Changes in Lipid Metabolism by Soy β-Conglycinin-Derived Peptides in HepG2 Cells

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In this study, HepG2 cells were treated with short peptides (7S-peptides) derived from highly purified soybean β-conglycinin (7S), which was free from lipophilic protein, and the effect of the peptide treatment on lipid metabolism was determined. 7S-peptide treatment suppressed the secretion of apolipoprotein B-100 from HepG2 cells into the medium. The 7S-peptides also suppressed the incorporation of ^{3}H-glycerol and ^{14}C-acetate into triacylglyceride but not into major phospholipids, such as phosphatidylcholine and phosphatidylethanolamine. Additionally, the synthesis of cholesterol esters was dramatically decreased for 2 h after the addition of the 7S-peptides, whereas the synthesis of cholesterol remained unchanged by 4 h and increased by 8 h after the addition of the 7S-peptides. The cleaved nuclear form of SREBP-2 increased 8 h after the addition of the 7S peptides, suggesting a decrease in intracellular cholesterol levels. Analysis of changes in mRNA expression after 7S-peptide treatment suggested that the 7S-peptides lower the level of cholesterol in the endoplasmic reticulum, increase the mRNA of genes related to β-oxidation of fatty acids, and increase the synthesis of cholesterol. From these results, it may be concluded that the peptides derived from 7S altered the lipid metabolism to decrease secretion of apolipoprotein B-100-containing lipoprotein from HepG2 cells.

KEYWORDS: Soy protein; β-conglycinin; peptides; HepG2 cells; apolipoprotein B-100; triacylglycerol

INTRODUCTION

Soy protein isolate, which is manufactured by acidifying soymilk, has shown several beneficial effects on human health, such as the ability to lower cholesterol and triglyceride levels in the blood (*1*). Studies on the cholesterol- and triglyceride-lowering ability of soy protein isolate indicate that the effects are principally exerted by proteins, although the isoflavone content also contributes to these effects (*2−4*). Because soy protein isolate was believed to be primarily composed of β-conglycinin (7S) and glycinin, past studies to identify the functional protein moieties in soy protein isolate were performed with either the 7S or the glycinin fraction. These fractions were believed to be comprised of relatively pure 7S or glycinin, which was demonstrated by polyacrylamide gel electrophroesis and total protein staining of the fractions; however, Samoto et al. showed that 7S and glycinin fractions prepared by conventional methods contain large amounts of lipophilic proteins (LP), which are difficult to stain with Coomassie Brilliant Blue on poly-

acrylamide gels (*5*). Because LPs also lower plasma cholesterol levels (*6*), the physiological effects of the 7S and glycinin cannot be directly tested using conventionally prepared 7S and 11S fractions due to the substantial LP content. The LP-free 7S fraction was further tested by supplementing the diets of adults with high plasma triglycerides with 5 g/day of LP-free 7S and plasma triacylglycerol (TG), and visceral fat amounts were monitored (*7*). The LP-free 7S normalized plasma TG levels and reduced visceral fat in participants. The LP-free 7S peptide did not show any hypocholesterolemic effects.

Very low-density lipoprotein (VLDL) particles are assembled in the endoplasmic reticulum (ER) of hepatocytes by adding primarily TG, as well as phospholipids, cholesterol, and cholesterol esters, to apolipoprotein B-100 (ApoB-100), and then, VLDL particles are secreted into blood (*8*). The TG component of circulating VLDL is degraded by lipoprotein lipase (LPL) in the blood, which supplies nonesterified fatty acids and 2-monoacylglycerol to other tissues (*9*). Therefore, the plasma TG level is controlled by both the amount of VLDL secreted from the liver and the rate of LPL-mediated VLDL-TG catabolism in blood. Given the previously observed beneficial effects of 7S on plasma TG levels, we aimed to evaluate the effects of highly purified, LP- and isoflavone-free 7S on lipid metabolism and on the expression of genes related

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to lipid metabolism in HepG2 cells, a human hepatocellular carcinoma cell line. Because of the large size of 7S, it is improbable that substantial amounts of undigested 7S will be able to cross the intestinal barrier and enter the bloodstream. Therefore, in this study, protease digestion of 7S was used to generate small peptides (7S-peptides) that are more likely to enter into circulation, and the effects of the 7S-peptides on HepG2 cell lipid metabolism were determined.

MATERIALS AND METHODS

Materials. The Enrei variety of soybean seeds was used in this study. The three bacterial proteases used to generate the 7S-peptides were Thermoase (Daiwa Kasei, Shiga, Japan), Bioprase (Nagase Chemtex Co., Osaka, Japan), and Sumizyme FP (Shin Nihon Chemical Co., Aichi, Japan). Fetal bovine serum was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Culture dishes coated with collagen I and microtiter plates were obtained from Asahi Technoglass Corp. (Tokyo, Japan). The Live-dead cell staining kit was obtained from BioVision (Mountain View, CA). Fish gelatin blocks were obtained from BioFX Laboratories (Owings Mills, MD). Human VLDL was purchased from Millipore (Billerica, MA). The anti-ApoB-100 monoclonal antibody 12G10 was obtained from Monosan (Uden, The Netherlands), and affinity purified antihuman ApoB-100 goat polyclonal antibodies were obtained from Rockland (Gilbertsville, PA). Antisterol regulatory element binding protein (SREBP)-2 goat polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated antigoat IgG donkey serum was obtained from Promega Corp. (Madison, WI), and a TMB microwell peroxidase substrate system was purchased from KPL (Gaithersburg, MD). [2-^{3}H]-glycerol (37 TBq/mmol), [1-^{14}C]-acetic acid (37 TBq/mmol), and [^{14}C]-oleoyl-CoA (1.96 GBq/mmol) were purchased from GE Healthcare, Bio-Sciences Corp. (Piscataway, NJ). [2-^{14}C]-Malonyl-CoA (1.85−2.22 GBq/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Oleoyl-CoA and sn-1,2-diacylglycerol (DG) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Silica gel 60 thin layer plates were purchased from Merck & Co., Inc. (Whitehouse Station, NJ). The SV total RNA isolation kit was obtained from Promega Corp.

