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Critical Role of Peroxisome Proliferator Activated Receptor-δ on Body Fat Reduction in C57BL/6J and Human Apolipoprotein E2 Transgenic Mice Fed Delipidated Soybean

Ji Hae Lee,[†] Hee-jin Jun,[†] Yaoyao Jia,[†] Wook Kim,[‡] Sung-Gil Choi,[§] and Sung-Joon Lee*[,†]

[†]Division of Food Bioscience and Technology and [‡]Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, South Korea

[§]Department of Food Science and Technology, Gyeongsang National University, Gyeongsang Namdo, Jinju, 660-701, South Korea

Supporting Information

ABSTRACT: The consumption of soy protein and fiber reduces body fat accumulation; however, the mechanism of this effect has not been clearly understood. We investigated the antiobesogenic effect of soy protein and fiber in two different mouse models. Normolipidemic nonobese C57BL/6J and hyperlipidemic obese human apolipoprotein E2 transgenic mice were fed either delipidated soybean (DLSB) containing soy protein and fiber or a control diet. The DLSB-fed mice showed a significant reduction in body weight gain and adiposity compared with controls, in both C57BL/6J and apoE2 mice. All metabolic parameters were significantly improved in the DLSB group compared with controls: total cholesterol, low-density lipoprotein cholesterol, insulin, and leptin levels were significantly reduced. Adiponectin concentrations were significantly elevated, and glucose tolerance was improved. In both types of DLSB-fed mice, the specific induction of PPAR-δ protein expression was evident in muscle and adipose tissues. The expression of PPAR-δ target genes in the DLSB-fed mice was also significantly altered. Acetyl-CoA carboxylase-1 and fatty acid synthase levels in adipose tissue were downregulated, and uncoupling protein-2 in muscle was upregulated. Intestinal expression of fatty acid transport protein-4, cluster of differentiation-36, and acyl-CoA synthetase were significantly downregulated. We propose that marked activation of PPAR-δ is the primary mechanism mediating the antiobesogenic effect of soybean and that PPAR-δ has multiple actions: induction of thermogenesis in muscle, reduction of fatty acid synthesis in adipose tissue, and reduction of fatty acid uptake in intestinal tissue.

KEYWORDS: delipidated soybean, obesity, peroxisome proliferator-activated receptor-δ, adiponectin, uncoupling protein

INTRODUCTION

The global epidemic of obesity is the fastest growing health issue worldwide. Obesity results in multiple severe clinical sequelae, including coronary heart disease, type 2 diabetes, cancer, and metabolic syndrome,[1,2] which eventually markedly shorten life expectancy.[3]

Several observational migration studies have suggested that the low prevalence of obesity in the population of Eastern Asia compared with Western societies is explained largely by dietary factors, particularly soybean consumption.[2] Accordingly, the potential health benefits of the major components of soybean, which contains protein (35−46%), dietary fiber (10−15%), unsaturated fatty acids (70−80% of total fatty acids), and bioactive phytochemicals such as isoflavone (0.1−0.3% dry weight),[4−7] have been intensively investigated.[7,8]

Obesity is strongly associated with excess energy intake and is characterized by the accumulation of excess lipid in both adipose and nonadipose tissues. Thus, the pharmacological reduction of plasma lipids and dietary lipid uptake is a potential strategy for the treatment of obesity.[9] The hypolipidemic and plasma triglyceride (TG) reducing activities of soybean have been well studied. A meta-analysis has suggested that the replacement of animal protein with soybean protein is associated with reduced plasma total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, and TG levels, and an elevated high-density lipoprotein (HDL) cholesterol level.[10] Furthermore, a decrease in plasma TG is correlated with reduced adiposity and reduced obesity.[11] The amino acid composition of soybean is lower in methionine and higher in arginine compared with animal proteins such as casein, and this has been suggested as the basis of its hypolipidemic activity.[7] Methionine is a precursor of *S*-adenosylmethionine, a key cofactor for hepatic biosynthesis of phosphatidylethanolamine, which facilitates the synthesis of hepatic very-low-density lipoprotein.[12,13] Accordingly, soy protein low in methionine may reduce plasma TG levels and adiposity. Arginine levels in soybean are on average 2-fold those in animal proteins and may mediate an antiobesogenic effect by enhancing mitochondrial biogenesis and fatty acid β-oxidation, thus reducing total adipose tissue mass.[14,15] Alternatively, antiobesogenic activity may be mediated by nitric oxide endogenously synthesized from arginine.[16] However, these mechanisms may be epiphenomenal, and the precise molecular mechanism of soy protein activity remains to be elucidated.

Evidence from *in vivo* studies showing that hepatic lipogenic enzyme activities (glucose-6-phosphate dehydrogenase, malic enzyme, fatty acid synthase, and acetyl-CoA carboxylase) were

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reduced by soy protein intake suggests that soy protein can reduce hepatic lipogenesis.[17] In addition, soybean intake has been shown to induce thermogenesis, although the mechanism remains unknown.

Soy protein may regulate lipid metabolism by activating peroxisome proliferator-activated proteins (PPARs), which are nuclear transcription factors that regulate the expression of genes involved in glucose homeostasis, lipid metabolism, and fatty acid oxidation.[18] Each of the three PPAR subtypes, PPAR-α, -γ, and -δ, binds to the peroxisome proliferator response element in a target gene promoter after heterodimerization with retinoid X receptor.[19,20] PPAR-γ coactivator-1 (PGC-1α) is subsequently recruited and regulates the transcription of the target gene.[21] The roles of PPAR-α and -γ have been widely investigated, and pharmacological ligands have been developed as therapeutic drugs.[22,23] PPAR-δ is not as well studied, but recent data have suggested a role in thermogenesis and β-oxidation in muscle and adipose tissue.[24]

The dietary fibers abundant in soybean may also contribute to its hypolipidemic and antiobesogenic effects through multiple mechanisms. Possible mechanisms include the induction of satiety, delay of digestion, and suppression of insulin secretion, which decreases intestinal absorption of nutrients and may increase dietary thermogenesis.[25] Soybean contains a significant quantity of dietary fiber (approximately 10−15%, w/w). Nevertheless, its importance in nutrition has not attracted much attention. Moreover, the combined effect of soy protein and fiber has not been investigated.

In the present study, we investigated the antiobesogenic effect of delipidated soybean, in which soy protein and fiber are the major components, in two different mouse models. The results indicate dramatic antiobesogenic effects.

