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High Molecular Weight Poly-Gamma-Glutamic Acid Regulates Lipid Metabolism in Rats Fed a High-Fat Diet and Humans

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We investigated the effect of high molecular weight poly-gamma-glutamic acid (hm γ-PGA) on adiposity and lipid metabolism of rats in the presence of an obesity-inducing diet. Thirty-two Sprague–Dawley rats were fed either a normal-fat (11.4% kcal fat, NFC) or high-fat (51% kcal fat, HFC) diet. After 5 weeks, half of each diet-fed group was treated with hm γ-PGA (NFP or HFP) for 4 weeks. The HFC group had significantly higher body weight, visceral fat mass, fasting serum levels of total cholesterol, LDL cholesterol, and leptin, and lower serum HDL cholesterol level compared with those of the NFC group ($p < 0.05$). Treatment with hm γ-PGA decreased body weight gain and perirenal fat mass ($p<0.05$), fasting serum total cholesterol, and mRNA expression of glucose-6-phosphate dehydrogenase (G6PD), regardless of dietary fat contents ($p < 0.01$). However, hm γ-PGA increased serum HDL cholesterol in the HFC group ($p < 0.05$). *In vitro*, 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase activity was suppressed by the addition of hm γ-PGA. In agreement with observations in animal study, the supplementation of hm γ-PGA (150 mg/day) to 20 female subjects in an 8-week double-blind, placebo-controlled study resulted in a tendency to decrease total cholesterol and LDL cholesterol concentrations. We thus conclude that dietary supplementation of hm γ-PGA may act as a hypocholestrolemic agent, secondary to its inhibitor effect on HMG-CoA reductase, and decrease abdominal adiposity by decreasing hepatic lipogenesis. The present study is an important first step in establishing the effect of hm γ-PGA on cholesterol levels in rats and humans.

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In recent decades, obesity has become an epidemic in many countries worldwide and is closely related to the development of diabetes, dyslipidemia, hypertension, and other metabolic diseases [15]. Atherosclerosis is one of the major risk factors for cardiovascular disease, and cholesterol may play an important role in its development [7]. Lipid metabolism, a complicated process that is highly regulated by various factors such as hormones and peptides, is influenced by diet [20, 31]. Numerous natural substances have been proposed as effective ways to regulate lipid metabolism in animal models and humans [1, 11, 14].

In recent years, there has been a considerable interest in the effects of soybean and soy-based products on human health [9, 33]. *Chungkookjang* and *natto*, traditional Korean and Japanese soybean products, respectively, are prepared by fermenting steamed soybeans with *Bacillus* species for 2 or 3 days without salt or other seasoning. During fermentation, *Bacillus* strains produce extracellular viscous materials in the stationary phase, along with the development of spores [16].

Studies of the bioactive effects of *chungkookjang* components have reported that *chungkookjang* increases insulin sensitivity, antioxidative capacity [22, 24, 25], and fibrinolytic activity, and possesses antidiabetic, antioxidative, and hypocholesterolemic properties [24, 26, 38]. The main source of the beneficial effects associated with *chungkookjang* and other fermented soybean foods is thought to be isoflavones. Glycoside isoflavones in soybeans are known to be hydrolyzed by β-glycosidase produced by microorganisms, thereby increasing aglycone isoflavones during fermentation [18]. Another study showed that poly-gamma-glutamic acid (γ-PGA) from water-soluble fractions of *chungkookjang*

and *natto* has antioxidative functions [17]. *Bacillus subtilis* (natto) and *B. subtilis* subsp. *chungkookjang* have been used as the starter strains of *natto* and *chungkookjang* and produce different sizes of γ-PGA depending on the bacterial strains used. The former produces the predicted molecular weight product, which ranges widely from 10 kDa to 200 kDa, in addition to various other components such as polysaccharides. Sung *et al.* [38] reported that PGA produced using *B. subtilis* subsp. *chungkookjang* as the biocatalyst led to the synthesis of high molecular weight γ-PGA (over 2,000 kDa) with effective purification of the polymer [38]. A recent study reported that high molecular weight γ-PGA of over 2,000 kDa (hm γ-PGA) enhanced immune function [21]. In addition, *chungkookjang* or *natto* powder was found to improve lipid metabolism in diabetic rats [38]. However, although hm γ-PGA is thought to play an independent physiological role, little research has been done on this topic.

In the present study, we explored the effects of hm γ-PGA produced using *B. subtilis* subsp. *chungkookjang* on lipid metabolism, adiposity, and hepatic lipogenic enzyme expression in rats fed a fat-rich diet. A pilot study was performed to examine the weight-loss and lipid-lowering effects of hm γ-PGA in humans.

Materials and Methods

Preparation and Molecular Weight Determination of Poly-γ-Glutamic Acid

High molecular weight γ-PGA (hm γ-PGA) derived from *Bacillus subtilis* subsp. *chungkookjang* was prepared at a pilot-scale plant (BioLeaders Corporation, Daejeon, Korea) as described previously [38]. Samples of γ-PGA with molecular masses of 1, 10, 50, 500, and 2,000 kDa were prepared by gel permeation chromatography (GPC) using polyacrylamide (1–9,000 kDa) as a standard material. γ-PGA solution was diluted with 0.1 M $NaNO_3$ and injected into a GPC system equipped with a ViscoGel $GMPW_{XL}$ column (7.8 mm × 30 cm; Viscotek, Houston, TX, USA) at 40°C and a flow rate of 0.8 ml/min. γ-PGA was detected with a Viscotek LR25 laser refractometer and polyacrylamide was used as the standard material for molecular weight determination. Viscosity was determined by a viscometer (DV+I; Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) at 25°C. The γ-PGA samples were used to confirm inhibitory effects in HMG-CoA reductase, and hm-γ-PGA (> 2,000 kDa) was used in rat and human clinical studies.