Preparation of 7S-Derived Peptides. 7S was prepared according to the method of Samoto et al. (*5*). Briefly, defatted soy flour was added to water and brought to pH 8.0 with 5 N NaOH. This mixture was stirred for 1 h at 20 °C and then centrifuged at 3000*g* for 10 min with a Microcentrifuge model 1710 (Kubota Corp., Tokyo). The supernatant was reserved, 1 mM Na$_2$SO$_3$ was added, and the pH was adjusted to 5.8 with 1.75 M H$_2$SO$_4$. The suspension was centrifuged at 3000*g* for 10 min. Again, the supernatant was reserved, brought to pH 5.0, and heated to 55 °C for 15 min. The pH was then adjusted to pH 5.5 with 1.75 M H$_2$SO$_4$, and precipitates were removed by centrifugation at 3000*g* for 10 min. The resulting supernatant was brought to pH 4.5 with 1.75 M H$_2$SO$_4$ to precipitate 7S, which was pelleted by centrifugation at 3000*g* for 10 min.

To remove isoflavones absorbed to 7S, 7S was suspended in 3 volumes of 70% cold acetone (−20 °C) and stirred for 30 min. The suspension was centrifuged at 3000*g* for 10 min, and the supernatant was decanted. The 70% cold acetone wash was repeated three times, and then, the 7S powder was air-dried. The isoflavone content of 7S was determined by the method of Kudou et al. (*10*). Briefly, three 70% ethanol extractions were performed on the highly purified 7S preparations. The extracts were analyzed by reverse-phase chromatography using an ODS column.

The 7S powder was suspended to a concentration of 3% with distilled water and then sequentially digested with three bacterial proteases, Thermoase, Bioprase, and Sumizyme FP, at 58 °C under conditions of optimal pH according to the methods of Maebuchi et al. (*11*). After digestion, the suspension was heated at 90 °C for 20 min to inactivate proteases and centrifuged at 7500*g* for 20 min at 4 °C. The resulting supernatant was filtered with a Centriplus YM-10 (Millipore Corp.) to remove proteases and polypeptides with a molecular mass greater than approximately 10000 Da. The peptide-containing filtrate (7S-peptides) was sterilized by further filtration with a syringe driven filter unit Millex-

GV PVDF (Millipore Co., Bedford, MA). To determine the concentration, and the peptide solution was lyophilized and weighed.

To determine the size distribution of the 7S-peptides, the 7S-peptide sample was separated on a Superdex Peptide 7.5/300 GL size exclusion column (Amersham Biosciences, Piscataway, NJ) that was equilibrated with 10 mM sodium phosphate buffer (pH 8) containing 1% (w/w) sodium dodecyl sulfate (SDS) and eluted with the same buffer at a flow rate of 0.25 mL/min. Elution of peptides was monitored by absorbance at 220 nm using a spectrophotometer (*11*).

Cell Culture. HepG2 cells were used throughout this study. The cells were propagated by seeding dishes with 1.27×10^4 cells/cm^2 and maintained in minimum Eagle's media α-modification (MEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO$_2$. 7S-Peptides were added to HepG2 cell cultures at 48 h postseeding. To determine the number of cells in a given culture, the cells were dispersed by trypsin treatment and counted using a hemocytometer. To determine cell viability, cells were cultured on collagen-coated cover glass slips and stained using the Live-dead cell staining kit. The specimens were examined on an MRC-1024 laser scanning confocal imaging system (Bio-Rad Laboratories, Hercules, CA).

Enzyme-Linked Immunosorbent Assay (ELISA) of ApoB-100. To quantify the amount of VLDL secreted from HepG2 cells, an antibody capture ELISA for ApoB-100 was used. Microtiter plates were coated with 100 μL of the capture antibody (anti-ApoB-100 monoclonal antibody) diluted 1:200 in phosphate buffer saline (PBS) (8 mM Na$_2$HPO$_4$, 1.5 mM K$_2$HPO$_4$, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) at 4 °C overnight. The plates were then washed twice with 300 μL/ well PBS and blocked with blocking solution (fish gelatin blocks diluted 1:10-fold with PBS) overnight at 4 °C. After the plates were washed twice with 300 μL/well PBS, 100 μL of either the VLDL standard (human VLDL solution) or the HepG2 culture medium diluted 1:30 with a 50/50 PBS-to-MEM mixture was added to the plates, and the plates were incubated for 2 h. The plates were washed with PBS, and 100 μL of anti-ApoB-100 polyclonal antibodies, diluted 1:1,000 in blocking solution, was added to each well. The plates were incubated for 2 h. After the plates were washed with PBS, 100 μL of a horseradish peroxidase-conjugated antigoat IgG serum, diluted 1:5000 in blocking solution, was added to each well, and the plates were incubated for 2 h. After they were washed with PBS, 100 μL of the TMB substrate solution was added to each well and allowed to develop for 5 min at 25 °C. The enzymatic reaction was stopped by the addition of 100 μL of 1 M H$_3$PO$_4$, and absorbance was measured at 450 nm in a microtiter plate reader model 680 (Bio-Rad Laboratories). The plates were washed four times in each wash step, and all incubation steps were carried out at room temperature, unless otherwise noted.