MATERIALS AND METHODS

Preparation of Soybean Samples. Soybean (*Glycine max* (L.) Merr.) was purchased from the Nonghyup market (Suanbo, Korea) and was ground to produce whole soybean powder. The lipids were removed from the whole soybean powder using a supercritical carbon dioxide extraction system (Ilshin Autoclave Co., Daejeon, Korea). Supercritical carbon dioxide, produced through a high-pressure pump and chiller, was used to extract lipids from approximately 530 g of whole soybean powder in an extraction vessel, maintained at 50 °C and a pressure of 300 bar. After extraction for 8 h, the processed delipidated soybean (DLSB) sample was collected from the extraction vessel. The extraction procedure was repeated five times, and more than 95% of the total lipids were removed. The final DLSB preparation was composed of 61% protein and 39% carbohydrate, which included 15% dietary fiber, and was used for the feeding studies. Isoflavone and soysaponins were not detected by HPLC.

Animals and Diets. C57BL/6J mice (8 weeks old) purchased from Samtako (Seoul, Korea) and human apolipoprotein (apo) E2 transgenic mice (10 weeks old) purchased from Taconic (Hudson, NY, USA) were used for the experiments. Only male mice were included in the feeding study. ApoE, a major apolipoprotein, has a key role in lipoprotein metabolism. Human apoE2 transgenic mice were produced by replacing the mouse *apoE* gene with human *apoE2*, which has markedly reduced affinity for the LDL receptor. These mice develop severe hypertriglyceridemia and obesity[26] and exhibit high total TC and TG concentrations.[27] Some humans homozygous for E2, but not all, develop type III hyperlipidemia; however, in mice, nearly 100% of apoE2 homozygous mice develop severe hypertriglyceridemia and obesity. Mice were maintained under a 12 h light/dark cycle. The mice were quarantined for 1 week and then randomly assigned into three groups (n = 8 per group). The C57BL/6J mice were fed either a control or DLSB diet for 10 weeks without any prior feeding of a Western diet. The apoE2 mice were fed an AIN-76A-based Western diet (40% of calories from fat and 0.15%, w/w, cholesterol) for 5 weeks to induce obesity and exaggerated hyperlipidemia, and then were either maintained on the AIN-76A diet or switched to an isocaloric DLSB diet. Body weight was measured twice per week. At the end of the feeding period, the mice were sacrificed, and tissue samples were removed from the liver, epididymal fat, perirenal fat, mesenteric fat, intestine, and skeletal muscle. All animal experiments were performed according to a protocol approved by the Animal Experimentation Committee of Korea University (Protocol No. KUIA-CUC-20090420-4).

Blood Lipid Analysis. Blood lipid analysis was performed once a month during the feeding period. Blood samples were collected retro-orbitally after 12 h of fasting, and plasma TG, total cholesterol (TC), LDL-cholesterol, and HDL-cholesterol levels were determined using an automated clinical chemistry analyzer (Cobas111, Roche, Basel, Switzerland).[28] Plasma insulin (Alpco, Salem, NH, USA), leptin (Millipore, Bedford, MA, USA), and adiponectin (Invitrogen, Carlsbad, CA, USA) concentrations were measured by ELISA, according to the manufacturer's instructions.

Oral Glucose Tolerance Test. After 10 weeks of feeding, the mice were fasted overnight and fed glucose (1.5 g/kg body weight in PBS) by oral administration. Blood glucose concentrations were measured using a portable glucose meter (Accu-Check Go; Roche) at 0, 15, 30, 60, 90, and 120 min after glucose feeding.

Histological Analysis of Adipose Tissue. Epididymal adipose tissue was fixed in 4% formaldehyde and stained with hematoxylin and eosin. The size of the stained fat pads was determined using an upright microscope and related software (Axio Imager M1; Carl-Zeiss, Oberkochen, Germany) at the Histopathology Department of Anam Korea University Hospital (Seoul, Korea).

Total RNA Extraction. Total RNA from white adipose tissue, intestine, and muscle was isolated using a total RNA extraction reagent (RNAiso Plus; Takara Bio Inc., Shiga, Japan). After the addition of 1 mL of RNA extraction reagent, the tissue samples were homogenized and incubated for 5 min at room temperature (RT). The samples were clarified by centrifugation at 12000g for 10 min, and the supernatants were collected. The samples were extracted with 200 μL of chloroform, the hydrophilic layer was collected, and the RNA was precipitated with isopropanol. The RNA pellets were washed with 75% ethanol and dried at RT. The RNA concentration was measured spectrophotometrically (Smart Spec Plus; Bio-Rad, Hercules, CA, USA).

Reverse Transcription and Quantitative PCR Analysis. For cDNA synthesis, 2 μg of total RNA was incubated with 1 μL of oligo(dT) primer (50 pmol) and 4 μL of dNTP mixture (2.5 nM) in RNase-free water at 65 °C for 15 min, and then mixed with 1 μL of reverse transcriptase (Mbiotech, Seoul, Korea), 5× buffer, and 5 μL of RNase-free water, followed by incubation at 42 °C for 60 min and 70 °C for 15 min. The synthesized cDNA was amplified by real-time PCR (rt-PCR; iCycler iQ5; Bio-Rad) using rt-PCR premix solution containing SYBR Green (SYBR Premix Ex *Taq*II; Takara) and the appropriate primers. Amplification was performed with an initial denaturation step at 95 °C for 30 s, followed by 60 cycles of denaturation at 95 °C for 10 s, annealing at 55−61 °C for 15 s, and extension at 68 °C for 20 s. The fluorescence signal was automatically detected at the end of each PCR cycle. Primer sequences and annealing temperatures are presented in Supplementary Table 1 in the Suppporting Information.

Protein Isolation and Immunoblot Analysis. Tissue samples were lysed in buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) containing a protease inhibitor cocktail (Biobasic, Ontario, Canada) at 4 °C. The lysate was clarified by centrifugation at 12000g for 10 min at

