Enzymatically-Synthesized Glycogen Induces Cecal Glucagon-Like Peptide-1 Production and Suppresses Food Intake in Mice

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Summary It is well known that dietary fiber stimulates the release of satiety hormones such as glucagon-like peptide-1 (GLP-1), which in turn suppresses appetite. In order to evaluate appetite regulating role of enzymatically synthesized glycogen (ESG, one of the resistant starch), we examined the effects of dietary supplementation of ESG on food intake and cecal *proglucagon* gene expression in normal and high fat diet-fed mice. Twenty four male ICR mice were weighed and assigned to four groups: normal diet group; normal diet containing 25% ESG group; high-fat diet (HFD) group; HFD containing 25% ESG group. Each group was fed the relevant diets for 3 wk. All data were analyzed by a two-way ANOVA with the main effects of HFD and ESG. ESG significantly decreased food intake and increased the weight of the cecum and cecal content. Plasma total short chain fatty acids concentration was significantly elevated by ESG. The mRNA levels of *proglucagon* in the cecum and plasma total GLP-1 concentration were significantly increased by ESG. The mRNA levels of appetite regulating neuropeptides such as neuropeptide Y, agouti-related protein, proopiomelanocortin, and cocaine- and amphetamine-regulating transcript in the hypothalamus were not influenced by ESG. There is no significant interaction between diet and ESG in any parameters. These results suggest that ESG-induced upregulation of GLP-1 production in the cecum suppresses food intake in mice and that fecal fermentation may be involved in the anorexigenic effect.

Key Word appetite, GLP-1, food intake, mice, HFD

Obesity is the major risk factor for several causes of diseases, including cardiovascular disease (1) and type 2 diabetes (2). The prevalence of overweight and obese people has globally increased in recent decades. For example, the number of individuals with a body mass index (BMI) of 25 kg/m² or greater increased from 857 million in 1980, to 2.1 billion in 2013 (3). Overall mortality was lowest in adults with a BMI of about 22.5–25 kg/m² in both sexes and at all ages, and above this range, each 5 kg/m² higher BMI was associated with about 30% higher all-cause mortality (1). Therefore, new drugs or food ingredients that effectively prevent obesity should be developed for a pharmacotherapy or alimentotherapy for obesity-related disorders.

Obesity results from the dysregulation of energy homeostasis including increased food intake and decreased energy expenditure. Therefore, appetite regulatory system is one of the therapeutic targets in obesity treatment (4). The hypothalamus is one of the best-studied brain region involved in the regulation of food intake. Orexigenic neuropeptide Y (Npy) and agouti-related peptide (Agrp)-expressing neurons and the anorexigenic proopiomelanocortin (Pomc)-expressing neurons in the hypothalamus play crucial roles in the central regulation of food intake (4). Recent findings suggest that dietary fiber influences appetite via hypothalamic neuropeptides including Npy, Agrp, Pomc, and

cocaine and amphetamine regulated transcript (Cart) (5). It is therefore possible that dietary fiber is a promising candidate for a food ingredient to prevent or ameliorate obesity.

Enzymatically synthesized glycogen (ESG) from starch using isoamylase (EC 3.2.1.68), branching enzyme (EC 2.4.1.18), and amylomaltase (EC 2.4.1.25) (6) showed various biological properties, including immunostimulating activity (7), indigestibility and intestinal environment improving effect (8). Moreover, the addition of ESG in a high fat diet resulted in significant decreases in total body fat mass in rats as well as visceral fat mass (9). Dietary ESG also significantly increased the amount of lipid in the feces in rats (9). The combination of dietary ESG and exercise effectively reduced body fat mass in high fat diet (HFD)-fed mice (10). Thus, ESG is the candidate for use as a food ingredient to prevent obesity in human.