Animals and Diets

Ten-week-old male Sprague–Dawley rats were obtained from Charles River Laboratory (Orient Bio Co., Sungnam, Korea) and fed a standard laboratory diet (Samyang Co., Seoul, Korea) for 1 week to allow all metabolic measurements to acclimate. Each cage contained one rat in a temperature- and humidity-controlled room set to a 12/12 h light/dark cycle (lights on from 6:00 AM to 6:00 PM) and maintained at 22 ± 1°C with 50 ± 10% humidity. After an adaptation period, rats were randomly divided into two groups: one group was raised on a normal-fat diet (NFC, 10% kcal fat), and the other was raised on a high-fat diet (HFC, 45% kcal fat) for 5 weeks to induce obesity. Rats from each group were then further divided into two groups and fed the same fat diet as they had been receiving (NFC or HFC) for an additional 5 weeks with or without hm γ-PGA [0.1% (w/w)]. On a caloric basis, the high-fat diet consisted of 51.2% fat, 29.2% carbohydrate, and 19.6% protein (5.09 cal/g), whereas the normal-fat diet contained 10.6% fat, 68.4% carbohydrate, and 21.0% protein (3.80 kcal/g). We added hm γ-PGA to the diet instead of a portion of the cornstarch. Experimental diet compositions are shown in Table 2. The total food intake of each rat was recorded three times weekly, and body weight was measured once weekly using a top-loading balance. At the end of the experimental period, rats were sacrificed by decapitation. Livers and epididymal and perirenal white adipose tissues were quickly excised and weighed. All organs were carefully cleaned from the surrounding muscular tissue and other adipose tissue prior to being weighed. Blood was collected after a 16 h fast and serum was separated and stored at −70°C until analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kookmin University and followed the National Research Council guidelines.

Serological Analysis

Serum levels of total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) were measured using an automatic blood chemical analyzer (Prime E automatic photometer; Asan Pharmaceutical Co., Hwaseong, Korea). Serum leptin was measured with a rat enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden) following the manufacturer's instructions.

Tissue G6PD Activity

Glucose-6-phosphate dehydrogenase (G6PD) activity was determined according to Bergmerye's method with slight modification [4]. Approximately 250 mg of liver tissue was homogenized at room temperature with 5 ml of buffer. The solution was then vortexed and the homogenate was immediately centrifuged at 12,000 ×*g* for 15 min at 4°C. The supernatant was assayed for G6PD activity. Total protein in the tissues was assayed by the Bradford method using bovine serum albumin (BSA) as a standard [41]. G6PD activity was expressed as μmol NADPH release/mg protein.

Quantification of Triglyceride and Cholesterol Content

Lipids in the liver tissue and feces were extracted using the Bligh and Dyer method [5]. Total lipids were extracted from frozen livers with chloroform-methanol [2:1 (v/v)]. The lipid extract was dried, dissolved in 2-propanol, and used for total lipid and cholesterol analysis. Total lipid and cholesterol content was measured using a commercially available kit (Asan Pharmaceutical Co).

mRNA Expression of G6PD Enzyme in the Liver

G6PD mRNA expression was assayed using real-time RT-PCR. Total RNA was isolated with TRI reagent (Sigma, St. Louis, MO, USA). cDNA was reverse transcribed from 1 μg of total RNA and oligo(dT) 12-18 primer using SuperScipt II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Real-time quantitative (RTQ) PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix. We designed primers using

Primer Express software based on gene sequences available from the GenBank database. The primer sequence for the rat G6PD gene was as follows: forward (F), 5'-GCAAACAGAGTGAGCCCTTC-3'; reverse (R), 5'-TGGVCTCTTGAGGTGCTTG-3'. The PCR reaction consisted of cDNA, 0.5 μM of each primer, 4 μl of the dNTP mix, $MgCl_2$, *Taq* DNA polymerase, and SYBR Green in a total volume of 20 μl. The thermal profile included the denaturation of the sample and activation of the *Taq* DNA polymerase at 95°C for 3 min, and 40 PCR cycles (denaturation at 95°C for 10 s, annealing at 56°C for 10 s, and elongation at 72°C for 10 s) followed by a melting curve. Bio RAD iQ5 software (ver. 2.0; Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify and analyze the results. The relative mRNA values were normalized with the levels of β-actin. The purity and specificity of G6PD were verified by analysis of the melting curve.

Histological Analysis

Perirenal white adipose tissue was fixed in 10% phosphate-buffered formalin for 1 day and processed in a routine manner to generate 5-μm-thick paraffin sections. Sections were stained with hematoxylin and eosin (H&E) and examined microscopically. Mean adipocyte size was determined by computerized image analysis. Ten random visual fields were photographed with a CCD camera (Axiocam; Carl Zeiss, Boerkochem, Germany). The images were converted into a binary format with the Image J tool kit. The total number and cross-sectional areas of adipocytes were calculated and expressed by the computer as square millimeters (mm^2). Because each millimeter of the digital image equaled 50 μm, the calculated area was multiplied by a conversion factor of 2,500 to determine the cross-sectional area of the adipocytes in square micrometers (μm^2) [8].

