduces liver PPARγ2 protein. Supplementation of β-conglycinin itself or active peptides embedded in β-conglycinin might be effective in preventing fatty liver and obesity in humans who ingest excessive amounts of sucrose and fat in their diets.

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