Effect of Dietary Porphyran from the Red Alga, *Porphyra yezoensis*, on Glucose Metabolism in Diabetic KK-Ay Mice

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Summary Porphyran (POR) from the red alga *Porphyra yezoensis* is a water soluble dietary fiber. In this study, we investigated the effect of dietary POR on glucose metabolism in KK-Ay mice (a model for type 2 diabetes). Mice were divided into 4 groups and fed a diet containing 5% cellulose (control), POR, POR Arg or POR K. After 3 wk of feeding, plasma insulin levels and the calculated homeostasis model assessment-insulin resistance (HOMA-IR) index were significantly lower in the POR group than in the control group. Compared with the control group, plasma adiponectin levels were significantly increased in the POR, POR Arg and POR K groups. These results suggest that dietary POR should improve glucose metabolism in diabetes via up-regulation of adiponectin levels. In addition, the amount of propionic acid in the cecum of the POR group was significantly higher than in the control group and the profile of bacterial flora was changed by dietary POR. In the cecum of the POR, POR Arg and POR K groups, *Bacteroides* was significantly increased and *Clostridium coccoides* was significantly decreased compared with in the control group. The effects of dietary POR on the hindgut environment might contribute to the improvement of glucose metabolism.
Key Words dietary fiber, insulin, adiponectin, algae, porphyran

Porphyra yezoensis is a red alga that is abundantly cultivated in eastern Asia. In Japan, *P. yezoensis* is generally used to prepare nori, which is a traditional marine food. Dried nori is well known to contain many nutritional and bio-functional components such as proteins, minerals, dietary fibers, polyunsaturated fatty acids, carotenoids and mycosporine-like amino acids (porphyra-334). Thus, dietary nori contributes to the intake of essential elements in the diet. In particular, phycoerythrin, porphyra-334 and porphyran are not found in terrestrial animals and plants. Phycoerythrin, the major light-harvesting pigment protein in *P. yezoensis*, shows an anti-inflammatory activity (*1*) and a hepatoprotective effect (*2*). We previously reported that porphyra-334 possesses an antioxidative activity and works synergistically with α-tocopherol (*3*).

Porphyran, one of the main components of *P. yezoensis*, is a sulfated polysaccharide comprising the hot-water soluble portion of the algal cell wall (*4*). Porphyran is related to agarose in that it contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked 3,6-anhydro-α-L-galactose, but differs in that some residues occur as the 6-sulfate and the 6-*O*-methyl derivatives. Porphyran usually accounts for nearly 40% of the total mass of dried nori. Therefore, the nutritional and physiological functions of porphyran are important, and several studies have demonstrated its beneficial effects including its antioxidative (*5*, *6*), anti-tumor (*7*), immuno-modulating (*8*–*10*), and lipid metabolism improving activities (*11*, *12*).

It is generally believed that water soluble dietary fibers improve life style-related diseases and contribute to human health (*13*, *14*). However, the effects of porphyran on glucose metabolism have not yet been reported. In this study, we evaluated the effects of dietary porphyran supplementation on glucose metabolism using type 2 diabetes model KK-Ay mice.

MATERIALS AND METHODS

Materials. Porphyran, porphyran arginine salt and porphyran potassium salt were provided by Shirako Co., Ltd. (Tokyo, Japan).

Animals. Four-week-old male KK-Ay mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All mice were kept under conditions of 25˚C and a 12 h light-dark cycle. All animal studies were performed in accordance with the guidelines of Kyoto University for the use and care of laboratory animals.

Experimental protocol. After acclimatization with a standard diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) for 1 wk, mice were divided into 4 groups (n=6/group) and were housed in individual cages. Mice were fed a high-fat diet (14% fat) based on AIN-93G with 5% cel-

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Table 1. Composition of experimental diets.

Ingredient (weight %)	Control	POR	POR Arg	POR K
β-Cornstarch	32.75	32.75	32.75	32.75
Casein	20.00	20.00	20.00	20.00
Dextrinized cornstarch	13.20	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00	10.00
Soybean oil	14.00	14.00	14.00	14.00
Cellulose powder	5.00			
Porphyran		5.00		
Porphyran arginine salt			5.00	
Porphyran potassium salt				5.00
AIN-93 mineral mix	3.50	3.50	3.50	3.50
AIN-93 vitamin mix	1.00	1.00	1.00	1.00
L-Cystine	0.30	0.30	0.30	0.30
Choline bitartrate	0.25	0.25	0.25	0.25
Butyl hydroxy toluene	0.0014	0.0014	0.0014	0.0014

Table 2. 16S rRNA gene-target group-specific primers used in this study.