Radiolabeling and Analysis of Cellular Lipids. Long-term labeling with ^{3}H-glycerol was carried out to measure the total amount of TG in cells. At 24 h postseeding, 185 kBq/mL ^{3}H-glycerol (144 mBq/mmol) was added to the HepG2 culture and incubated for 24 h. The cells were then either mock-treated or treated with 3 mg/mL 7S-peptides and incubated for an additional 24 h. Pulse labeling of lipids with ^{3}H-glycerol or ^{14}C-acetic acid was carried out to assay synthetic activity of TG and phospolipids or TG, cholesteryl ester (CE), and cholesterol in cells. At 48 h postseeding, HepG2 cells were either mock-treated or treated with 7S-peptides (3 or 6 mg/mL) for 2, 4, or 8 h. The culture medium was then supplemented with 180 kBq/mL ^{3}H-glycerol (7.2 GBq/mmol) or 42 kBq/mL ^{14}C-acetic acid (37 TBq/mmol) for 1 h.

After labeling, the total lipids were extracted from cells according to the method of Bligh and Dyer (*12*). Radiolabeled TG, cholesterol, CE, and DG were separated by thin-layer chromatography using hexane/ diethylether/acetic acid (100:100:2) as a developing solvent. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were separated by thin-layer chromatography using chloroform/methanol/acetic acid/ distilled water (90:75:15:5). Individual lipid spots were visualized by exposure to iodine vapors. Radioactivity in the scraped spot was measured by liquid scintillation counting.

Immunoblot Analysis of SREBP-2. Cells were mixed with SDS-loading buffer, heated for 10 min at 95 °C, and then subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was probed with anti-SREBP-2

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antibodies, followed by a horseradish peroxidase-conjugated IgG secondary antibody, using Western Lightning Chemiluminescence Reagent (Perkin-Elmer Life Science, Boston, MA).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). HepG2 cells were either mock-treated or treated with 3 or 6 mg/mL of 7S-peptides for 2 or 4 h. Total cellular RNAs were extracted using the SV total RNA isolation kit. Quantization of specific mRNAs was carried out by real-time RT-PCR using a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc., Shiga, Japan). The primers used for the detection of specific mRNA molecules were as follows. Angiopoietin-like 3 (ANGPTL3): forward, 5′-TTCCAAGCCAAGAG-CACCAAG-3′, and reverse, 5′-AAACTTTGAGAGTTGCTGGGTCT-GAT-3′; SREBP-1c: forward, 5′-GGCTCCTGCCTACAGCTTCT-3′, and reverse, 5′-CAGCCAGTGGATCACCACA-3′; and β-actin: forward, 5′-TGGCACCCAGCACAATGAA-3′, and reverse, 5′-CTAAGT-CATAGTCCGCCTAGAAGCA-3′. SYBR Premix Ex Taq (TaKaRa Bio Inc.) was the polymerase used for these assays. Real-time PCR was performed under conditions described below: denaturing at 95 °C for 10 s (first cycle) and 5 s for additional cycles; annealing and elongation at 60 °C for 30 s; the total number of cycles was 50.

Measurement of Total DG Acyltransferase Activity. To measure the DG acyltransferase activity, microsomal membranes of the HepG2 cells had to be isolated. Briefly, HepG2 cells (9×10^6 cells) that were either mock-treated or treated with 6 mg/mL of 7S-peptides for 8 h were collected and homogenized by sonication. The homogenate was centrifuged at 25000*g* for 15 min at 4 °C. The resulting supernatant was centrifuged again at 100000*g* for 1 h at 4 °C. The resultant pellets were resuspended in buffer composed of 0.1 M sucrose, 50 mM KCl, 30 mM EDTA, and 40 mM potassium phosphate, pH 7.2. The DG acyltransferase (EC 2.3.1.20) activity was measured by the modified methods described previously (*13*). Briefly, 200 μL of a reaction mixture containing 200 μM DG in acetone, 25 μM [^{14}C]-oleoyl-CoA, 5 mM MgCl$_2$, 200 μg of bovine serum albumin, and 15 μg of microsomal protein in 175 mM Tris-HCl buffer, pH 8.0, was prepared and incubated for 30 min at 25 °C. The reaction was stopped by adding 1.5 mL of a 50/50 heptane:2-propanol solution (2:8). The heptane layer was washed twice with alkaline ethanol. An aliquot of the heptane phase was then used to determine the amount of ^{14}C-labeled TG by liquid scintillation counting.

Measurement of Fatty Acid Synthase. HepG2 cells (9×10^6 cells) either mock-treated or treated with 3 or 6 mg/mL 7S-peptides for 8 h were collected and frozen at −80 °C. The frozen cells were thawed, suspended in an aqueous buffer (1 mM DTT, 1 mM EDTA, and 20 mM Tris-HCl, pH7.5), and incubated at 37 °C for 15 min. Fatty acid synthase activity was measured by the methods of Swinnen et al. (*14*).