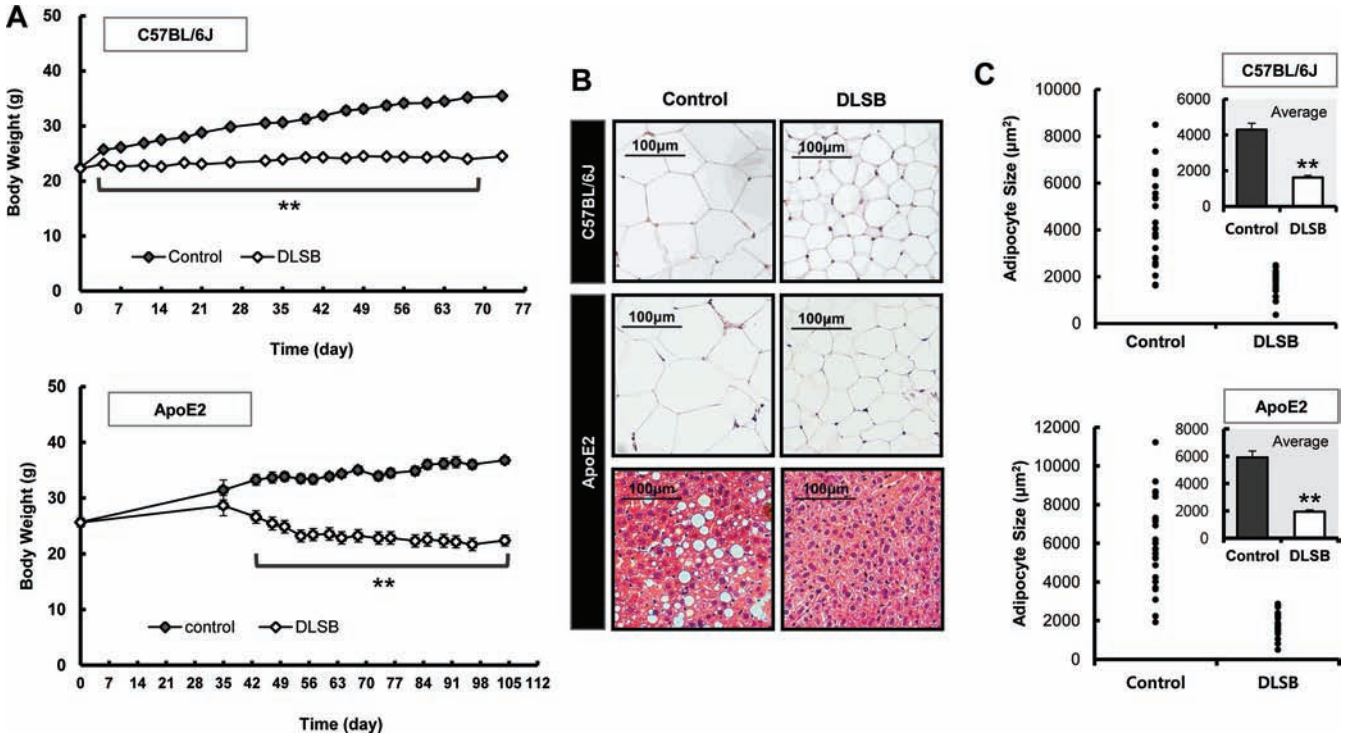

Figure 1. Body weight, adipocyte size, and liver histology of C57BL/6J and apoE2 mice after DLSB feeding. (A) Average body weight change of C57BL/6J and apoE2 mice in the control and DLSB groups during the 10-week feeding period. (B) Representative images of adipocytes in the control and DLSB groups of C57BL/6J and apoE2 mice after 10 weeks. (C) Average adipocyte size. Twenty-four adipocyte cells were selected randomly, and the cell surface area was quantified by image analysis. Adipocyte size distributions of each group are shown, and the average values for the corresponding mice are inset. Values are means ± SEM. *$P < 0.05$, **$P < 0.005$ vs controls.

4 °C, and the protein concentration was determined using Bradford reagent (Bio-Rad) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard. Protein samples (50 μg) in Laemmli sample buffer were boiled for 5 min and resolved by 10% SDS−PAGE. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell Bioscience, Dassel, Germany) at 100 V for 1 h. Nonspecific binding was blocked with 5% nonfat dry milk in TBS-T buffer for 1 h at RT, and the membranes were incubated with primary antibody overnight at 4 °C. The membranes were washed in TBS-T for 30 min and then incubated with secondary antibody overnight at 4 °C. Immunoreactive protein bands were detected using enhanced chemiluminescence (Animal Genetics, Seoul, Korea), imaged using a ChemiDoc XRS+ System (Bio-Rad), and quantified with Gel-Pro Analyzer software.[29]

Statistical Analysis. All data are expressed as means ± SEM. Student's *t*-test was performed for comparisons between two groups. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Body Weight, Body Fat Content, Adipocyte Size, and Food Intake. The body weights of the C57BL/6J and apoE2 mice were similar prior to the initiation of DLSB feeding. The control group of each mouse type continuously gained body weight, whereas the DLSB group showed either marginal weight gain (C57BL/6J) or reduced weight (apoE2) (Figure 1A). The weight difference between the control and DLSB group increased gradually for both mouse types. The total body weight changes at 10 weeks in the control and DLSB groups were +13.0 ± 1.6 and +2.0 ± 1.1 g, respectively, in C57BL/6J mice and +5.3 ± 1.5 and −7.9 ± 1.8 g, respectively, in apoE2 mice. Representative images of animals in each group are shown in Supplementary Figure 1A in the Suppporting Information. Body fat mass and average adipocyte size were dramatically reduced in the DLSB group compared with the control group (Figure 1B,C; Supplementary Figure 1B in the Suppporting Information). Thus, DLSB consumption significantly decreased total body fat content.

In both mouse types, the liver weight was significantly reduced in the DLSB group compared with the control group. Liver weight in apoE2 mice fed the Western fat diet was significantly increased; however, the liver-to-body weight ratio was not significantly different between control C57BL/6J and control apoE2 mice (Supplementary Table 2 in the Suppporting Information). Total adipose tissue weight was dramatically reduced after DLSB feeding in both types of mice (4.6 ± 0.2 and 1.2 ± 0.1 g, respectively, in apoE2 control and DLSB groups; $P < 0.005$, tissue images are shown in Supplementary Figure 1B in the Suppporting Information). In both types of mice, the average adipocyte size was significantly smaller in the DLSB group than in the control group (38% and 33% in C57BL/6J and apoE2 mice; Figure 1B,C). ApoE2 livers developed overt steatosis, and this was dramatically improved after DLSB feeding, as demonstrated by the disappearance of lipid droplets in the liver sections (Figure 1B, bottom). Food intake did not differ significantly between the DLSB and control groups in the C57BL/6J mice (2.8 ± 0.1 and 2.6 ± 0.1 g/day, respectively) or the apoE2 mice (3.0 ± 0.1 and 2.9 ± 0.1 g/day, respectively).