***In Vitro* Hydroxymethylglutaryl-CoA (HMG-CoA) Reductase Activity**

The effect of hm γ-PGA on HMG-CoA reductase activity was analyzed using the HMG-CoA reductase assay kit from Sigma-Aldrich (St. Louis, MO, USA). An incubation mixture containing 400 μM of HMG-CoA and 400 μM of NADPH was preincubated in the presence of 5 μl of γ-PGA (10 mg/ml) in a final volume of 1 ml for 5 min at 37°C. Next, 5 μl of HMG-CoA reductase (0.52 mg protein/ml) was added and the incubation continued. Catalytic activity was determined by following A_{340} as a measure of NADPH utilization with a kinetic program.

Subjects and Ethical Approval

The study was approved by the Institutional Review Board (Ethics Committee) of Seoul Paik Hospital, Inje University, Seoul, Korea. The protocol for each procedure was fully explained to the subjects, who provided written informed consent before any procedure was performed. Subjects did not use any medication, and those exhibiting any significant poor condition were excluded.

Study Design and Supplements

An 8-week double-blind, placebo-controlled trial was conducted using human subjects. These subjects were divided into control and experimental groups and consumed placebo or test supplements (150 mg/day). Routine blood tests and counseling sessions with a physician and a registered dietitian about improving blood lipid levels were performed at baseline (week 0) and weeks 4 and 8 for both groups. A researcher at the Center at the Institute for Clinical Nutrition, Paik Hospital, Seoul, monitored subjects' compliance, side effects, and any other problems once a week. A registered dietitian provided dietary advice and education to the subjects. Serum total cholesterol, TG, and HDL cholesterol were determined after 10 h of fasting in the initial and sequel studies using an automatic analyzer (Olympus AU5400; Olympus Diagnostics, Japan). All samples were analyzed on the same day.

Statistical Analysis

Weight gain, visceral fat-pad weight, liver weight, food intake, and serum and hepatic biochemical analyses were expressed as means ± SEM of eight rats. Real-time PCR data were presented as means from duplicate analyses of RNA samples from eight rats per group. Treatment effects (dietary fat and hm γ-PGA) were analyzed by a two-way analysis of variance (ANOVA), using SPSS for Windows (ver. 14.0; SPSS Inc., Chicago, IL, USA). When treatment effects were significantly different ($p < 0.05$), means were tested using Duncan's multiple comparison tests. In the human pilot study, variation in lipid levels was analyzed using a paired Student's t-test. Differences between groups were compared using an unpaired Student's t-test.

RESULTS

Molecular Weight and Viscosity of γ-PGA

The molecular weights of γ-PGA samples were measured as 1, 10, 50, 500, and 2,000 kDa by GPC using polyacrylamide (1–9,000 kDa) as a standard material (Table 1). The viscosity of the materials increased in proportion to molecular weight.

Food Intake, Body Weight, Liver Weight, and Fat Weight

At the start of the experiment, the mean body weight of all animals was 348.8 ± 7.2 g. Body weights and body weight gains during the experimental diet feeding period are shown in Table 3. The high-fat diet promoted a significant increase in body weight. In addition, significant differences in body weight among groups became noticeable in the fifth week of feeding the high-fat diet (NFD 434.9 g *vs.* HFD 498.7 g, NFP 438.2 g *vs.* HFP 490.3 g). All rats fed a high-fat diet showed 14.6% higher body weights as compared with rats fed a normal-fat diet ($p < 0.01$) even though the initial body weights were similar between the NFC and

Table 1. Molecular weight, polydispersity, and viscosity of various sizes of γ-PGA.

Test sample	GPC (kDa)[1]	PD[2]	Viscosity (cP)[3]
1 kDa	1.3	1.2	N.D.
5 kDa	5.5	1.4	2
50 kDa	53	1.8	8
500 kDa	540	2.2	54
2,000 kDa	2,500	3.8	220

[1]Values were determined by using GPC (column: Viscotek $GMPW_{XL}$).
[2]Values were determined by Mw/Mn.
[3]Values were determined with Brookfield DV+I Viscometer.

Table 2. Composition of experimental diets.

Ingredient	NFC[1]	NFP	HFC	HFP
		(g/kg diet)		
Casein	200	200	250	250
Cornstarch	549.48	548.48	148.69	147.69
Sucrose	100	100	200	200
Soybean oil	45	45	0	0
Cholesterol	0	0	10	10
Shortening	0	0	290	290
Cellulose powder	45	45	50	50
AIN-93M mineral MIX[2]	45	45	35	35
AIN-93 vitamin MIX[3]	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone	0.02	0.02	0.06	0.06
L-Cysteine	3	3	3.75	3.75
γ-PGA[4]	0	1	0	1
Total mass	1,000	1,000	1,000	1,000