Measurement of Malic Enzyme (EC 1.1.1.40) and Carnitine Palmitoyltransferase (EC 2.3.1.21). HepG2 cells (8×10^6 cells) either mock-treated or treated with 3 or 6 mg/mL 7S-peptides for 8 h were collected and sonicated in 0.3 M sucrose. The disrupted cell suspension was centrifuged at 18000*g* for 10 min at 4 °C. The supernatant was then centrifuged at 100000*g* for 1 h at 4 °C. The resulting supernatant was used for enzyme assays. The malic enzyme activity was assayed by the method of Hsu and Lardy (*15*). Carnitine palmitoyltransferase activity was assayed by the methods of Maekwell (*16*).

Protein Measurement. The protein concentrations were measured using an RC DC protein assay kit (Bio-Rad Laboratories), which is a colorimetric assay for protein determination in the presence of reducing agents and detergents. The assay was carried out using γ-immunoglobulin as an internal standard as described in the manufacturer's instructions.

Statistical Analysis. The results are expressed as the means ± standard deviations for three experiments, and the Student's *t* test was used to determine the statistical significance of the results.

RESULTS AND DISCUSSION

Suppression of ApoB-100 Secretion by 7S-Peptides Treatment of HepG2 Cells. Previous studies indicated that 7S lowers plasma levels of TG in humans (*7*). It appears possible that 7S exerts its plasma-TG lowering effects by suppressing secretion

Table 1. Content of Isoflavones in 7S or 7S Washed with Acetone[a]

	content (mg/100 g dry weight)	
isoflavone	7S	7S washed with acetone
daidzin	7.7	0.3
glycitin	5.6	0.1
genistin	17.3	0.4
malonyl daidzin	84.9	0.6
malonyl glycitin	20.5	0.2
malonyl genistin	141.0	1.7
acetyl daidzin	3.0	ND
acetyl glycitin	2.7	ND
acetyl genistin	4.4	ND
daidzein	30.9	0.2
glycitein	5.4	ND
genistein	31.7	ND
total	355.1	3.5

[a] Isoflavones were measured as described in the Materials and Methods. ND, not detected.
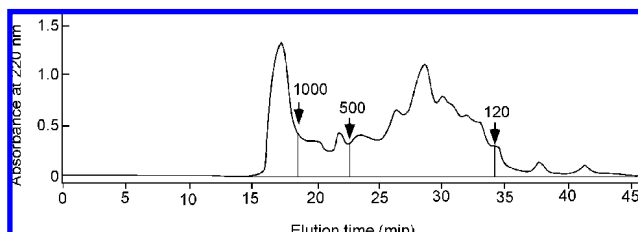

Figure 1. Size distribution of the 7S-peptides. The 7S-peptides were separated by gel filtration column chromatography as described in the Materials and Methods. Arrows indicate the elution time for peptides with molecular masses of 1000, 500, or 120 Da.

of VLDL from hepatocytes. Therefore, we sought to determine the effect of 7S-peptides treatment on the accumulation of ApoB-100, a protein component of VLDL, in culture medium of HepG2 cells. 7S-Peptides were prepared by digesting highly purified 7S, which was nearly free of isoflavone and LP contamination, with proteases (**Table 1**). The size distribution of the resulting 7S-peptides was measured by gel filtration chromatography using a 1% SDS phosphate buffer as the running buffer (**Figure 1**). The 7S-peptides were heterogeneous in size, with 86% of peptides having a molecular mass less than 1000 Da, and 68% of peptides having a molecular mass less than 500 Da. HepG2 cells treated with 6 mg/mL of the 7S-peptides showed a 30% reduction in the accumulation of ApoB-100 in the medium, as compared to the mock-treated cells (**Figure 2A**). This reduction was not due to aberrant cell death induced by the 7S-peptides, because cells treated with 6 mg/mL of the 7S-peptides showed no signs of toxicity (**Figure 2B,C**). Thus, the reduction in ApoB-100 accumulation may be due to 7S-peptide-induced alterations of lipid metabolism in HepG2 cells.