Target	Primer (5′-3′)	Production length (bp)	Annealing temp (°C)
All bacteria	GCCTAACACATGCAAGTCGA GTATTACCGCGGCTGCTGG	472	58
Lactobacillus spp.	TGGAAACAGRTGCTAATACCG GTCCATTGTGGAAGATTCCC	232	58
Bifidobacterium spp.	AGGGTTCGATTCTGGCTCAG CATCCGGCATTACCACCC	156	62
Eubacterium spp.	AGAGTTTGATCCTGGCTCAG GCCTTAAACCCTRCGCTT	492	55
Clostridium coccoides group	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	440	55
Bacteroides spp.	GTCAGTTGTGAAAGTTTGC CAATCGGAGTTCTTCGTG	127	60

lulose, porphyran (POR), porphyran arginine salt (POR Arg) or porphyran potassium salt (POR K) and were allowed free access to food and water for 3 wk (Table 1). The body weight of each mouse was measured 3 times each week and food intake was measured every day. At the end of the 3 wk of feeding, the mice were euthanized by withdrawing blood under isoflurane anesthesia after 12 h of fasting. The blood was centrifuged at 1,000 ×*g* for 15 min at 4°C to separate the plasma. The liver, kidney, spleen, adipose tissue (epididymal, perirenal, and mesenteric) and cecum of each animal were excised and cecal contents were collected. Samples were frozen immediately in liquid nitrogen and were stored at −80°C until analyzed.

Analyses of plasma and liver. Glucose, triacylglycerol, total cholesterol, free cholesterol, high-density lipoprotein (HDL) cholesterol, phospholipid, and free fatty acid of each plasma sample were determined using enzymatic assay kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Insulin, adiponectin and leptin in the plasma were determined using an Insulin Mouse ELISA Kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), an Adiponectin Mouse ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) and a Leptin Mouse/Rat ELISA Kit (Bio Vendor Laboratory Medicine, Inc., Brno, Czech Republic), respectively. For liver lipid analysis, the total lipids were extracted with 3 mL chloroform-methanol (2 : 1, v/v) from 0.9 mL of a 10% liver homogenate. After dissolving the liver total lipids in Triton X-100, triacylglycerol, total cholesterol and free cholesterol were determined using the same kits described above for plasma analysis (*15*).

Determination of short chain fatty acids in the cecum. Short chain fatty acids (SCFAs) in the cecum were determined by gas chromatography (*15*). Briefly, 100 μL diethyl ether and 5 μL 35% HCl were added to 100 μL 15% cecal contents homogenate containing 0.95 mmol/L 2-ethylbutyric acid as an internal standard. After centrifugation at 1,500 ×*g* for 20 min at 4°C, the diethyl ether layer (upper layer) was collected. The ether extract sample (1 μL) was injected into a gas chromatograph (GC-14B, Shimadzu Co., Kyoto, Japan) equipped with a DB-FFAP capillary column (15 m× 0.535 mm i.d. with a 0.5 mm film, Agilent Technologies, Santa Clara, CA, USA). Injector and detector temperatures were 145°C and 175°C, respectively. The initial oven temperature was 80°C for 1 min, and was then increased by 10°C/min, then held at 130°C for 1 min.

Analysis of bacterial flora in the cecum. A real-time PCR assay was used to quantify *Lactobacilli*, *Bifidobacte-*

Table 3. Body and tissue weights in mice fed experimental diets.

	Control	POR	POR Arg	POR K
Body weight (g)				
Before	24.65±0.64	24.22±0.66	24.13±0.73	24.15±0.67
After	38.02±2.62	37.61±1.44	36.09±2.27	36.59±1.58
Tissue weight (mg/g body weight)				
Liver	48.0±3.9	40.6±2.4	45.0±4.0	47.2±6.6
Kidney	13.6±0.9	12.9±1.1	13.4±1.2	13.0±1.6
Spleen	3.40±0.80^{a}	2.40±0.50^{b}	2.94±0.40^{a,b}	3.00±0.55^{a,b}
Adipose tissue				
Epididymal	42.8±3.9	43.2±3.1	37.0±6.5	38.6±3.2
Perirenal	18.9±1.8	17.7±2.5	16.9±3.7	17.8±1.9
Mesenteric	21.9±5.6	22.8±5.2	17.1±3.4	18.3±2.0
Total	83.7±8.7	83.8±6.5	71.0±12.6	74.8±5.9
Cecum	2.19±0.41^{a}	3.59±0.66^{b}	3.96±0.43^{b}	3.46±0.27^{b}
Cecal contents	4.48±1.72^{a}	7.91±2.34^{b}	5.97±1.57^{a,b}	5.17±2.23^{a,b}