[1]NFC (10% fat diet), NFP (10% fat plus 0.1% high molecular γ-PGA diet), HFC (50% fat diet), HFP (50% fat plus 0.1% high molecular γ-PGA diet).
[2]Mineral mixture provides calcium carbonate, 12.5 g; sodium chloride, 2.59 g; potassium citrate, 2.48 g; potassium phosphate, 6.86 g; potassium sulfate, 1.63 g; magnesium oxide, 0.85 g; manganous carbonate, 0.02 g; ferric citrate, 0.21 g; zinc carbonate, 0.06 g; cupric carbonate, 0.01 g; potassium iodate, 0.00035 g; sodium selenite, 0.00036 g; chromium potassium sulfate, 0.01 g; ammonium paramolybdate, 0.0003 g; sodium meta-silicate, 0.05 g; libitium chloride, 0.0006 g; boric acid, 0.003 g; sodium fluoride, 0.002 g; nickel carbonate, 0.001 g; ammonium vanadate, 0.0002 g; sucrose finely powdered, 7.73 g.
[3]Vitamin mixture provides thiamin HCl, 0.006 g: riboflavin, 0.006 g: pyridoxine HCl, 0.007 g: nicotinic acid, 0.03 g: calcium pantothenate, 0.016 g: folic acid, 0.002 g; D-biotin, 0.0002 g; vitamin B_{12}, 0.025 g; vitamin A palmitate, 0.008 g; DL-alpha tocopheryl, 0.15 g; vitamin D_3, 0.002 g; vitamin K, 0.00075 g; sucrose finely ground, 9.75 g.
[4]γ-PGA: poly-gamma-glutamic acid having a molecular size of over 500 K.

HFC groups. Total food intake during the experimental period was greater in the NFC rats than in the HFC rats ($p < 0.001$). The high-fat diet was calorically denser than the normal-fat diet (5.1 kcal *vs.* 3.8 kcal). Therefore, the cumulative energy intake was similar in the HFC-fed and NFC-fed rats. These data clearly show that the high-fat diet induced a profound accumulation of energy in the form of body fat, including liver, epididymal, and perirenal fat masses. There were no significant differences in daily energy intake between rats fed the same diet with or without hm γ-PGA.

Although the weight of epididymal adipose tissues in rats supplemented with hm γ-PGA was not significantly different regardless of dietary fat, the relative weight of perirenal adipose tissue of rats fed a high-fat diet plus hm γ-PGA was significantly reduced (Table 4). The relative epididymal fat pad size of these rats showed a decreasing trend as compared with rats given the high-fat diet alone.

Table 3. Total food intake, energy intake, initial and final body weights, and body weight gain[1].

Group	Food intake (g/day)	Energy intake (kcal/4 wk)	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
NFD	24.6 ± 1.1[a]	2,867.2 ± 427.1	434.9 ± 15.5	500.1 ± 18.9	85.2 ± 14.0
NFP	25.5 ± 0.7[a]	3,191.3 ± 76.7	438.2 ± 13.7	505.7 ± 19.3	67.6 ± 9.9
HFD	18.3 ± 0.8[b]	3,253.8 ± 145.8	498.7 ± 20.4	575.3 ± 26.3	82.9 ± 10.3
HFP	17.2 ± 0.7[b]	3,148.1 ± 106.0	490.3 ± 17.3	568.7 ± 20.4	72.9 ± 7.6
			2 × 2 ANOVA[2]		
Fat	S	NS	NS	NS	NS
γ-PGA	NS	NS	NS	S	S
Fat × γ-PGA	NS	NS	NS	NS	NS

[1]Values are expressed as means, n = 8; Duncan's multiple comparison tests were used to determine differences between groups after ANOVA. Different letters in a column indicate significant differences ($p < 0.05$).
[2]ANOVA: effect significant, S ($p < 0.05$) or non-significant, NS ($p > 0.05$).

Table 4. Serum triglycerides, cholesterol, HDL cholesterol, LDL cholesterol, glucose, insulin, and leptin[1].

Group	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	Glucose (mg/dl)	Insulin (U/l)	Leptin (ng/l)
NFD	80.7 ± 6.3^{b}	87.6 ± 2.7^{ab}	50.2 ± 2.8^{b}	21.0 ± 3.2^{a}	125.6 ± 6.3	0.38 ± 0.04^{a}	4.03 ± 2.14^{b}
NFP	84.9 ± 4.8^{b}	79.7 ± 2.6^{a}	45.0 ± 4.0^{ab}	19.3 ± 1.40^{a}	118.2 ± 3.0	0.48 ± 0.05^{a}	6.30 ± 1.90^{b}
HFD	55.5 ± 4.4^{a}	99.1 ± 5.8^{b}	19.7 ± 3.3^{a}	50.0 ± 5.6^{b}	119.6 ± 3.0	1.07 ± 0.24^{b}	5.12 ± 1.10^{b}
HFP	55.3 ± 4.7^{a}	91.5 ± 5.3^{b}	31.0 ± 2.2^{b}	48.0 ± 2.6^{b}	120.0 ± 2.2	0.97 ± 0.13^{b}	2.25 ± 0.5^{a}
				2 × 2 ANOVA			
Fat	S	S	S	S	NS	S	NS
Hm γ-PGA	NS	S	NS	NS	NS	NS	NS
Fat × Hm γ-PGA	NS	NS	S	NS	NS	NS	S

[1]Values are expressed as means ± SEM, n = 8; Duncan's multiple comparison tests were used to determine differences between groups after ANOVA. Different letters in a column indicate significant differences ($p < 0.05$).

[2]ANOVA: effect significant, S ($p < 0.05$) or non-significant, NS ($p > 0.05$). Two-way ANOVA showed that the HFD had a significant effect on triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, and insulin, whereas γ-PGA had a significant effect on total cholesterol. The interaction between dietary fat and hm γ-PGA was significant for HDL cholesterol and leptin.

Marked fatty liver observed in rats fed a high-fat diet was prevented with hm γ-PGA supplementation.