Lipid Analysis. DLSB feeding showed potent hypolipidemic effects in apoE2 mice; the plasma TG level was 70% lower in the DLSB group compared with the control group at week 10. In

Table 1. Concentrations of Plasma Lipids and Hormones[a]

		plasma lipid						plasma hormone		
group	plasma glucose (mg/dL)	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	HDL/TC	HDL/LDL	insulin (ng/dL)	leptin (ng/dL)	adiponectin (μg/dL)
C57BL/6J										
control	87 ± 10.4	65.8 ± 9.8	141.0 ± 4.2	120.1 ± 3.5	16.0 ± 1.6	0.8 ± 0.1	7.5 ± 0.5	0.31 ± 0.02	0.52 ± 0.07	33.69 ± 1.04
DLSB	69.5 ± 5.0	63.5 ± 3.4	111.6 ± 2.9**	100.6 ± 2.1**	9.4 ± 0.6**	1.0 ± 0.0*	10.7 ± 0.7**	0.26 ± 0.00**	0.12 ± 0.01**	41.01 ± 2.80*
AopE2										
control	154.4 ± 9.8	344.7 ± 16.6	539.4 ± 32.1	149.1 ± 2.7	280.9 ± 13.5	0.3 ± 0.0	0.5 ± 0.0	0.18 ± 0.03	21.53 ± 1.44	28.90 ± 0.19
DLSB	98.4 ± 3.7**	104.3 ± 14.5**	196.2 ± 11.8**	108.3 ± 6.5**	53.5 ± 6.7**	0.6 ± 0.0**	2.0 ± 0.4**	0.09 ± 0.00**	3.62 ± 0.52**	61.15 ± 0.67**

[a] Data were calculated after the 10 week feeding period. Values are means ± SEM, *P < 0.05, **P < 0.005 vs control.

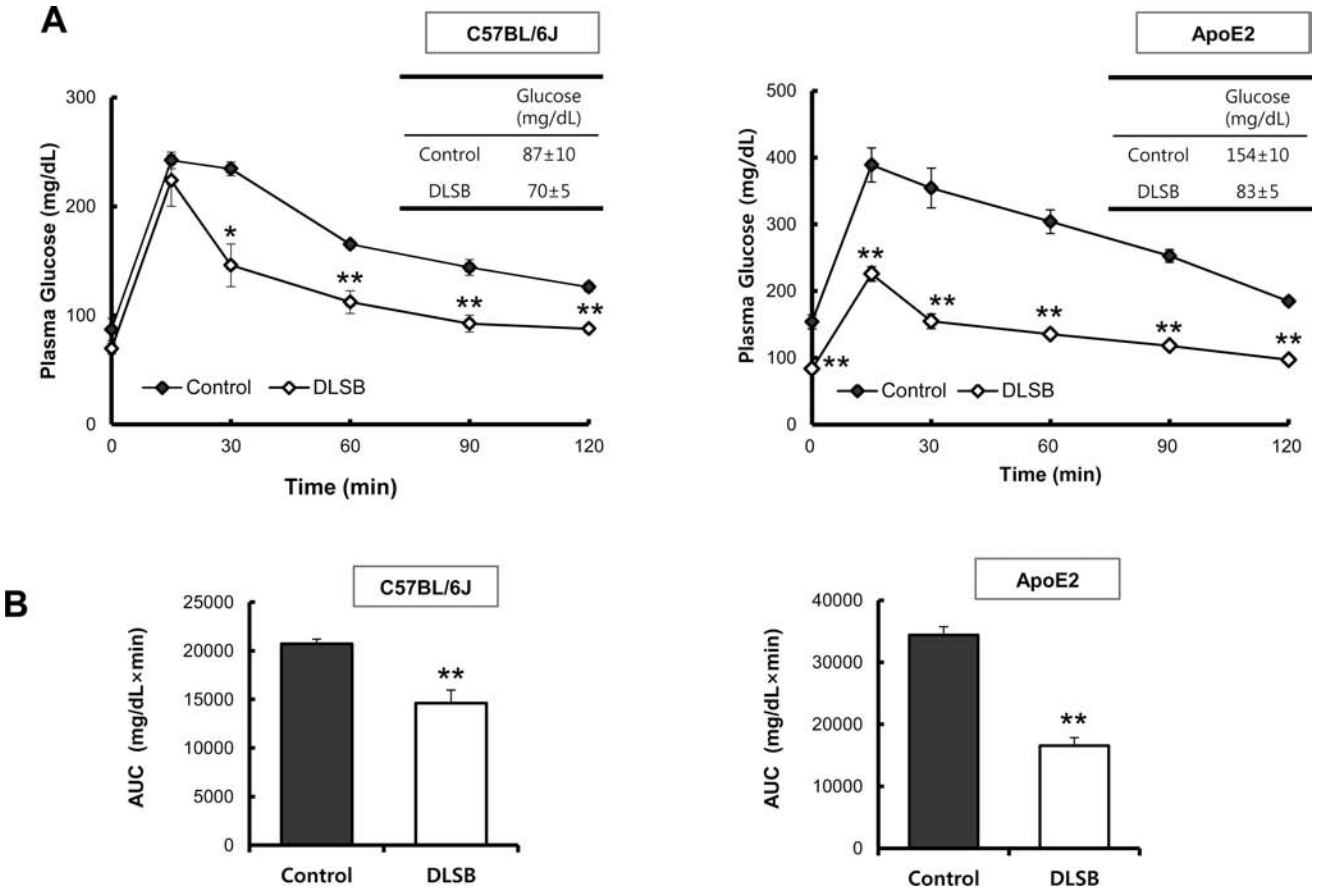

Figure 2. Improved glucose tolerance in C57BL/6J and apoE2 mice after DLSB feeding. (A) Oral glucose tolerance testing of the control and DLSB groups of C57BL/6J and apoE2 mice. Values in the table are the glucose levels at time zero. (B) Area under the curve calculated for the oral glucose tolerance test curve of the control and DLSB-fed C57BL/6J and apoE2 mice. Values are means ± SEM. *P < 0.05, **P < 0.005 vs controls.

contrast, DLSB feeding did not alter the TG concentration in C57BL/6J mice (Table 1). The TC and LDL-cholesterol concentrations were 21 and 42% lower, respectively, in the apoE2 DLSB group and 64 and 81% lower, respectively, in the C57BL/6J DLSB group compared with the respective control groups. For both mouse types, the HDL-cholesterol level was reduced after 10 weeks in the DLSB group compared with the control group. Nevertheless, the ratios of HDL-cholesterol to TC and HDL-cholesterol to LDL-cholesterol were significantly increased in the DLSB group compared with the control group for both types of mice (Table 1).