7S-Peptides Reduce Incorporation of Glycerol and Acetic Acid into TG. Unless a full compliment of TG is assembled onto ApoB-100 in the ER, mature VLDL particles will not be formed, and ApoB-100 is destroyed by the ubiquitin-mediated proteasomal degradation pathway (*17*). Therefore, the decrease in ApoB-100 secretion by 7S-peptide-treated HepG2 cells may be due to a decrease in TG synthesis. To determine the effect of the 7S-peptides on lipid synthesis in HepG2 cells, the incorporation of radioactive glycerol or acetic acid into cellular lipids was measured. HepG2 cell lipids were metabolically labeled with [2-^{3}H]-glycerol for 48 h, such that at 24 h postaddition of the ^{3}H-glycerol, the cells were either mock-treated or treated with the 7S-peptides, which were maintained in the culture medium for the last 24 h of the lipid labeling.
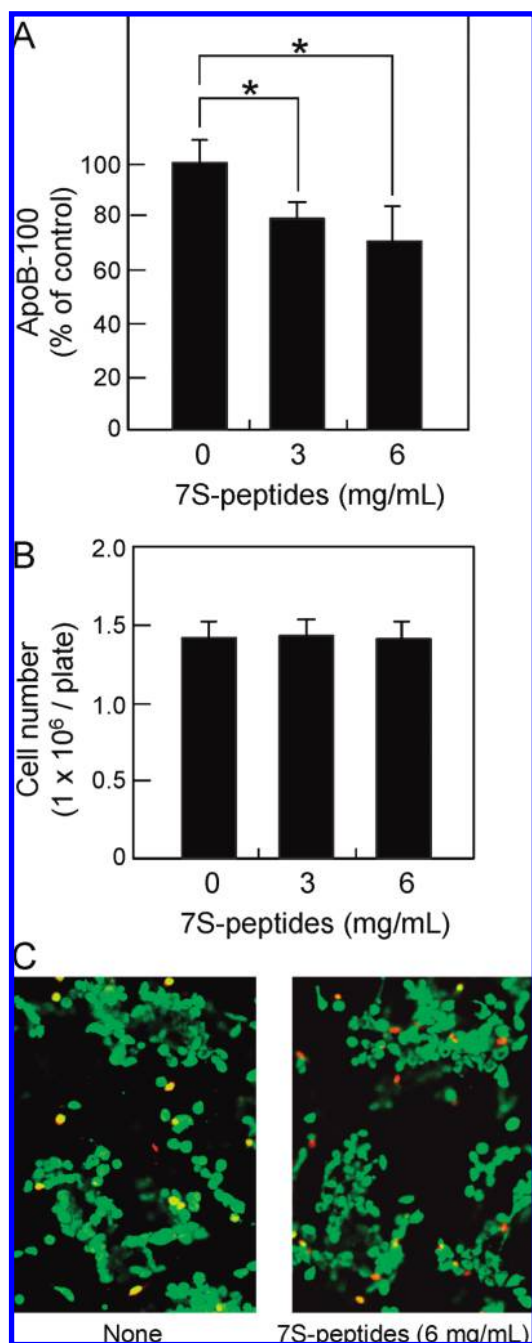
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Figure 2. Decrease in the secretion of ApoB-100 from HepG2 cells by 7S-peptide treatment. (**A**) The 7S-peptides were added to the culture medium at 3 or 6 mg/mL. At 24 h postpeptide addition, the amount of ApoB-100 secreted in medium was measured by ELISA. (**B**) Cells were counted 24 h after the addition of the 7S-peptides. Values are presented as the percentage of the value obtained from the mock-treated cells. Bars represent the means ± standard deviations of three experiments. *, $p <$ 0.05 as compared with the control. (**C**) Cells were treated with 6 mg/mL of the 7S-peptides, and at 24 h postpeptide addition, the cells were stained with using the Live-dead cell staining kit and observed by confocal microscopy. Green and red fluorescence indicate live and dead cells, respectively.

The amount of ^{3}H-glycerol incorporated into PC, which is a major phospholipid, in either the absence or the presence of the 7S-peptide treatment, reached a plateau at 36−48 h, so radioactivity incorporated into each glycerolipid at 48 h was supposed to be equivalent to the amount of each glycerolipid. The amount of radioactivity incorporated into TG and DG in

7S-peptide-treated cells (3 mg/mL) was about 30% lower than that in mock-treated cells. In contrast, the amount of radioactivity incorporated into the major phospholipids, PC and PE, was not significantly different between the treated and the untreated cells (**Figure 3A**). Next, to determine the effects of 7S-peptide treatment on the rate of glycerolipid synthesis, cells were pulse-labeled for 1 h with ^{3}H-glycerol after treatment with the 7S-peptides for 2, 4, or 8 h. As before, the amount of radioactivity incorporated into PC and PE did not differ significantly between treated and untreated cells (**Figure 3B**). However, at all of the time points, the incorporation of ^{3}H-glycerol into TG was significantly lower in 7S-peptide-treated cells than in mock-treated cells (**Figure 3B**). In a similar assay, the incorporation of ^{14}C-acetic acid into TG also was decreased at all of the time points in the 7S-peptide-treated HepG2 cells relative to the control cells (**Figure 3C**). The reduced synthesis of TG could be caused by reduced DG acyltransferase activity, which catalyzes the final reaction of TG synthesis. However, the activity of this enzyme was not altered by treatment of HepG2 cells with the 7S-peptides (**Table 2**). The rate of TG synthesis also depends on the intracellular level of fatty acyl-CoA, and these results suggested that either fatty acid synthesis was decreased and/or β-oxidation was accelerated by the 7S-peptide treatment. The enzyme AMP-activated protein kinase (AMPK) is a central regulator for the metabolism of lipids and carbohydrates and is known to stimulate β-oxidation in mitochondria through the phosphorylation of acetyl-CoA carboxylase in the liver (*18*). To examine the possibility that the 7S-peptide treatment activated this system, we tested the effect of the AMP-activated protein kinase inhibitor, compound C, on the 7S-peptide-induced suppression of ApoB-100 secretion. However, the 7S-peptides were able to suppress VLDL secretion, even in the presence of compound C (data not shown).