Values are mean±SD, n=6. Values in rows with different letters are significantly different (p<0.05).

Table 4. Plasma and hepatic lipids in mice fed experimental diets.

		Control	POR	POR Arg	POR K
Plasma					
Triacylglycerol	(mg/dL)	152±28	158±38	138±22	139±45
Total cholesterol	(mg/dL)	96.0±21.0	86.8±16.8	79.8±10.9	87.5±26.5
Free cholesterol	(mg/dL)	21.3±11.4	17.0±3.5	15.6±4.4	18.7±7.6
HDL cholesterol	(mg/dL)	103.0±19.6	86.4±16.0	88.9±7.0	91.9±23.2
Phospholipids	(mg/dL)	208±32	176±28	183±13	183±38
Free fatty acids	(mEq/L)	1.96±1.99	1.71±0.60	1.64±1.12	1.74±1.05
Liver					
Triacylglycerol	(mg/g liver)	69.8±13.6	53.9±26.3	79.3±15.0	88.5±40.0
Total cholesterol	(mg/g liver)	11.8±1.4	10.7±0.7	11.7±0.8	10.4±0.8
Free cholesterol	(mg/g liver)	8.58±0.47	8.92±0.30	8.73±0.53	8.97±0.41

Values are mean±SD, n=6.

ria, *Bacteroides*, *Eubacteria* and *Clostridium coccoides* and all bacteria in the cecum. The bacterial-specific 16S rRNA gene primers are listed in Table 2 (*16–20*). External standards used to determine DNA copies of bacteria were prepared using the genomic DNA from RIKEN BioResource Center standard strains, which were *Lactobacillus acidophilus* (JCM1034), *Bifidobacterium pseudocatenulatum* (JCM 1200^{T}), *Eubaterium cylindroides* (JCM 7786), *Clostridium coccoides* (JCM 1395^{T}) and *Bacteroides thetaiotaomicron* (JCM5827^{T}). Briefly, each powdered bacterial sample was suspended in TE buffer at 95˚C for 5 min, and was then kept on ice for 1 min. Two microliters of each extract was used as a template to amplify the target-specific DNA sequence using a PCR KOD FX kit (Toyobo, Osaka, Japan). Amplifications were done with the following temperature profiles: one cycle at 95˚C (3 min), 40 cycles of denaturation at 95˚C (30 s), primer annealing (30 s), extension 72˚C (1 min 30 s) and one final cycle at 72˚C (10 min). The copy number of each standard gene was estimated on the basis of DNA fragment sizes and concentrations were measured using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA).

DNA was extracted from 0.05 g of each cecal specimen using a commercial DNA Stool Mini Kit (Qiagen, Hilden, Germany). The real-time PCR assays were performed in a volume of 20 μL containing 10 μL iQ SYBR Green Realtime PCR supermix (Bio-Rad), 5 nmol of each primer, 2 μL extracted DNA or each standard, and adjusted up to 20 μL with Milli-Q purified water. The PCR was conducted with a MyiQ Real Time PCR Detection System (BioRad). The real-time amplification was performed as follows: the first step was an internal preheating for 3 min at 95˚C, followed by 40 denaturing cycles at 95˚C for 15 s, annealing for 30 s, and 80˚C for 30 s. The standard curves were generated as the linear regression between Ct and $\log_{10}$ starting copy number of standard DNA. Copy numbers of bacteria-specific genes in the cecum extract were calculated using the standard curves.

Statistical analysis. Data are shown as means±SD. Statistical analyses were carried out using one-way ANOVA with Scheffe's test for comparison among the four groups. Statistically significant difference was set at p<0.05.

Table 5. Effect of dietary porphyran on insulin resistance parameter in mice.