There is evidence that dietary ESG also increased the amounts of cecal short chain fatty acids (SCFA) and the viable counts of fecal *Bifidobacterium* and *Lactobacillus* in rats (8). It is therefore likely that dietary ESG functions like dietary fiber. Dietary fiber-increased colonic SCFA stimulates the release of satiety hormones such as glucagon-like peptide-1 (GLP-1), which in turn suppresses appetite and energy intake (5). Lines of evidence suggest that appetite regulating neuropeptides in the hypothalamus are involved in the anorexigenic action of GLP-1 (11–13). However, the effect of ESG on the

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Table 1. Composition of the experimental diets (%).

	ND	ND+ESG	HFD	HFD+ESG
Casein	20	20	25.6	25.6
L-Cystine	0.3	0.3	0.36	0.36
Corn starch	39.7486	24.7386	16	1.5
ESG	—	25	—	25
Maltodextrin	13.2	8.21	6	0.5
Sucrose	10	10	5.5	5.5
Cellulose	5	—	6.61	1.61
Soybean oil	7	7	2	2
Lard	—	—	33	33
Calcium carbonate	—	—	0.18	0.18
Mineral mixture ¹	3.5	3.5	3.5	3.5
Vitamin mixture ²	1	1	1	1
Choline bitartrate	0.25	0.25	0.25	0.25
<i>tert</i> -Butylhydroquinone	0.0014	0.0014	—	—
Crude protein (%)	18	18	23	23
Energy (kcal/100 g)	387.7	387.7	518.2	518.2

¹ AIN-93G mineral mixture (Japan SLC, Inc., Shizuoka, Japan).

² AIN-93 vitamin mixture (Japan SLC, Inc.).

appetite-related factors including cecal GLP-1 production, plasma GLP-1 levels, and hypothalamic appetite regulating neuropeptides have not been investigated.

In this study, we aimed to evaluate the effect of ESG on GLP-1 release and food intake in HFD-fed mice. Our results suggest that dietary ESG suppresses appetite by the induction of cecal GLP-1 production.

MATERIALS AND METHODS

Animals and diets. The animal experiment in the present study was approved by the Institutional Animal Care and Use Committee (Permission number: 28-02-01) and carried out according to the Kobe University Animal Experimentation Regulations. Male ICR mice, 5-wk-old, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed individually in plastic cages at 25°C in a room with an automatically controlled 12 h light:dark cycle (lights on from 1500 to 0300). Upon arrival, the mice were fed a nonpurified diet (DC-8, 23.1% crude protein and 3,590 kcal/kg, CLEA Japan, Inc., Tokyo, Japan) and acclimated to the facility for 6 d before feeding of the experimental diets. The mice had free access to water and assigned diets throughout the experimental period. Feed intake was measured three times a week. Body weight was measured every week.

Experimental design. Twenty four male ICR mice were weighed and assigned to four groups ($n=6$): normal diet group (ND); normal diet containing 25% ESG group (ND+ESG); high-fat diet group (HFD); HFD containing 25% ESG group (HFD+ESG). The composition of experimental diets was shown in Table 1. The composition of ND is the same as that of AIN93G (14), and the composition of HFD is the same as that of commercial HFD (HFD60, Oriental Yeast Co., Ltd., Tokyo, Japan). ESG was obtained from Ezaki Glico Co., Ltd. (Osaka,

Japan). The dietary fiber content of glycogen was estimated to be approximately 17–22% from analyses data (15), so 20% of ESG was replaced with cellulose, and 80% was replaced with cornstarch and maltodextrin to balance the calorie in the diet in each diet group. Each group was fed the relevant diets for 3 wk. At the end of the feeding period, mice were anesthetized by inhalation of isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) under an ad libitum feeding condition in a dark period, and their blood was collected from the abdominal vein by using a 27G needle and a 1 mL syringe. Aprotinin (500 kIU/mL of blood) and EDTA (1 mg/mL of blood) were used as a protease inhibitor and anticoagulant, respectively. Plasma was separated immediately by centrifugation at $3,000 \times g$ for 10 min at 4°C, frozen by liquid nitrogen and stored at -80°C for plasma components analysis. The liver, epididymal adipose tissues, perirenal adipose tissues, gastrocnemius muscles, cecum, and cecum content were weighed. The liver and perirenal adipose tissues were then immediately frozen in liquid nitrogen for storage at -80°C before real-time PCR analysis. The diencephalons and cecum were collected and preserved in RNAlater[®] tissue storage reagent (Sigma-Aldrich Co., St. Louis, MO, USA). The hypothalamus was dissected from the diencephalon based on reference to a brain atlas (16).