In addition to a decrease in TG synthesis, 7S-peptide treatment of HepG2 cells also caused a drastic decrease in the incorporation of ^{14}C-acetic acid into CE at 2 h after the addition of 7S-peptides (**Figure 4A**). This 7S-peptide-induced decrease in CE synthesis indicated that there was a rapid decrease in ER cholesterol pool after the addition of the 7S-peptides, because acyl-CoA:cholesterol acyltransferase is allosterically activated by excess cholesterol in the ER cholesterol pool (*19*). This conclusion was supported by the observation that incorporation of ^{14}C-acetic acid into cholesterol was increased 8 h after the addition of 7S-peptides (**Figure 4B**). This elevation in cholesterol synthesis is likely stimulated by the initial decrease in the ER cholesterol pool. Depletion of the ER cholesterol pool stimulates the release of the transcription factor SREBP-2 from the ER membrane, which translocates to the nucleus, activates the genes involved in the synthesis and uptake of cholesterol, and decreases the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, a key enzyme for sterol synthesis (*20*). All of this leads to a net increase in cholesterol synthesis activity. The increase in cholesterol synthesis observed at 8 h after the addition of the 7S-peptides was also associated with increased transcription of genes involved in cholesterol synthesis and the uptake of cholesterol (Supporting Information). Coincident with the up-regulation of these genes, the cleaved nuclear form of SREBP-2 increased 8 h after the addition of the 7S-peptides (**Figure 5**). The mechanism underlying the initial decrease in the ER cholesterol pool induced by the 7S-peptide treatment remains to be determined. Determination of accurate amounts of the ER cholesterol was hard since complete separation of the other organella membranes, especially plasma membranes containing cholesterol, from the ER fraction was difficult. In
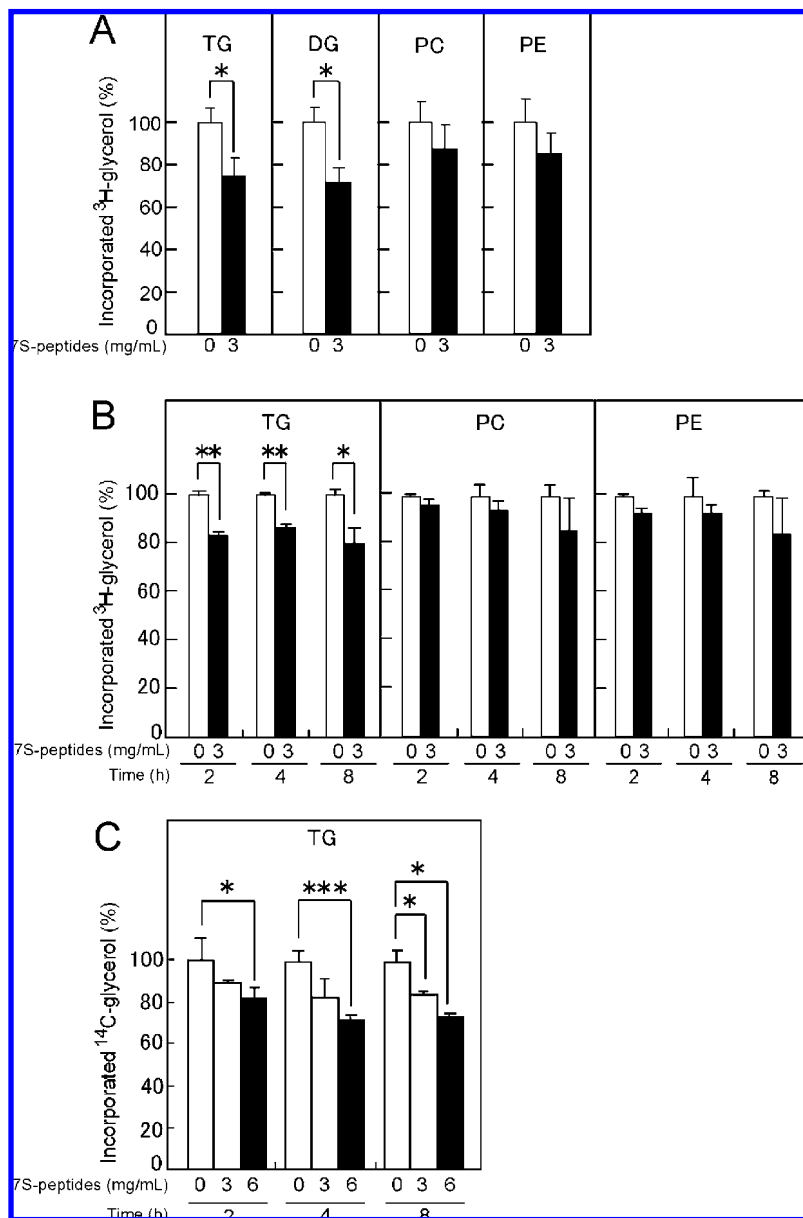
Figure 3. Effects of the 7S-peptide treatment of HepG2 cells on the incorporation of [2-³H]-glycerol and [¹⁴C]-acetic acid into glycerolipids. (**A**) HepG2 cells were incubated for 24 h in the presence of [2-³H]-glycerol and then incubated in the presence of [2-³H]-glycerol with or without 3 mg/mL of the 7S-peptides for an additional 24 h. The amounts of radioactivity incorporated into HepG2 cell TG, DG, PC, and PE were measured. (**B**) HepG2 cells were incubated for 2, 4, or 8 h in the presence or absence of 3 mg/mL of the 7S-peptides and then incubated in the presence of [2-³H]-glycerol for 1 h. The amount of radioactivity incorporated into the HepG2 cell TG, PC, or PE was measured. (**C**) Hep G2 cells were incubated for 2, 4, or 8 h in the presence or absence of 3 or 6 mg/mL of the 7S-peptides and then incubated in the presence of [¹⁴C]-acetic acid for 1 h. The amount of radioactivity incorporated into the HepG2 cell TG was measured. Values are presented as the percentage of the value obtained from the mock-treated control cells. Bars represent the means ± standard deviations of three experiments. *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.001$ as compared to control.

addition, participation of other factor(s) than cholesterol cannot be excluded.