	Control	POR	POR Arg	POR K
Glucose (mg/dL)	401±107	273±110	366±116	370±123
Insulin (ng/mL)	1.65±0.44[a]	1.00±0.12[b]	1.10±0.30[a,b]	1.12±0.39[a,b]
HOMA-IR	40.8±11.4[a]	17.6±8.3[b]	24.7±8.2[a,b]	25.4±11.3[a,b]

Values are mean±SD, $n=6$. Values in rows with different letters are significantly different ($p<0.05$).

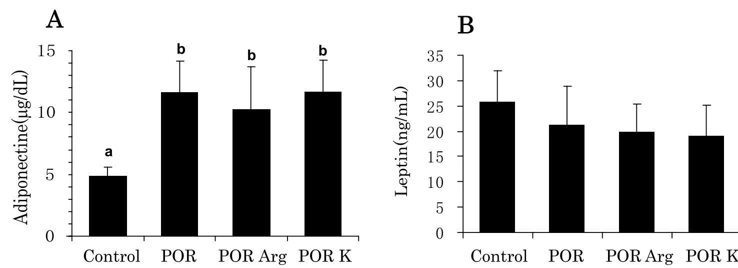

Fig. 1. Effect of porphyran on plasma adiponectin (A) and leptin (B) levels in mice. Values are means±SD ($n=6$). Means not sharing a common letter are significantly different among the groups at $p<0.05$, as determined by a one-way ANOVA with Scheffe's test.

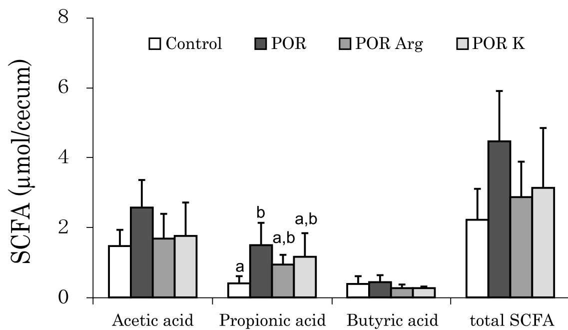

Fig. 2. Effect of porphyran on cecal short chain fatty acids (SCFAs) in mice. Values are means±SD ($n=6$). Means not sharing a common letter are significantly different among groups at $p<0.05$, as determined by a one-way ANOVA with Scheffe's test.

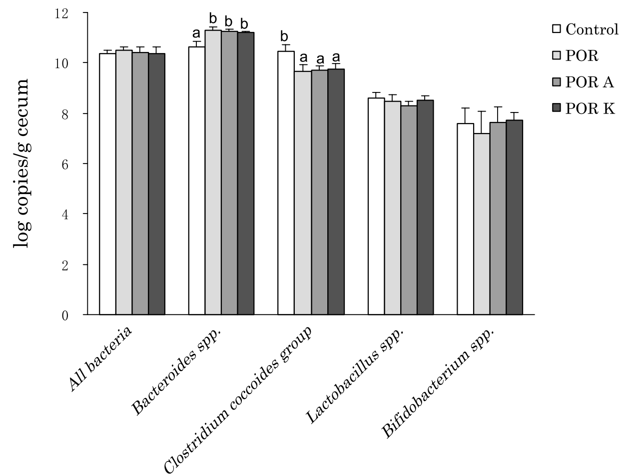

Fig. 3. Microbial flora populations in the cecum of mice fed experimental diets. Values are means±SD ($n=6$). Means not sharing a common letter are significantly different among groups at $p<0.05$, as determined by a one-way ANOVA with Scheffe's test.

RESULTS

The daily food intake was similar among the four groups of animals during the feeding study, and the body weight gain was not significantly different among the four groups (Table 3). There were no differences in liver, kidney or adipose tissue weights among the four groups, but the spleen weight in the POR group was significantly lower than in the control group. The cecum weights in the POR, POR Arg and POR K groups were significantly higher than in the control group.

As shown in Table 4, plasma and liver lipid levels did not differ among the four groups. Although there were no significant differences in plasma glucose levels among the groups, the plasma insulin level and the calculated homeostasis model assessment-insulin resistance (HOMA-IR) index in the POR group was significantly lower than in the control group, and those values in the POR Arg and K groups tended to be lower than in the control group (Table 5). Plasma adiponectin levels in the POR, POR Arg and POR K groups were significantly higher than in the control group (Fig. 1). There were no significant differences in plasma leptin levels among the four groups. These results suggest that dietary porphyran should improve insulin resistance in diabetic mice fed a high-fat diet.