Plasma components analysis. Plasma glucose, non-esterified fatty acid (NEFA), and triglyceride were measured by using commercial kits (LabAssay[™] Glucose, LabAssay[™] NEFA, and LabAssay[™] Triglyceride, respectively, FUJIFILM Wako Corporation, Osaka, Japan). Plasma lactic acid and SCFA were measured by using a short chain fatty acid labeling kit (XSREAR01, YMC Co. Ltd., Kyoto, Japan) and HPLC column (YMC-Pack FA, YMC Co. Ltd.). Plasma insulin (LBIS[®] Mouse Insulin

Table 2. Primer sequences for real-time PCR analysis.

Gene		Primer sequences	Accession number ¹
<i>Proglucagon</i>	sense	5'-CCA AGA GGA ACC GGA ACA AC-3'	NM_008100
	antisense	5'-CCC TTC AGC ATG CCT CTC AA-3'	
<i>Npy</i>	sense	5'-TCC GCT CTG CGA CAC TAC ATC-3'	NM_023456
	antisense	5'-GGG CTG GAT CTC TTG CCA TA-3'	
<i>Agrp</i>	sense	5'-CGG AGG TGC TAG ATC CAC AGA-3'	NM_001271806
	antisense	5'-GGA CTC GTG CAG CCT TAC ACA-3'	
<i>Pomc</i>	sense	5'-CTC CTG CTT CAG ACC TCC ATA GA-3'	NM_001278581
	antisense	5'-GGA TGC AAG CCA GCA GGT T-3'	
<i>Cart</i>	sense	5'-AGC TGA TCG AAG CGT TGC A-3'	NM_001081493
	antisense	5'-CGT AGA TCG GAA TGC GTT TAC TC-3'	
<i>Gpl</i>	sense	5'-TCC TGG CTC TGC CTT ACG A-3'	NM_133198
	antisense	5'-GGC GCA TAG TGT TCA CAG TGT T-3'	
<i>Gs2</i>	sense	5'-CCA ACG ACG GAT GGC TTT AA-3'	NM_145572
	antisense	5'-GAT CCT GAC GGA GAA GGT GGT A-3'	
<i>Pc</i>	sense	5'-AGC AGG ACA CAG GGC AGA TG-3'	NM_001162946
	antisense	5'-GGC CAC GGC CAA TAA GGT A-3'	
<i>Pdk4</i>	sense	5'-CCC AAA CTG TGA TGT GGT AGC A-3'	NM_013743
	antisense	5'-GGT CGC AGA GCA TCT TTG C-3'	
<i>Pepck1</i>	sense	5'-CCA CAG CTG CTG CAG AAC AC-3'	NM_011044
	antisense	5'-GAA GGG TCG CAT GGC AAA-3'	
<i>Pfk1</i>	sense	5'-TCA ACG CTG CAA TGG AGA GT-3'	NM_008826
	antisense	5'-ATG AAC ACA CGC CGC TTT G-3'	
<i>Accα</i>	sense	5' -CGC TCA GGT CAC CAA AAA GAA T-3'	NM_133360
	antisense	5' -GTC CCC GCC ACA TAA CTG AT-3'	
<i>Fas</i>	sense	5'-TCC TGG AAC GAG AAC ACG ATC T-3'	NM_007988
	antisense	5'-GAG ACG TGT CAC TCC TGG ACT TG-3'	
<i>Cpt1a</i>	sense	5'-GAA CCC CAA CAT CCC CAA AC-3'	NM_013495
	antisense	5'-TCC TGG CAT TCT CCT GGA AT-3'	
<i>Rps17</i>	sense	5'-CCG GGT CAT CAT CGA GAA GT-3'	NM_009092
	antisense	5'-GCG CTT GTT GGT GTG GAA GT-3'	