To investigate the relationship between the observed changes in HepG2 cell lipid metabolism and the gene expression profile induced by the 7S-peptides, a DNA microarray analysis was carried out. Among the 54675 probe sets, the transcription of 753 was significantly increased, and 949 were significantly decreased in HepG2 cells treated with 3 mg/mL of the 7S-peptides for 8 h as compared to mock-treated cells. Among the transcripts of which a change call was either "increase" or "decrease", genes related to lipid metabolism are shown in the Supporting Information. The expression of the majority of genes related to the synthesis or uptake of fatty acids remained unchanged. Rather, the mRNA levels of acetyl-CoA carboxy-

Table 2. Enzyme Activities of HepG2 Cells Supplemented with or without 7S-Peptides[a]

	activity (nmol/min/mg protein)		
enzyme	none	3 mg/mL 7S-peptides	6 mg/mL 7S-peptides
DG acyltransferase	0.31 ± 0.05	not determined	0.32 ± 0.03
fatty acid synthase	9.73 ± 0.95	10.42 ± 0.31	10.69 ± 2.48
malic enzyme	3.04 ± 0.10	3.37 ± 1.11	3.62 ± 2.63
carnitine palmitoyltransferase	3.12 ± 0.64	4.12 ± 1.60	3.95 ± 2.69

[a] Enzyme activities were measured at 8 h after addition of 7S-peptides as described in the Materials and Methods. Results shown represent the means ± SDs of three experiments.
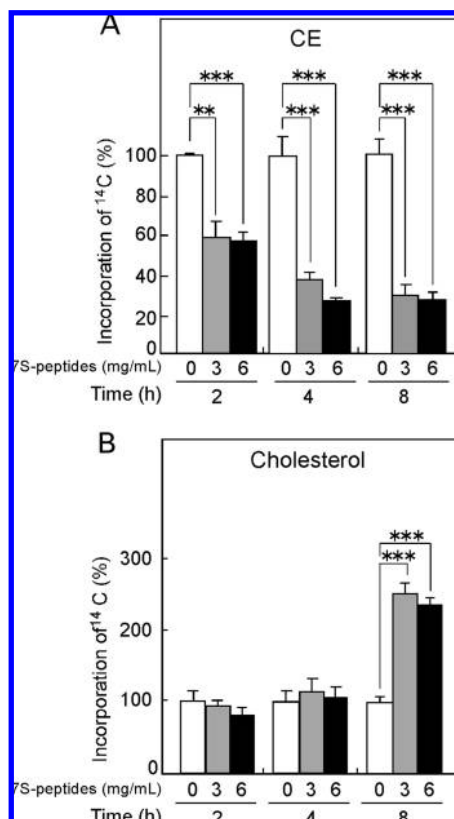
Figure 4. Effect of 7S-peptide treatment on the incorporation of [^{14}C]-acetic acid into CE and cholesterol of HepG2 cells. Hep G2 cells were incubated for 2, 4, or 8 h in the presence or absence of 3 or 6 mg/mL of the 7S-peptides and then incubated in the presence of [^{14}C]-acetic acid for 1 h. The amount of radioactivity incorporated into the HepG2 cell CE (**A**) or cholesterol (**B**) was measured. Values are presented as the percentage of the value obtained from the mock-treated control cells. Bars represent the means ± standard deviations of three experiments. **, $p < 0.005$; ***, $p < 0.001$ as compared to control.
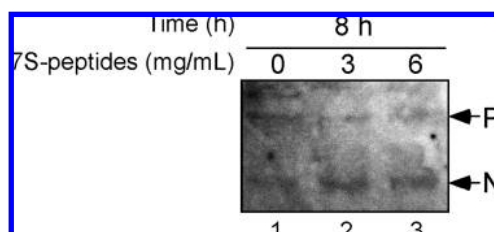

Figure 5. Increase in the cleaved nuclear form of SREBP-2 by treatment with 7S-peptide. HepG2 cells were incubated for 8 h (lanes 1–3) in the presence or absence of 3 or 6 mg/mL of 7S-peptides. SREBP-2 was detected by immunoblot analysis. N and P denote the cleaved nuclear form and the uncleaved precursor forms of SREBP-2, respectively.