We examined the effect of dietary porphyran on the level of SCFAs in the cecum because SCFAs formed by bacterial flora in the hindgut are highly dependent on the type of dietary fiber. The propionic acid content in the cecum of the POR group was significantly higher than in the control group (Fig. 2). Total SCFAs tended to increase in the POR group compared with the control group ($p=0.07$). In addition, we also examined the effect of dietary porphyran on the microbial population

in the cecum by quantitative PCR assay using 16S rRNA gene-targeted primers (Fig. 3). The levels of *Bacteroides* spp. in the cecum of the POR, POR Arg and POR K groups were significantly higher than the level in the control group. On the other hand, the levels of *Clostridium coccoides* in the cecum of the POR, POR Arg and POR K groups were significantly lower than the level in the control group. This change of the bacteria profile in the cecum by dietary porphyran may be related to the increase of propionic acid.

DISCUSSION

In the present study, we demonstrated that dietary porphyran improves the HOMA-IR and increases plasma adiponectin levels in type 2 diabetes mellitus model KK-Ay mice. It is well documented that adiponectin improves insulin sensitivity and plays a role in the suppression of metabolic derangements that may result in diabetes mellitus, obesity and atherosclerosis (*21*). However, the complete regulatory mechanisms for adiponectin expression have yet to be identified. It has been reported that the intake of dietary fiber is associated with higher plasma adiponectin levels in humans (*22*, *23*). Porphyran might improve the glucose metabolism via the enhancement of adiponectin expression.

It is well known that soluble dietary fibers are substrates for fermentation that produce SCFAs, primarily acetate, propionate and butyrate, as end products. The production of SCFAs is determined by a number of factors, including the numbers and types of microflora present in the colon, the substrate source and the gut transit time (*24*). In the present study, the content of propionic acid in the cecum of the porphyran-treated groups was significantly higher than in the control group and the profile of cecum bacterial flora was affected by the dietary porphyran. This change in the bacterial profile by dietary porphyran, especially the increase of *Bacteroides*, might be related to the increase of propionic acid. The propionic acid content in POR Arg and POR K groups tended to be increased, but not significantly different from that in the control group. Arginine and potassium might affect the production of SCFA by bacterial flora.

SCFAs produced in the hindgut are rapidly absorbed from the lumen and drain through the visceral tissues into the portal vein. It has been recently shown that SCFAs, including propionic acid, are ligands for the G protein-coupled receptors GPCR41 and GPCR43. Both GPCR41 and GPCR43 are present in adipose tissue (*25*, *26*), which suggests that adipose tissue is an important target for SCFAs. In contrast to our results, Al-Lahham et al. found that propionic acid significantly stimulated leptin mRNA expression and secretion by human adipose tissue in culture, whereas it had no effect on adiponectin (*27*). It is important to clarify the specific mechanism of adipocytokine production via GPCRs by SCFAs in vivo. In the present study, the plasma adiponectin concentrations in the POR, POR Arg and POR K groups were almost the same. In the POR Arg and POR K groups, however, HOMA-IR tended to be increased, but not significantly different from that in the control group. The reduction of HOMA-IR by dietary porphyran might be due to not only an increased level of adiponecitin but also elevated production of propionic acid, as well as other factors.

Tsuge et al. reported that porphyran lowers renal adipose tissue weight and serum cholesterol levels, and enhances the excretion of fecal cholesterol, while agar consisting of sugar components similar to porphyran had no effect on Sprague-Dawley rats fed a normal diet (*11*). However, the results of this study show that there are no effects of dietary porphyran on the lipid profile of plasma or liver in KK-Ay mice fed a high-fat diet. It seems that experimental conditions, especially the content of fat in the diet, affect the interference with the absorption of cholesterol by porphyran.

Generally, the quality of nori is highly correlated with its black color. A significant amount of cultivated nori is discarded because of its low quality, due to its reduced black color. Elucidation of the biological functions of dietary porphyran should provide an effective utilization of low quality nori. In this study, we compared the effects of dietary POR, POR Arg and POR K on KK-Ay mice, because porphyran salt forms are easy to prepare by an industrial procedure. It seems that the dietary POR Arg and POR K may improve the glucose metabolism, but the effect was slightly weaker than for the free form of porphyran.

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