Glucose Tolerance. At week 10, the fasting glucose level was significantly reduced by 46% in the DLSB group compared with the control group of hyperglycemic apoE2 mice, whereas the level was unaltered in the normoglycemic C57BL/6J mice (Table 1). Based on the oral glucose tolerance test, both types of DLSB-fed mice showed faster removal of glucose from the circulation compared with the control groups (Figure 2A,B). In the apoE2 mice, the glucose concentration in DLSB mice at 30 min was 50% of the peak value at 15 min, whereas in the control group, it took nearly 2 h to achieve a 50% reduction in the peak glucose concentration at 15 min (Figure 2A). Accordingly, the

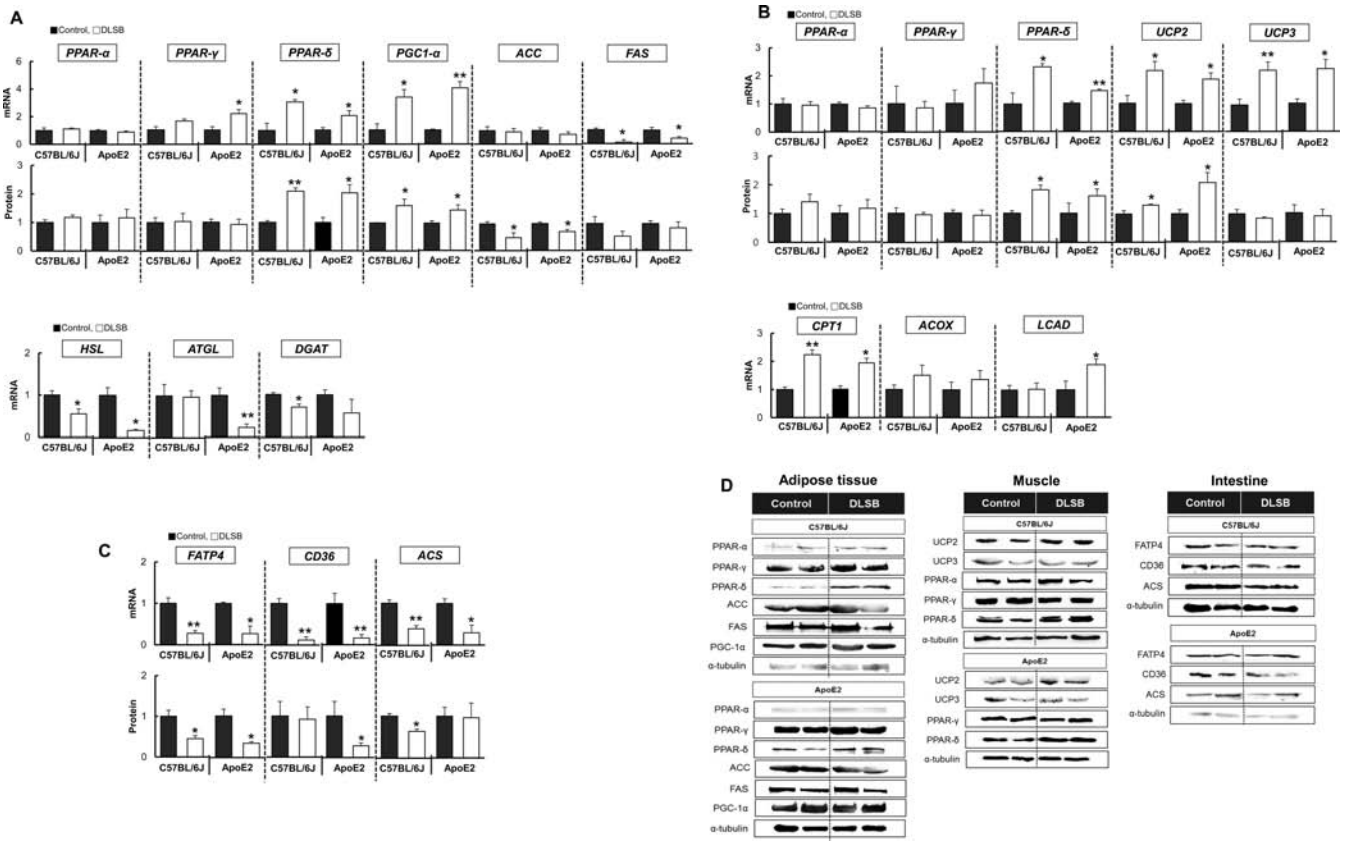

Figure 3. Activation of PPAR-δ expression and regulation of its target genes in adipose, muscle, and intestine tissues of C57BL/6J and apoE2 mice after DLSB feeding. mRNA and protein expression levels were assessed by real-time PCR and immunoblot analysis respectively. Expression of (A) PPAR-α, PPAR-γ, PPAR-δ, PGC-1α, HSL, ATGL, ACC, FAS, and DGAT in adipose tissue; (B) PPAR-α, PPAR-γ, PPAR-δ, CPT1, ACO, LCAD, UCP2 and UCP3 in muscle tissue; and (C) FATP4, CD36, and ACS in intestinal tissue. (D) Immunoblot images. Values are means ± SEM. *P < 0.05, **P < 0.005 vs controls.

area under the oral glucose tolerance test curve showed significant reductions in the DLSB groups (71% and 48% for C57BL/6J and apoE2 mice, respectively) compared with the control groups (Figure 2B).

Insulin, Leptin, and Adiponectin. Plasma insulin and leptin concentrations were significantly reduced and adiponectin levels were significantly elevated in the mice fed DLSB compared with the control mice. In the apoE2 mice, the insulin, leptin, and adiponectin levels changed by −50, −83%, and +112%, respectively, compared with the control levels (P < 0.05 for all; Table 1). The C57BL/6J mice showed a similar trend.

The mRNA and Protein Expressions in Adipose Tissue. The expression levels of several genes in fatty acid and lipid metabolism were quantified in the adipose, muscle, and intestinal tissues of both C57BL6/J and apoE2 mice after DLSB feeding. In adipose tissues, the expression levels of three PPAR isoforms, PGC-1α, and their target genes were assessed by quantitative real-time PCR (Figure 3A). PPAR-α expression was similar between the control and DLSB groups (+11% and −11% in the C57BL/6J DLSB and apoE2 DLSB groups, respectively, compared with the corresponding controls), and PPAR-γ gene expression was induced in both DLSB groups (+61%, P = 0.12 and +114%, P = 0.03 in the C57BL/6J DLSB and apoE2 DLSB groups, respectively, compared with the corresponding controls). However, PPAR-δ expression showed dramatic induction in the adipose tissues of the DLSB group in both types of mice, with 3.03-fold (P = 0.02) and 2.0-fold (P = 0.05) induction in C57BL/6J and apoE2 mice, respectively, compared with the controls. PGC-1α was also upregulated in adipose tissues of the DLSB groups in both mouse types: +225% and +296% in the C57BL/6J (P = 0.04) and apoE2 (P = 0.003) DLSB groups, respectively, compared with the corresponding controls. The expression levels of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL/desnutrin/PNPLA2) were downregulated by 45 and 3%, respectively, in the C57BL/6J DLSB group (P = 0.04, 0.77) and by 83 and 77%, respectively, in the apoE2 DLSB group (P < 0.05 for both), compared with the corresponding control groups. This downregulation may be attributable to a reduction of stored lipids in adipose tissue. The expression levels of the lipogenesis genes acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and acyl-CoA:diacylglycerol acyltransferase-1 (DGAT-1) were downregulated by 10, 90, and 30% in adipose tissues of C57BL/6J DLSB mice (P = 0.8, 0.02, and 0.03) and by 29, 60, and 43% in apoE2 DLSB mice (P = 0.1, 0.02, and 0.15) compared with controls.