lase, stearoyl-CoA desaturase, and fatty acid binding protein 1 increased slightly in cells treated with the 7S-peptides. There was also no difference in expression of the SREBP-1c mRNA between mock-treated and cells treated with the 7S-peptides (**Figure 6B**). No difference in the enzymatic activity of fatty acid synthase or malic enzyme was detected in either the mock or the 7S-peptide-treated cells (**Table 2**). Therefore, fatty acid synthesis may have been only slightly altered by the 7S-peptide treatment. Expression of the low density lipoprotein (LDL) receptor mRNA increased by a factor of 1.87 after 7S-peptide treatment. An increase in LDL receptor expression may contribute the observed decrease in ApoB-100 secretion, since
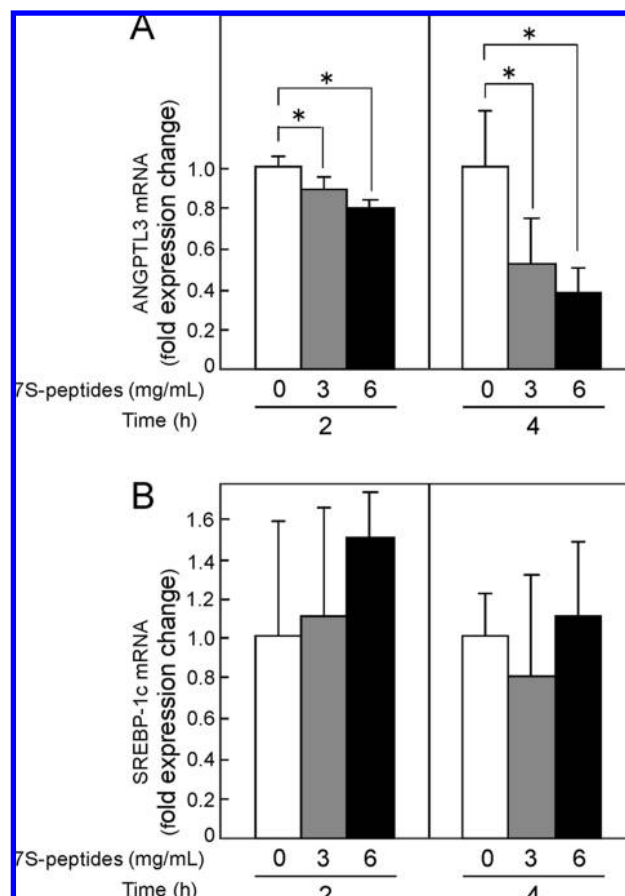

Figure 6. Decrease in mRNA levels of ANGPTL3, but not SREBP-1c, induced by the 7S-peptide treatment. HepG2 cells were incubated for 2 or 4 h in the presence or absence of 3 or 6 mg/mL of the 7S-peptides. The copy numbers of the ANGPTL3 mRNA (**A**) and SREBP-1c mRNA (**B**) were measured by real-time RT-PCR. The values for ANGPTL3 and SREBP-1c mRNA were standardized to the β-actin mRNA level in the respective total cellular RNA preparation. Fold expression change was calculated as the ratio of the mRNA in 7S-peptide-treated vs the mock-treated HepG2 cells. Bars represent the means ± standard deviations for three experiments. *, $p < 0.05$ as compared with control.

the LDL receptor promotes post-translational degradation of ApoB-100 and thereby reduces VLDL particle secretion (*21*).

Interestingly, a drastic decline in the ANGPTL3 mRNA (0.09-fold) by 7S-peptide treatment was found by the microarray analysis. In addition, a substantial decrease in the ANGPTL3 mRNA levels was observed using real-time quantitative RT-PCR analysis even at 2 and 4 h after the addition of the 7S-peptides (**Figure 6A**). ANGPTL3 is secreted from the liver into the bloodstream and directly inhibits lipoprotein lipase activity, resulting in an increase in plasma TG levels (*22*). Furthermore, it has been suggested that ANGPTL3 activates lipolysis to stimulate the release of free fatty acid and glycerol from adipocytes (*23*). The KK/Snk mouse, which is a mutant strain of the KK obese mouse, has a mutation in the ANGPTL3 gene and has been reported to have significantly lower plasma TG and free fatty acid levels (*24*). Furthermore, transcription of the ANGPTL3 mRNA has been shown to depend on oxysterol-mediated signaling through the liver X receptor (LXR) (*25*). Previous studies indicated that oxysterol-induced and LXR-mediated increases in plasma TG levels are attributable to increases in ANGPTL3 expression (*26*). Therefore, it is likely that the decreased cholesterol levels, and therefore decreased oxysterol levels, in HepG2 cells caused by the 7S-peptide

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treatment down-regulated the expression of ANGPTL3 mRNA. Finally, it would be interesting to determine if the plasma levels of ANGPTL3 are decreased in humans eating a diet supplemented with 7S.

The mRNA of hepatocyte nuclear factor-4a, known as a transcription factor that increases plasma TG levels (*27*), was also decreased after 7S-peptide treatment. However, the beneficial effect of this is unclear, since the expression levels of both apolipoprotein C-III and microsome TG transfer protein, which are activated by hepatocyte nuclear factor-4α (*28*), were not altered by the 7S-peptide treatment.

In conclusion, this study indicated that peptides from highly purified and isoflavone-free 7S decreased TG synthesis and secretion of lipoprotein particles containing ApoB-100 from HepG2 cells. We also demonstrated that the 7S-peptide treatment lead to changes in the gene expression profile that are consistent with the reduction of hypertriglyceridemia seen in 7S-fed human volunteers. Additional studies are needed to identify the specific active peptides in the 7S-peptide preparations. In-depth analysis of these peptides will provide a more detailed molecular mechanism about beneficial aspects of 7S.

ABBREVIATIONS USED

7S, β-conglycinin; ANGPTL3, angiopoietin-like 3; ApoB-100, apolipoprotein B-100; CE, cholesteryl ester; DG, diacylglycerol; ER, endoplasmic reticulum; LDL, low-density lipoprotein; LP, lipophilic proteins; PBS, phosphate buffer saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RT-PCR, reverse transcription-polymerase chain reaction; SREBP, sterol regulatory element binding protein; TG, triacylglycerol; VLDL, very low-density lipoprotein.

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Supporting Information Available: Table of lipid metabolism-related genes that were up- or down-regulated in HepG2 cells by the addition of 7S-peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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