Metabolic Markers

Changes in serum lipid levels of metabolic markers in the four groups are summarized in Table 5. Compared with normal-fat-fed rats, cholesterol levels were elevated in high-fat-fed rats. Treatment with hm γ-PGA induced a significant reduction in total cholesterol in rats fed a normal-fat diet, and a slight, but not significant, reduction in rats fed a high-fat diet. Serum fasting LDL cholesterol levels were increased by the high-fat diet. The intake of hm γ-PGA did not change LDL cholesterol levels regardless of dietary fat. HDL cholesterol levels were decreased by the high-fat diet. Treatment with hm γ-PGA increased HDL cholesterol in rats fed high-fat diet to levels comparable with those in normal-fat-fed rats ($p < 0.05$). Triglyceride levels were decreased by the high-fat diet and were unaffected by hm γ-PGA intake.

Fasting glucose levels in all experimental groups remained within normal reported values. The HFC groups showed normal blood glucose levels combined with severely increased insulin levels. The intake of hm γ-PGA tended to decrease insulin secretion to maintain normal blood glucose levels in the HFC group.

As shown in Table 6, hepatic triglycerides and cholesterol levels in the high-fat-fed rats were higher than in normal-fat-fed rats. Treatment with hm γ-PGA tended to decrease hepatic lipids ($p = 0.052$) in normal-fat-fed rats; however, it did not affect total cholesterol content in the liver. Therefore, hepatic fat accumulation was decreased by treatment with hm γ-PGA in the normal-fat diet.

The high-fat diet induced increased fecal excretion of total triglycerides and cholesterol as compared with the normal-fat diet. Fecal lipid excretion was not altered by hm γ-PGA intake.

Table 5. The relative weights of liver, epididymal fat, and perirenal fat[1].

Group	RLS[2]	RES	RPS
NFD	2.5 ± 0.1^{a}	2.0 ± 0.1^{a}	2.4 ± 0.2^{a}
NFP	2.4 ± 0.1^{a}	1.9 ± 0.2^{a}	2.2 ± 0.2^{a}
HFD	5.3 ± 0.2^{c}	2.7 ± 0.2^{b}	3.9 ± 0.4^{b}
HFP	4.8 ± 0.2^{b}	2.1 ± 0.1^{ab}	2.6 ± 0.1^{a}
	2 × 2 ANOVA[3]		
Fat	S	S	S
Hm γ-PGA	NS	NS	S
Fat × Hm γ-PGA	S	NS	S

[1]Values are expressed as means ± SEM, n = 8; Duncan's multiple comparison tests were used to determine differences between groups after ANOVA. Different letters in a column indicate significant differences ($p < 0.05$).

[2]RLS: relative liver size = (liver weight × 100)/body weight; RES: relative epididymal fat size = (epididymal fat weight × 100)/body weight; RPS: relative perirenal fat size = (perirenal fat weight × 100)/body weight.

[3]ANOVA: effect significant, S ($p < 0.05$) or non-significant, NS ($p > 0.05$). Two-way ANOVA showed that the HFD had a significant effect on RLS, RES, and RPS, whereas hm γ-PGA had a significant effect on RPS. The interaction between dietary fat and hm γ-PGA was significant for RLS and RPS.

G6PD Activity and mRNA Expression

As shown in Table 7, the high-fat diet significantly decreased the activity of hepatic G6PD as compared with the normal-fat diet. Treatment with hm γ-PGA reduced hepatic G6PD activity in normal-fat-fed rats. Whereas G6PD activity was decreased by the high-fat diet, hm γ-PGA intake did not lead to a further reduction of G6PD activity in the liver. We performed a real-time RT–PCR analysis to determine whether hm γ-PGA regulates the mRNA expression of G6PD. As

Table 6. Liver and fecal triglycerides and cholesterol[1].

Group	Liver				Feces			
	Cholesterol		Triglycerides		Cholesterol		Triglycerides	
	(mg/total)	(mg/ g wet tissue)	(mg/total)	(mg/ g wet tissue)	(mg/total)	(mg/ g feces)	(mg/total)	(mg/ g feces)
NFD	3.1 ± 0.5[a]	0.31 ± 0.02[a]	347.4 ± 50.0[a]	3.1 ± 0.5[a]	0.43 ± 0.06[a]	0.22 ± 0.02[a]	26.1 ± 3.3[a]	49.9 ± 7.4[a]
NFP	3.0 ± 0.6[a]	0.29 ± 0.02[a]	254.8 ± 25.2[a]	3.0 ± 0.6[a]	0.49 ± 0.04[a]	0.22 ± 0.01[a]	23.9 ± 1.9[a]	54.8 ± 5.9[a]
HFD	56.7 ± 4.6[b]	1.98 ± 0.09[b]	3,102.6 ± 220.6[b]	56.7 ± 4.6[b]	2.99 ± 0.37[b]	1.20 ± 0.07[b]	66.3 ± 2.6[b]	163.2 ± 34.2[b]
HFP	54.8 ± 5.0[b]	2.00 ± 0.11[b]	2,948.4 ± 214.8[b]	54.8 ± 5.0[b]	3.07 ± 0.43[b]	1.79 ± 0.53[b]	61.8 ± 3.6[b]	164.6 ± 23.9[b]
				2 × 2 ANOVA[2]				
Fat	S	S	S	S	S	S	S	S
Hm γ-PGA	NS	NS	NS	NS	NS	NS	NS	NS
Fat × Hm γ-PGA	NS	NS	NS	NS	NS	NS	NS	NS

[1]Values are expressed as means ± SEM, n = 8; Duncan's multiple comparison tests were used to determine differences between groups after ANOVA. Different letters in a column indicate significant differences ($p < 0.05$).