Protein expression was quantified by immunoblot analysis. The protein levels were generally similar to the gene expression patterns (Figure 3A). PPAR-α and PPAR-γ protein levels in adipose tissue of DLSB-fed mice were not significantly different from those of control mice for both types of mice, mirroring the qPCR data. However, PPAR-δ and PGC-1α were significantly upregulated in adipose tissue in the DLSB group compared with

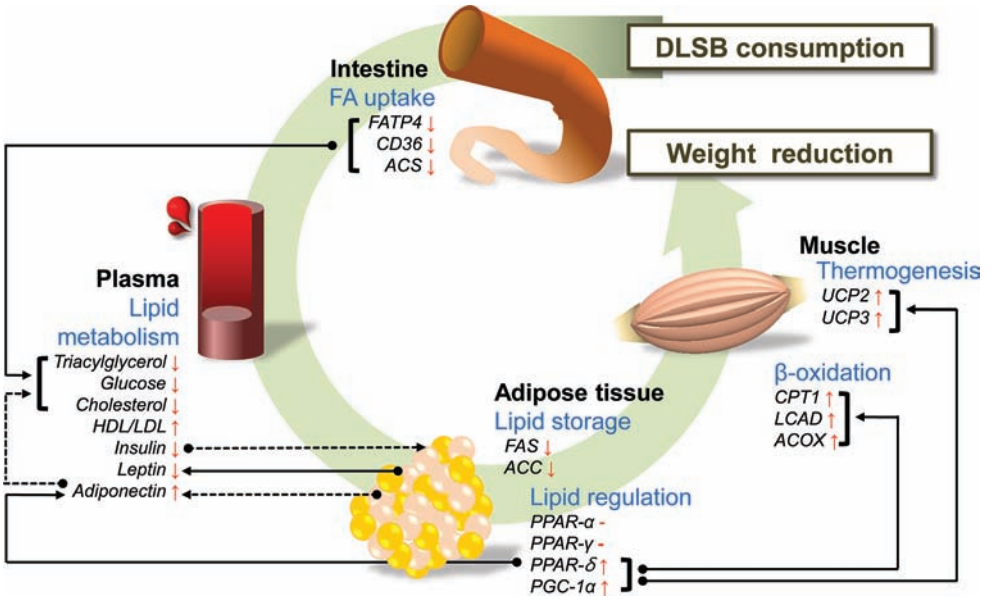

Figure 4. Proposed mechanism of the antiobesogenic effects of DLSB. Solid lines indicate positive regulation of the corresponding target proteins, and dotted lines indicate negative regulation.

controls in both mouse types; PPAR-δ expression in C57BL/6J and apoE2 DLSB mice was induced by 2.1-fold ($P = 0.003$) and 2.0-fold ($P = 0.04$), respectively, and PGC-1α expression showed a similar trend in C57BL/6J (1.6-fold, $P = 0.05$) and apoE2 mice (1.5-fold, $P = 0.05$). The protein levels of ACC, which is involved in adipocyte lipogenesis, were reduced in adipose tissue in the DLSB group compared with controls in C57BL/6J mice (51%, $P = 0.05$) and in apoE2 mice (30%,$P = 0.04$).

The mRNA and Protein Expressions in Muscle Tissue. The expression levels of three PPAR isoforms and six key fatty acid metabolism genes were quantified in muscle tissue (Figure 3B). The pattern of PPAR isoform expression in muscle was similar to the pattern in adipose tissue. PPAR-δ was upregulated in both C57BL/6J (2.4-fold; $P = 0.02$) and apoE2 (1.4-fold $P < 0.005$) DLSB mice compared with controls. The PPAR-α and PPAR-γ expression levels were marginally altered, with respective changes of −4% ($P = 0.8$) and −15% ($P = 0.7$) in C57BL/6J DLSB mice and −14% ($P = 0.07$) and +70% ($P = 0.4$) in apoE2 DLSB mice compared with the corresponding controls. The increases in acyl-CoA oxidase-1 (ACOX) expression (50%) and long-chain acyl-CoA dehydrogenase (LCAD) expression (3%) were not significant in C57BL/6J DLSB mice ($P = 0.16$, 0.09, respectively, vs controls), whereas carnitine palmitoyl transferase-1 (CPT1) expression was significantly increased (+140%; $P < 0.005$ vs controls). Additionally, the expression of uncoupling proteins (UCPs) was dramatically upregulated in C57BL/6J DLSB mice, with UCP2 and UCP3 levels that were 116 and 131% of control levels, respectively, in the muscle tissue of DLSB-fed mice. In apoE2 mice, the expression levels of several genes involved in fatty acid β-oxidation were higher in the DLSB group compared with the control group: CPT1 was increased by 92% ($P = 0.03$); and LCAD, by 90% ($P = 0.04$). UCP expression was also increased in DLSB-fed apoE2 mice, by 1.9-fold (UCP2) and 2.2-fold (UCP3) ($P = 0.04$ for both vs controls).

The PPAR-α and PPAR-γ protein levels in muscle tissues of DLSB-fed mice did not differ significantly from those of the control in C57BL/6J mice (1.4-fold and 0.9-fold, respectively; $P = 0.2$, 0.8 vs control) or apoE2 mice (1.2-fold and 0.9-fold, respectively; $P = 0.7$, 0.6 vs control), whereas PPAR-δ levels were significantly higher in the DLSB group of C57BL/6J mice (1.8-fold; $P = 0.005$ vs control) and apoE2 mice (1.6-fold; $P = 0.03$ vs control). The UCP2 protein level was higher in the DLSB group for both types of mice (+32 and +101% in C57BL/6J and apoE2 mice, respectively; $P = 0.005$, 0.04 vs control). Compared with the levels in the control groups, the UCP3 protein levels were not significantly altered in the DLSB groups of C57BL/6J and apoE2 mice, respectively ($P = 0.4$, 0.8 vs controls).