¹ Refer to GenBank accession number. Abbreviations: *Npy*, neuropeptide Y; *Agrp*, agouti related peptide; *Pomc*, proopiomelanocortin; *Cart*, cocaine and amphetamine regulated transcript; *Gpl*, glycogen phosphorylase; *Gs2*, glycogen synthase 2; *Pc*, pyruvate carboxylase; *Pdk4*, pyruvate dehydrogenase kinase 4; *Pepck1*, phosphoenolpyruvate carboxykinase 1; *Pfk1*, phosphofructokinase 1; *Accα*, acetyl-CoA carboxylase α ; *Fas*, fatty acid synthase; *Cpt1a*, carnitine palmitoyltransferase 1a; *Rps17*, ribosomal protein S17.

Table 3. Effects of a high fat diet and enzymatically synthesized glycogen on body and tissue weights in mice.

	ND	ND+ESG	HFD	HFD+ESG	Diet	ESG	Interaction
Body weight (g)	40.2±1.2	39.4±0.4	39.2±1.1	38.5±0.6	—	—	—
Food intake (g)	90.7±3.5	84.0±2.3	66.9±1.8	62.6±1.6	*	**	—
Energy intake (kcal)	351.6±13.6	325.7±8.9	346.7±9.3	324.4±8.3	—	*	—
Liver (g)	2.31±0.10	2.18±0.10	1.99±0.10	1.92±0.10	**	—	—
Gastrocnemius (g)	0.37±0.01	0.35±0.01	0.34±0.01	0.34±0.01	—	—	—
Epididymal adipose tissue (g)	1.20±0.11	1.16±0.12	1.31±0.06	1.36±0.20	—	—	—
Perirenal adipose tissue (g)	0.50±0.04	0.46±0.02	0.51±0.05	0.57±0.07	—	—	—
Cecum (g)	0.12±0.01	0.15±0.00	0.09±0.00	0.13±0.01	**	**	—
Cecal content (g)	0.28±0.04	0.47±0.04	0.23±0.03	0.29±0.03	**	**	—

Values are means±SE of six mice in each group. *, **, and — indicate $p<0.05$, $p<0.01$, and $p\geq 0.05$, respectively.

ELISA Kit (T-type), FUJIFILM Wako Shibayagi Corporation, Osaka, Japan), glucagon (Glucagon ELISA Kit Wako, FUJIFILM Wako Shibayagi Corporation), total GLP-1 (GLP-1 ELISA Kit Wako, FUJIFILM Wako Corporation), and active GLP-1 (LBIS® GLP-1 (Active) ELISA

Kit, FUJIFILM Wako Shibayagi Corporation) were measured by using commercial kits.

Real time-PCR analysis. The mRNA levels of gut hormones and hypothalamic neuropeptide genes were analyzed by real time PCR analysis as described previ-

Table 4. Effects of a high fat diet and enzymatically synthesized glycogen on plasma parameters in mice.

	ND	ND+ESG	HFD	HFD+ESG	Diet	ESG	Interaction
Glucose (mg/dL)	207±8	195±12	184±5	202±6	—	—	—
Non-esterified fatty acid (mEq/dL)	0.39±0.04	0.34±0.04	0.57±0.06	0.68±0.11	**	—	—
Triglyceride (mg/dL)	176±20	173±31	119±11	179±37	—	—	—
Lactic acid (mM)	5.33±0.32	5.24±0.54	2.98±0.20	3.60±0.21	**	—	—
Acetic acid (μM)	84.5±1.6	86.5±1.2	58.2±0.8	91.2±10.1	—	—	—
Propionic acid (μM)	23.7±2.2	36.0±2.9	70.1±9.5	80.5±11.8	**	—	—
Butyric acid (μM)	30.7±7.5	31.0±5.4	41.3±4.0	54.4±6.0	**	—	—
Total short chain fatty acid (μM)	139±15	153±12	170±8	226±22	**	*	—
Insulin (pmol/L)	607±171	320±56	366±114	171±44	—	*	—
Glucagon (pmol/L)	15.1±2.9	16.0±2.0	8.1±1.7	10.7±2.1	—	*	—
Insulin/glucagon ratio	41.1±8.4	21.7±4.4	52.3±14.0	15.7±4.3	—	**	—

Values are means±SE of six mice in each group. *, **, and — indicate $p<0.05$, $p<0.01$, and $p\geq 0.05$, respectively.

ously (10). Primer sequences for real time PCR are shown in Table 2. Ribosomal protein S17 was amplified an internal standard.