[2]ANOVA: effects significant, S ($p < 0.05$) or non-significant, NS ($p > 0.05$). Two-way ANOVA showed that the HFD had a significant effect on triglycerides and cholesterol of liver and feces.

Table 7. G6PD activity and relative mRNA expression[1,2].

Group	Activity (mU/mg protein)	mRNA (%)
NFD	11.39 ± 2.03[c]	100.0 ± 32.3[c]
NFP	5.51 ± 1.06[b]	30.1 ± 8.5[b]
HFD	1.08 ± 0.12[a]	41.7 ± 5.6[b]
HFP	1.71 ± 0.21[a]	20.5 ± 4.7[a]
	2 × 2 ANOVA[3]	
Fat	S	S
Hm γ-PGA	NS	S
Fat × Hm γ-PGA	NS	NS

[1]Values are expressed as means ± SEM, n = 8, Duncan's multiple comparison tests were used to determine differences between groups after ANOVA. Different letters in a column indicate significant differences ($p < 0.05$).

[2]G6PD: glucose-6-phosphate dehydrogenase.

[3]ANOVA: effect significant, S ($p < 0.05$) or non-significant, NS ($p > 0.05$). Two-way ANOVA showed that the HFD had a significant effect on G6PD activity and mRNA expression, whereas hm γ-PGA had a significant effect on G6PD mRNA expression.

Table 8. Area of perirenal white adipocytes[1].

Group	Area (μm^2)
NFD	29,673.6 ± 1,840.3[b]
NFP	11,465.4 ± 877.8[a]
HFD	45,287.1 ± 1,507.9[c]
HFP	12,990.7 ± 523.2[a]
	2 × 2 ANOVA[2]
Fat	S
Hm γ-PGA	S
Fat × Hm γ-PGA	NS

[1]Values are expressed as means ± SEM, $n = 8$; Duncan's multiple comparison tests were used to determine differences between groups after ANOVA. Different letters in a column indicate significant differences ($p < 0.05$).

[2]G6PD: glucose-6-phosphate dehydrogenase.

[3]ANOVA: effect significant, S ($p < 0.05$) or non-significant, NS ($p > 0.05$). Two-way analysis of variance showed that the HFD or hm γ-PGA had a significant effect on the area of perirenal adipocytes.

shown in Table 7, hm γ-PGA significantly inhibited hepatic G6PD mRNA expression in the liver regardless of the amount of fat in the diet ($p < 0.05$).

Histological Analysis of Adipocyte Size

Although the total white adipose tissue mass of perirenal fat pads was significantly larger under a high-fat diet, this increase was inhibited by 32.4% in hm γ-PGA-supplemented rats (Table 4). Histological analyses of perirenal fat pads after fixation and quantification of adipocyte size revealed that adipocytes from rats fed a high-fat diet were significantly larger than those of rats fed a normal-fat diet (Fig. 1, Table 8). However, adipocyte size in rats fed an hm γ-PGA-

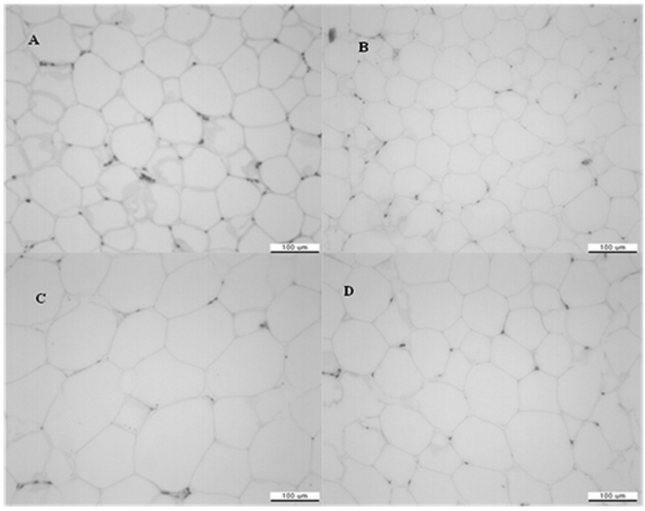

Fig. 1. Histological images of adipose tissue in rat.
Section from perirenal fat and stained with H&E. Images captured at 10× magnification. A: Normal-fat; B: Normal-fat plus hm γ-PGA; C: High-fat; D: High-fat plus hm γ-PGA.

supplemented diet was significantly smaller than that of adipocytes from rats without γ-PGA, regardless of dietary fat composition.

In Vitro HMG-CoA Reductase Activity

To examine the manner in which hm γ-PGA regulates lipid metabolism, the effect of hm γ-PGA on HMG-CoA reductase, the rate-controlling enzyme in cholesterol biosynthesis, was examined *in vitro*. As shown in Fig. 2, HMG-CoA reductase activity was inhibited by hm γ-PGA. The inhibitory potential increased linearly in a molecular weight-dependent manner.

Anthropometric and Lipid Profiles in Human Subjects

A total of 20 human subjects (10 in each group) participated in the clinical trial; 14 of these subjects (6 in the 0.15 g intake group, 8 in the placebo group) completed the 8 weeks of the trial. Serum glucose, triglyceride, waist circumference, and GPT showed no decreasing trend in the experimental group, as compared with the control group. No significant difference between test and control groups was observed for compliance in taking the supplement. Changes in blood pressure and pulse rate did not differ significantly between groups during the 8 weeks of the trial. However, total cholesterol and LDL cholesterol showed a clear decreasing trend in the group receiving 0.15 g of hm γ-PGA (Table 9).