The mRNA and Protein Expressions in Intestine Tissue. The expression levels of fatty acid transport protein-4 (FATP4), CD36, and acyl-CoA synthetase-1 (ACS1), which are all genes involved in intestinal fatty acid uptake, were quantified in intestinal tissues (Figure 3C). Compared with the levels in the controls, the expression of FATP4, CD36, and ACS in the intestines was significantly lower in DLSB-fed C57BL6/J and apoE2 mice, with respective changes of −73, −88, and −60% in C57BL6J DLSB mice ($P < 0.005$ vs control for all) and changes of −73, −84, and −71% in apoE2 mice ($P = 0.03$, $P < 0.005$, and $P = 0.01$, respectively, vs controls). The protein levels of these three were lower in DLSB-fed mice than in the controls. In C57BL/6J mice, FATP4 (0.45-fold; $P = 0.03$) and ACS (0.64-fold; $P = 0.01$) protein expression was downregulated in the DLSB group; however, CD36 protein expression was not significantly different in the DLSB group (−0.9-fold; $P = 0.86$ vs control). In apoE2 mice, CD36 protein expression was significantly lower in the DLSB group compared with the control (0.28-fold; $P = 0.05$), but the ACS protein was not (0.96-fold; $P = 0.93$). The change in the FATP4 protein level (0.34-fold; $P = 0.02$ vs control) was similar to that in C57BL/6J mice. The immunoblots are shown in Figure 3D.

DISCUSSION

We investigated the combined antiobesogenic effect of soy protein and fiber *in vivo* using two mouse models, normolipidemic

nonobese C57BL/6J and hyperlipidemic obese apoE2 mice (Figure 4). The effect of dietary soy protein has been studied by several groups, and the data consistently suggest that soy protein feeding reduces body fat. Replacing animal protein with soy protein has been shown to lower body fat content in both animals and humans. The mechanism by which soy protein exerts its beneficial effects on obesity is not clear, although several lines of evidence suggest that soy protein may favorably affect intestinal lipid absorption,[30] insulin resistance,[31] fatty acid metabolism in hepatocyte and adipocytes,[32] and other hormonal, cellular, or molecular changes associated with adiposity.[33]

In line with previous results, the C57BL/6J and apoE2 mice fed a DLSB diet showed markedly reduced body fat content and improved insulin sensitivity, a hallmark of obesity, compared with controls. Total body fat mass and average adipose cell size were significantly reduced. Plasma levels of adipokines, which are common indicators of obesity,[34] were improved with DLSB feeding. The upregulation of PPAR-δ along with alterations in the expression of its target genes and proteins in muscle and adipose tissues was also upregulation in the DLSB group. As far as we know, this is the first *in vivo* demonstration of the critical role of PPAR-δ in soy protein feeding.

The activation of PPAR upon ligand binding facilitates dimerization with retinoid X receptor (RXR), and this dimer mediates transcriptional activation of target genes involved primarily in energy metabolism. PPAR-α and PPAR-γ were highly expressed in liver and adipose tissues, respectively, whereas PPAR-δ was ubiquitously expressed. Thus, the activation of PPAR-δ may have a powerful metabolic effect on fatty acid metabolism and biogenesis in a variety of tissues.[35]

The effect of a DLSB diet in the present study was greater than previously reported effects. This may be attributable to the combined actions of soy protein and fiber, each of which has independent antiobesogenic mechanisms. The effect of soy protein on weight reduction has been reported to be due primarily to its amino acid.[7,36] As described in the Introduction, soy protein contains low methionine and high arginine levels, which may regulate plasma TG synthesis and cellular fatty acid oxidation, respectively. Soy fiber acts mainly in the GI tract to enhance satiety and reduce lipid uptake, possibly by inhibiting bile acid resorption and cholesterol and fatty acid uptake.[37] Some studies have suggested that soy isoflavone reduces body fat content and plasma lipid concentrations through multiple mechanisms, including the modulation of AMP-activated protein kinase activity[38] and the increase of fatty acid oxidation and PGC-1α-dependent mitochondrial biogenesis.[39] However, soy isoflavone was not detected in the DLSB; thus we were able to rule out potential effect from isoflavone.

Our data suggest that soy protein and fiber have additive effects on body weight reduction in mice. Interestingly, soy fiber appears to be highly effective in weight control compared with the cellulose fiber in the control diet. Several studies, including ours, have demonstrated the potent hypocholesterolemic effect of soy fiber;[40] however, dietary fiber has thus far not been the focus of biological activity studies with soybean. Our results suggest that soy fiber contributes significantly to body fat reduction and thus the efficacy of soy fiber should be investigated further, more specifically in comparison with fibers from other sources.

DLSB feeding lowered TC and LDL-cholesterol in both mouse types and reduced the ratios of HDL-cholesterol to TC and HDL-cholesterol to LDL-cholesterol, suggesting an improved lipoprotein profile in the DLSB group. In addition, DLSB significantly increased adiponectin levels while reducing leptin concentrations in both C57BL/6J and apoE2 mice. Leptin, a major hormone for satiety control in the hypothalamus, suppresses energy intake and increases energy expenditure in peripheral tissues. Indeed, reduced plasma leptin levels have been associated with improved leptin sensitivity, weight loss, and reduced adiposity.[41] Plasma adiponectin functions somewhat opposite to leptin in the regulation of adiposity; an elevated plasma adiponectin level is associated with improved symptoms in metabolic syndrome, insulin resistance, obesity, type 2 diabetes, and heart disease.[42] Alterations in the levels of these two adipokines with DLSB feeding, whether an epiphenomenal result of weight reduction or a direct effect of DLSB, would contribute to the improved insulin sensitivity in C57BL/6J and apoE2 mice. Insulin promotes lipogenesis while inhibiting lipolysis. Thus, the reduction in plasma insulin levels in DLSB-fed mice probably contributed to decreased weight and reduced adiposity. These hormonal changes indicate that DLSB feeding improved lipid and glucose metabolism in both types of mice.

It has been suggested that soybean intake can suppress appetite and food intake in humans owing to its high satiating and thermogenic characteristics. The secretion of peptide YY (PYY), a known inhibitor of food intake in humans and rodents, has been shown to increase after soy protein consumption in humans.[43] Soy protein has been reported to suppress appetite and food intake more efficiently than animal protein in both animals and humans;[44,45] however, our data did not show a significant change in food intake with DLSB, suggesting that the changes in metabolism and body composition in our study were due to DLSB itself and were not a result of reduced energy intake.

To define the effect of DLSB on body weight regulation, we investigated the expression of PPAR and its target genes in adipose and muscle tissue. As described previously, PPAR-α and PPAR-γ induction was relatively small compared with PPAR-δ upregulation in the DLSB group. PPAR-δ was ubiquitously expressed; thus, the activation of PPAR-δ may have a powerful metabolic effect on fatty acid metabolism and biogenesis in a variety of tissues.[35]

Somewhat similar to PPAR-α, PPAR-δ induces the transcription of genes involved in fatty acid oxidation and fat burning, but to a higher degree. This potent effect, which was confirmed in our results, may be partly attributable to the high expression levels of PPAR-δ in various tissues. The expression of genes involved in fatty acid β-oxidation and thermogenesis, which promote fat burning and thereby contribute to weight loss,[46] were notably induced in adipose and muscle tissues of the DLSB-fed mice. In a previous study, the administration of a PPAR-δ agonist prevented diet-induced obesity and insulin resistance by increasing the β-oxidation and reduction of lipids in muscle tissue.[47] To investigate the antiobesogenic effect of soybean, we focused on the expression of PPAR-δ and PPAR-δ target genes that regulate β-oxidation and thermogenesis in adipose and muscle tissue.