Statistical analysis. Data were analyzed by a two-way ANOVA with the main effects of diet and ESG. All statistical analyses were performed using a commercial software package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

RESULTS

As shown in Table 3, ESG significantly decreased food intake and energy intake. The weights of body, liver, abdominal adipose tissues, and gastrocnemius muscles were not influenced by dietary ESG. Food intake and liver weight were significantly decreased by HFD feeding. ESG significantly increased the weight of the cecum and cecal content, suggesting that ESG function as dietary fiber. These results clearly demonstrated that ESG suppresses food intake in mice.

As shown in Table 4, plasma concentration of glucose, NEFA, and TG were not influenced by dietary ESG. HFD significantly increased plasma NEFA concentration and decreased plasma lactic acid concentration. Plasma concentrations of propionic acid, butyric acid, and total SCFA were significantly increased by HFD. Total SCFA concentration in plasma was also significantly increased by ESG. Plasma concentration of insulin was significantly decreased by ESG. In contrast, plasma concentration of glucagon was significantly increased by ESG. Thus, ESG decreased insulin/glucagon ratio in plasma.

The mRNA level of proglucagon in the cecum and plasma total GLP-1 concentration were significantly increased by ESG, whereas plasma active GLP-1 concentration was not influenced by ESG (Fig. 1). The mRNA levels of appetite regulating neuropeptides in the hypothalamus were not influenced by ESG (Fig. 2). These findings suggest that ESG induced cecal GLP-1 production but did not influence the production of appetite regulatory neuropeptides in the hypothalamus.

Figure 3 shows the effects of HFD and ESG on the mRNA levels of carbohydrates and lipid metabolism related genes in the liver. Dietary ESG significantly

Fig. 1. Effects of enzymatically synthesized glycogen and high fat diet on the mRNA levels of cecal proglucagon and plasma concentrations of total and active glucagon in mice. Values are means±SE of six mice in each group. *, **, and — indicates $p<0.05$, $p<0.01$, and $p\geq 0.05$, respectively.

Fig. 2. Effects of enzymatically synthesized glycogen and high fat diet on the mRNA levels of hypothalamic neuropeptides in mice. Values are means $\pm$ SE of six mice in each group. * and — indicates $p < 0.05$ and $p \geq 0.05$, respectively. Abbreviations: *Npy*, neuropeptide Y; *Agrp*, agouti related peptide; *Pomc*, proopiomelanocortin; *Cart*, cocaine and amphetamine regulated transcript.

increased the mRNA levels of *glycogen phosphorylase* (*Gpl*). Dietary ESG also significantly increased the mRNA levels of *pyruvate carboxylase* (*Pc*), *phosphoenolpyruvate carboxykinase 1* (*Pepck1*), and *pyruvate dehydrogenase kinase 4* (*Pdk4*) in the liver, suggesting that ESG stimulates gluconeogenesis. On the other hand, dietary ESG did not influence gene expression of lipid metabolism-related genes in the liver and adipose tissue. HFD significantly decreased lipogenic genes, such as *acetyl-CoA carboxylase α* (*Acc α*) and *fatty acid synthase* (*Fas*), and significantly increased a lipolytic gene, such as *carnitine palmitoyltransferase 1a* (*Cpt1a*), in the liver. These results suggest that HFD suppressed de novo lipogenesis and induced fatty acid oxidation in mice.