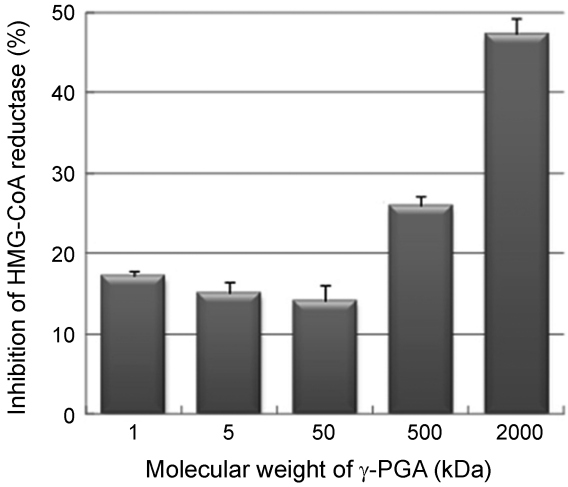

Fig. 2. Inhibition of HMG-CoA reductase by different molecular weights of γ-PGA *in vitro*.
Reactions, containing 20 μl of NADPH (to obtain a fixed final concentration of 400 μM), 60 μl of HMG-CoA substrate (to obtain a fixed final concentration of 400 μM), and 5 μl of γ-PGA solution (10 mg/ml) in a final volume of 1 ml of assay buffer, were initiated by the addition of 5 μl of the catalytic domain of human recombinant HMG-CoA reductase (0.52 mg protein/ml).

Table 9. Anthropometric measurements and serum lipid levels of human subjects.

Content		Placebo group (n = 8)	0.15 g group (n = 6)	p (t-test)
Weight (kg)	0 wk	70.28 ± 1.41	67.90 ± 1.09	
	8 wk	69.29 ± 1.59	67.27 ± 1.12	
	8 wk−0 wk	-0.99 ± 0.33	-0.63 ± 0.62	0.597
	p (paired)	0.020	0.352	
BMI (kg/m^2)	0 wk	26.65 ± 0.40	26.83 ± 0.83	
	8 wk	26.28 ± 0.46	26.58 ± 0.80	
	8 wk−0 wk	-0.38 ± 0.13	-0.25 ± 0.25	0.639
	p	0.020	0.366	
Cholesterol (mg/dl)	0 wk	179.38 ± 8.49	228.33 ± 14.93	
	8 wk	173.88 ± 9.07	214.83 ± 13.19	
	8 wk−0 wk	-5.50 ± 6.69	-13.50 ± 11.85	0.543
	p	0.438	0.306	
LDL cholesterol (mg/dl)	0 wk	109.38 ± 7.71	152.0 ± 11.29	
	8 wk	104.88 ± 8.45	138.67 ± 9.13	
	8 wk−0 wk	-4.50 ± 4.89	-13.30 ± 7.01	0.307
	p	0.388	0.116	
HDL cholesterol (mg/dl)	0 wk	59.00 ± 3.95	63.33 ± 6.45	
	8 wk	58.63 ± 2.89	59.67 ± 7.42	
	8 wk−0 wk	-0.38 ± 2.45	-3.67 ± 3.17	0.419
	p	0.883	0.300	
TG (mEq/l)	0 wk	94.50 ± 12.57	106.50 ± 14.67	
	8 wk	78.00 ± 9.53	89.50 ± 14.98	
	8 wk−0 wk	-16.50 ± 14.00	-17.00 ± 13.48	0.980
	p	0.276	0.263	

DISCUSSION

Excess accumulation of abdominal fat (*i.e.*, visceral fat obesity) is believed to elicit metabolic syndrome, which is a cluster of several metabolic and cardiovascular disease risk factors. Metabolic syndrome includes dyslipidemia, glucose intolerance, insulin resistance, and hypertension, and is becoming a major public health problem. Studies examining dietary components that can regulate body fat accumulation and related metabolic syndrome have great significance. Soybean or fermented soybean products have been reported to possess antidiabetic and antilipidemic abilities due to their isoflavonoids, as well as fiber and soy protein [6, 25, 29]. Since the fermentation of soybean changes the content and structure of isoflavones and proteins, cooked soybean and fermented soybean have different biological functions. In addition, γ-PGA is a polymer that is synthesized from glutamate monomers by *Bacillus subtilis* (natto) or *B. subtilis* subsp. *chungkookjang* during fermentation [34]. γ-PGA is the main component of the mucilage of fermented soybean products such as *chungkookjang* (a traditional Korean food) and *natto* (a traditional Japanese food). Although γ-PGA has been reported to have a variety of biological effects including antitumor immunity, scientific data on its hypocholesterolemic effect are scarce. Therefore, we investigated γ-PGA as a potential candidate for use as a food supplement to reduce abdominal fat and modify lipid metabolism in high-fat-fed animals.