We quantified PPAR-δ lipogenesis- and lipolysis-associated target gene expression in adipose and muscle tissue, because their combined activity may determine stored fat content *in vivo*. The DLSB-fed mice exhibited downregulation of lipogenesis genes and upregulation of lipolysis genes *via* PPAR-δ induction. This resulted in smaller adipocytes and reduced fat tissue content in the DLSB group compared with controls, in both C57BL/6J and apoE2 mice. ACC, FAS, and DGAT-1 play key roles in lipogenesis. ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA,

which is the rate-limiting step in fatty acid synthesis;[48] FAS is a protein complex that catalyzes the synthesis of saturated fatty acids from malonyl-CoA;[49] and DGAT-1 produces TG from diacylglycerol stored in adipose tissues.[50] On the other hand, the lipolysis-associated ATGL and HSL proteins hydrolyze TG and liberate fatty acids and glycerol for energy production.[51]

In the ligand-dependent PPAR activation, the activating function-2 (AF-2) helix of the ligand binding domain of PPAR has been shown to close on the ligand binding site, and the resulting active form of the receptor binds a coactivator for transcriptional activation. The ligand binding domain of PPARs possesses a large cavity with a total volume of 1300−1400 Å^3, which is substantially larger than those found in other nuclear receptors. This may facilitate the binding of various compounds with low affinity. Specifically, the cavity of PPARs is Y-shaped and includes a linear extension with two pockets, the polar small arm I and the nonpolar large arm II. Arm II plays a key role in binding to natural hydrophobic ligands.[52]

PPAR ligands are small hydrophobic molecules with molecular weights of 280−580 g/mol and commonly have a hydrophilic head and hydrophobic tail. All known natural PPAR ligands are lipid molecules such as fatty acids, phospholipids, and eicosanoids. Known synthetic ligands are fatty acid- or L-tyrosine-based small hydrophobic molecules.[53]

In DLSB, no substantial quantities of neutral fat or fatty acids were present, and thus it is not likely that fatty acids or their derivatives were interacting with PPAR-δ. As sugar molecules derived from carbohydrates are hydrophilic, they would not interact with PPAR-δ, and dietary fiber is not delivered via the circulation. Soy isoflavones were undetected in DLSB. Additionally, our preliminary BIAcore 3000 results based on the surface plasmon resonance technique showed that soy isoflavones did not exhibit a significant affinity against the ligand binding domain of PPARs (data not shown). Accordingly, we cannot rule out the possibility that a soy-derived small peptide may function as a ligand for PPAR-δ to regulate fatty acid metabolism and induce thermogenesis. Small peptides containing two to four amino acids, especially hydrophobic amino acids such as aromatic or branched amino acids, may be able to interact with the ligand binding domain cavity of PPAR-δ. Our preliminary data from an *in silico* modeling experiment using a series of small peptides and the ligand binding domain of PPAR and data from other group[54] suggest this hypothesis, and the possibility will be examined in the future. Alternatively, it is possible that soy protein or fiber intake could indirectly activate PPAR-δ by promoting the synthesis of an endogenous ligand.

With DLSB feeding, PGC-1α expression was also upregulated in adipose tissue. Originally discovered as a cold-inducible coactivator of PPAR-γ in brown adipose tissue, PGC-1α is a potent coactivator of a number of transcription factors, including PPAR-α and PPAR-δ.[55] In this way, PGC-1α regulates energy expenditure, resulting in increased fatty acid and glucose oxidation.[56] Thus, the upregulation of PGC-1α together with PPAR-δ may have a strong effect on cellular lipid metabolism, especially in muscle and adipose tissue, and may thereby reduce body fat content. As a transcription coactivator, PGC-1α is a key regulator of processes involved in energy metabolism, including mitochondrial biogenesis, fatty acid oxidation, and glucose metabolism.[57] PPAR-δ, but not PPAR-α, activates PGC-1α by binding to the peroxisome proliferator response element in the promoter site.[58] Therefore, by upregulating PGC-1α, PPAR-δ further induces mitochondrial biogenesis and respiration, causing an increase in energy consumption.

Additionally, we assessed the expression of the intestinal fatty acid uptake genes FATP4, CD36, and ACS. In the intestine, fatty acids can be taken up into cells directly by FATP4 or CD36, or fatty acids bound to CD36 can be transferred to FATP4 for uptake.[59] In the cytosol, ACS esterifies fatty acids to coenzyme A, and the overexpression of ACS has been shown to enhance fatty acid uptake.[60]

Our data indicate that FATP4, CD36, and ACS expression was significantly reduced in the intestines of DLSB-fed C57BL/6J and apoE2 mice. This may explain, at least in part, the observed reduction in body weight.

In conclusion, we propose that the activation of PPAR-δ, especially in adipocytes and muscle, is the key mechanism underlying the antiobesogenic effect of soybean protein and fiber and that PPAR-δ has multiple effects, including the induction of thermogenesis in muscle, the reduction of fatty acid synthesis in adipose tissue, and the reduction of fatty acid uptake in intestinal tissue.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary Figure 1 depicting representative images of fat tissues in C57BL/6J and apoE2 mice after DLSB feeding: (A) Images of representative mice in each group. (B) Adipose fat pad. Black arrows indicate fat pads. Supplementary Tables 1 (sequences of real-time PCR primers) and 2 (liver and adipose tissue weights). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: junelee@korea.ac.kr. Tel: 82-2-3290-3029.

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■ ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase-1; ACS, acyl-CoA synthetase-1; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyl transferase-1; DGAT, acyl-CoA:diacylglycerol acyltransferase-1; DLSB, delipidated soybean; FAS, fatty acid synthase; FATP4, fatty acid transport protein-4; HDL, high-density lipoprotein cholesterol; HSL, hormone sensitive lipase; LCAD, long-chain acyl-CoA dehydrogenase; LDL, low-density lipoprotein cholesterol; PGC, peroxisome proliferator-activated receptor gamma coactivator; PPAR, peroxisome proliferator-activated receptor; PYY, peptide YY; RXR, retinoid X receptor; TC, total cholesterol; TG, triacylglycerol; UCP, uncoupling proteins

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