DISCUSSION

Dietary fiber is fermented by resident microbiota, elevating the production of SCEA (5). Dietary ESG significantly increased cecal SCEA in rats (8). SCEA could stimulate hormonal signals that would exert an anorectic effect (5). For example, SCEA significantly increased the mRNA levels of proglucagon in vitro (17, 18). In the present study, ESG increased the mRNA levels of proglucagon in the cecum and total GLP-1 and SCEA levels in plasma. In addition, ESG significantly decreased food intake. All these findings suggest that ESG-increased the production of satiety signal GLP-1, which in turn, results decrease of food intake.

Lines of evidence suggest that GLP-1 directly influences the expression of appetite regulating neuropep-

tides in the hypothalamus (11–13) or modulates appetite via the stimulation of intestinal and hepatic vagal afferents that are integrated in the nucleus of the solitary tract (NTS) of the brainstem (19). In the present study, ESG did not influence the mRNA levels of hypothalamic *Npy*, *Pomc*, *Agrp*, and *Cart*. Since active GLP-1 concentration in plasma was not increased by ESG, plasma GLP-1 could not function as a humoral satiety signal to the hypothalamus. Only 25% of the secreted GLP-1 reaches the portal circulation, and a further 40–50% of GLP-1 is destroyed in the liver (19). Thus, only 10–15% of secreted GLP-1 enters the systemic circulation (19). In the present study, total GLP-1 in plasma and cecal proglucagon mRNA levels were significantly increased, suggesting that active GLP-1 in the cecum or liver seems to be increased by ESG, which in turn results anorexia via NTS.

In the present study, ESG significantly increased the mRNA levels of gluconeogenic gene in the liver. In addition, ESG decreased insulin/glucagon ratio in plasma. There is evidence that insulin downregulates gene expression of *Pc* and *Pepck*, whereas glucagon upregulates them (20–22). Park et al. (23) reported that hepatic *Pdk4* expression is enhanced by glucagon or cAMP treatment in mice hepatocytes. It has long been known that the hepatic cyclic AMP concentration is regulated by the insulin/glucagon ratio (24, 25). Kalra and Gupta reported that the insulin/glucagon ratio acts as a physiological fulcrum, balancing two opposing ends of the metabolic spectrum to provide energy when

Fig. 3. Effects of enzymatically synthesized glycogen and high fat diet on the mRNA levels of carbohydrate and lipid metabolism-related genes in the liver in mice. Values are means $\pm$ SE of six mice in each group. *, **, and — indicates $p<0.05$, $p<0.01$, and $p\geq 0.05$, respectively. Abbreviations: *Gpi*, glycogen phosphorylase; *Gs2*, glycogen synthase 2; *Pc*, pyruvate carboxylase; *Pfk1*, phosphofructokinase 1; *Pdk4*, pyruvate dehydrogenase kinase 4; *Pepck1*, phosphoenolpyruvate carboxykinase 1; *Accα*, acetyl-CoA carboxylase α ; *Fas*, fatty acid synthase; *Cpt1a*, carnitine palmitoyltransferase 1a.

needed or conserve it when not needed (26). Hepatic glucose production by insulin is partly explained by suppression of glucagon secretion (27). All these findings suggest that ESG-induced upregulation of the mRNA levels of gluconeogenic genes in the liver is due to the changes of plasma insulin and glucagon levels.

Effects of food ingredients can be affected by the composition of experimental diets. For example, soy protein suppressed serum TG concentration in normal diet-fed rats, but not in high cholesterol diet-fed rats (28). Soy protein prevented adipocyte hypertrophy in high-fat diet-fed rats, but not in normal diet-fed rats (29). In the present study, there was no significant interaction between diet and ESG in any parameters. Our findings suggest that the effects of ESG may not be influenced by the dietary fat level in mice.

In conclusion, dietary ESG induced the expression of

proglucagon in the cecum, which in turn results suppression of food intake in mice.

Authorship

Research conception and design: KH; experiments: KH and AY; statistical analysis of the data: KH and AY; interpretation of the data: KH, TS, and HK; writing of the manuscript: KH, TS, and HK.

Disclosure of state of COI

No conflicts of interest to be declared.

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