Treatment with hm γ-PGA decreased body weight gain regardless of dietary fat content ($p < 0.05$). When rats were fed a diet of high fat plus hm γ-PGA, relative liver size and relative perirenal fat pad size were significantly decreased ($p < 0.05$). The relative perirenal fat pad size of rats fed a high-fat diet plus hm γ-PGA was decreased to the level of normal-fat-fed rats. Although no significant effects of hm γ-PGA on food intake or calorie intake were observed, body weight gain and perirenal fat mass were lower in rats treated with hm γ-PGA as compared with rats not treated with hm γ-PGA. These data indicate that calorie consumption does not reflect changes in the amount of adipose tissue. Ali *et al*. [1] showed that soy isoflavones significantly reduce body weight gain and fat deposition in obese and lean rats. Back *et al*. [2] reported that supplementation with *chungkookjang* improves lean body mass and visceral fat areas. Several studies have also suggested that an isoflavone-rich diet improves lipid metabolism and has antiobese effects [3, 30]. Based on previous data, isoflavones or soy proteins have been reported to have antiobese properties, and in the present study, hm γ-PGA treatment showed the same effects.

Researchers have shown that dietary obesity causes metabolic abnormalities, and reductions in body weight and abdominal fat mass can ameliorate these changes [32, 37]. A high-fat diet was shown to accelerate the impairment of lipid metabolism [19]. Leptin affects energy homeostasis by inhibiting food intake and stimulating energy expenditures. Serum total cholesterol and leptin were higher in rats fed a high-fat diet than in rats fed a normal-fat diet. Obesity is generally accompanied by high serum leptin concentrations, which is termed leptin resistance. In the present study, treatment with hm γ-PGA reduced serum leptin in high-fat-fed rats, suggesting that hm γ-PGA prevents body fat gain, in part by inhibiting leptin resistance induced by the high-fat diet.

Since a high-fat diet induces metabolic abnormalities, key factors could, in theory, be identified through interventions that aim to correct these dyslipidemic conditions. *Chungkookjang* has been reported to decrease plasma total cholesterol and LDL cholesterol in human subjects [36]. Yang *et al*. [39] observed that the intake of *chungkookjang* along with cholesterol loading for 6 weeks significantly decreased LDL cholesterol and triglyceride levels by increasing fecal bile excretion. Whereas serum total cholesterol was significantly increased in this study, HDL cholesterol was decreased in high-fat-fed rats. The hypocholesterolemic effects of hm γ-PGA were observed at doses of 0.1% in the diet (hm γ-PGA intake of 15 mg/d corresponding to the amount of γ-PGA in 200 g of *natto* in human subjects) [27].

The hm γ-PGA obtained from culture filtrate of *B. subtilis* subsp. *chungkookjang* is known to have a high molecular weight (> 2,000 kDa) and higher viscosity in aqueous condition. Many viscous soluble dietary components are capable of increasing fecal excretion of cholesterol and triglycerides, but decrease hepatic accumulations of cholesterol and triglyceride in both normal and hyperlipidemic rats [40]. In the present study, we found that fecal excretions of cholesterol and total triglycerides were not affected by hm γ-PGA treatment (Table 5). Therefore, it appears that the hypocholesterolemic effects of hm γ-PGA might be closely related to the reduction in hepatic lipogenesis. The accumulation of both cholesterol and triglycerides in the liver of rats fed a high-fat diet is consistent with similar changes found by others using diets containing a wide range of cholesterol and fat levels [12, 13]. Under these conditions, triglyceride and cholesterol syntheses in the liver are extremely decreased [28, 35]; however, the high levels of cholesterol and triglycerides in the circulation seem to enhance liver uptake. We selected a high-fat diet in order to induce an obese condition. The decreased lipid absorption in the diet has been reported to affect serum lipid levels and lipid accumulation in adipose tissue and liver. The data presented in Table 3, Table 7, and Fig. 1 indicate that the decrease in lipid accumulation in the adipose tissues of rats fed a high-fat diet plus hm γ-PGA was not due to the increase in fecal lipid excretion.

Ingestion of a high-fat diet has been reported to markedly inhibit hepatic lipogenesis [10]. In the present study, G6PD activity in rats fed a high-fat diet was lower than in rats fed

a normal-fat diet. This result has also been observed in other studies using high-fat-fed rats [19]. As seen in Table 6, lipogenic enzyme activity was inhibited by hm γ-PGA (a rate-limiting enzyme of lipogenesis). G6PD activity was significantly inhibited by hm γ-PGA treatment in normal-fat-fed rats. G6PD mRNA expression was inhibited by hm γ-PGA in both high-fat and normal-fat diet fed rats. Thus, it is possible that improvements in lipid metabolism by hm γ-PGA are attributable to the inhibition of lipogenesis in hepatic tissue. These results indicate that hm γ-PGA intake facilitates lipid metabolism in rats regardless of the amount of fat in the diet; however, a normal-fat diet with hm γ-PGA is more effective than a high-fat diet. This result is consistent with previous studies that have reported that reduced total body weight and adiposity correct dietary-induced dyslipidemia [23, 32].

In the human clinical study, hm γ-PGA had no effect on weight loss, but total cholesterol and LDL cholesterol showed clear decreasing trends in the group receiving 0.15 g of hm γ-PGA. Although not statistically significant, this difference was meaningful. Because glucose, triglycerides, waist circumference, and GPT did not decrease in the experimental group, as compared with the control group, the cholesterol-lowering effect may be due to a mechanism (inhibited intestinal cholesterol absorption or hepatic cholesterol metabolism) other than the decrease of visceral fat or functional improvement of the metabolic syndrome. We found no statistically significant difference in cholesterol reduction between groups, owing to the small sample size. Thus, a larger clinical trial is needed to identify the cholesterol-lowering effect of hm γ-PGA.

From the results obtained in these studies, we conclude that hm γ-PGA possesses hypolipidemic effects by inhibiting hepatic lipogenesis and decreasing adiposity. Further studies will be required to explore the detailed mechanisms of action.

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