Effects of Dietary Korean Proso-Millet Protein on Plasma Adiponectin, HDL Cholesterol, Insulin Levels, and Gene Expression in Obese Type 2 Diabetic Mice

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We investigated the effect of dietary Korean proso-millet protein concentrate (PMP) on glycemic responses, plasma lipid levels, and the plasma level and gene expression of adiponectin in obese type 2 diabetic mice under normal and high-fat feeding conditions. The findings were that the feeding of PMP clearly elevated plasma high-density lipoprotein cholesterol (HDL cholesterol) and adiponectin levels and brought about effective reduction in the levels of glucose and insulin in mice under high-fat diet conditions as compared with a control diet. Gene expression study revealed that the diet up-regulated expression of adiponectin and down-regulated tumor necrosis factor-α (TNF-α). Considering the central role of adiponectin and HDL cholesterol in improving and ameliorating type 2 diabetes, obesity, and cardiovascular disease, our findings imply that PMP may have potential for therapeutic intervention in type 2 diabetes.

Key words: Korean proso-millet protein; adiponectin; high-density lipoprotein cholesterol (HDL cholesterol); type 2 diabetes

It is well established that diet and lifestyle affect the incidence of type 2 diabetes.1,2) Obesity and diabetes are the most common human health problems worldwide.3) Obesity is a complex metabolic disorder that results from an imbalance of energy intake and energy expenditure, leading to excess accumulation of fat in various adipose tissues and organs. Further, it is well known that the development of obesity is associated with hyperinsulinemia, insulin resistance, type 2 diabetes, and abnormalities in lipid metabolism.4) Type 2 diabetes mellitus has become a major health concern worldwide.3) It is caused by an impaired response to insulin in liver and peripheral tissues4) and the development of obesity.5,5)

Recent studies have shown that adipose tissue plays a crucial role in the development of insulin resistance and type 2 diabetes.6–8) In fact, adipose tissue is now well established as an endocrine organ because it releases many biologically active substances known as adipocytokines, such as adiponectin and tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1, interleukin-6, leptin, and resistin in response to specific extracellular stimuli and to variations in metabolic conditions.7–9) In addition, in type 2 diabetes, it has been reported that circulation of adiponectin is reduced, whereas the release of TNF-α is increased.9,10) It is also well documented that adiponectin is an insulin-sensitizing hormone,11) and that plasma adiponectin levels are highly associated with insulin sensitivity and type 2 diabetes.11–13) These reports demonstrate that adiponectin is an important adipocytokine in preventing the development of type 2 diabetes associated with obesity and vascular disease.6)

Some dietary proteins, such as soy and cod proteins, have been found to have favorable effects on glycemic responses and plasma adiponectin levels and gene expression.14,15) However, the relationship between dietary protein and the effect on glycemic control, plasma adiponectin levels and gene expression is not fully documented. Thus it would be of value to study how specific dietary protein acts on glycemic control and circulating levels of adipocytokines.

Millet is a vitally important food source for humans in Asia and Africa,16,17) but studies on the health benefits of millet are scarce. We have reported that feeding
of protein concentrate of proso-millet, cultivated in Japan, increases plasma HDL cholesterol levels and the HDL₂ subfraction in rats and mice.¹⁸–²⁰ Further, we found that proso-millet protein concentrate has protective effects against D-galactosamin-induced liver injury in rats.²¹

In South Korea, a considerable amount of foxtail-millet is consumed.²² We have also reported that the feeding of protein concentrate of Korean foxtail-millet significantly elevated plasma adiponectin and HDL cholesterol levels, whereas it caused major decreases in insulin levels relative to a casein diet in type 2 diabetic mice,²³ but no large-scale study of the potential health benefits of Korean proso-millet has been done.

The present study was undertaken to examine the effects of intake of Korean proso-millet protein on plasma concentrations of lipids, adiponectin, and insulin, and the gene expression of adiponectin and TNF-α in adipose tissue in obese type 2 diabetic mice under normal and high-fat feeding conditions. Our results show a clear increase in plasma levels of adiponectin and HDL cholesterol, while those of glucose and insulin decrease, accompanied by enhanced expression of adiponectin and a reduction in TNF-α. This is the first report on the beneficial effects of Korean proso-millet on plasma concentrations of lipids, adiponectin, and insulin and the gene expression of adipocytokines.

Materials and Methods

Materials. Alpha-amylase (Lactase SR-140) and glucoamylase (Magunax JW-203) were obtained from Rakuto Kasei Industries (Ohtsu, Japan). All other laboratory chemicals were of the highest available grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Korean proso-millet protein concentrate. Proso-millet (Panicum miliaceum L.) harvested in 2005 was obtained from a farmer in Yeosu, Korea, and the milled grain was used in this study. Proso-millet protein concentrate (PMP) was prepared according to previously reported methods¹⁸ such that the diet contained 20% protein. In brief, a mixture of α-amylase and glucoamylase was used to digest the starch at 60°C for 24 h (pH 7.0). Then after removal of the soluble fraction, the resulting material was freeze-dried and defatted with n-hexane. Table 1 shows the composition of the milled grain and PMP. The amino acid composition of the milled grain and PMP was analyzed with an amino acid analyzer (JLC-500, JEOL, Tokyo). Tryptophan content was analyzed by a photometric procedure²⁹ after alkaline hydrolysis.²⁵ The contents of cystine and methionine were determined according to the method of Moore.²⁶

Animals and diets. Two separate experiments in which animals were fed a normal diet or a high-fat diet were carried out using genetically obese type 2 diabetic male KK-Ay mice. Male mice (5 weeks old) weighing 24–26 g were purchased from Clea Japan (Tokyo, Japan) and were housed individually in stainless steel cages in an air-conditioned room controlled at 22 ± 1°C at 55% relative humidity under a 12:12-h dark-light schedule. Animals freely received diet and water.

In experiment 1, the effects of dietary PMP were investigated in mice ingesting a normal diet. After a period of adaptation, mice were divided into two experimental groups of 6–7 animals and given a diet containing casein or PMP diet containing high fat for 3 weeks. For details of diets, see "Materials and Methods."
by administering 1.5 g glucose/kg body weight by stomach tube after 16 h of fasting. Blood glucose was determined at 0, 15, 30, 60, 90, and 120 min after administration. On day 21, they fasted for 6 h, and blood was collected from the abdominal vena cava under anesthesia with diethyl ether. Blood was quickly centrifuged at 3,550 g for 15 min at 4 °C to obtain plasma. The liver was perfused with saline and quickly removed. Kidneys and adipose tissues were also excised. These samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

In experiment 2, the effects of dietary PMP were investigated in mice ingesting a high-fat diet. Animals were divided into two groups of 6–7 animals each and given a high-fat diet containing casein or PMP as protein sources for 3 weeks (Table 2). These diets with high-fat are referred to as CF and MPF respectively. The PMF diet was supplemented with lysine, as described in experiment 1. Animals were thereafter subjected to the procedures indicated in experiment 1.

All animal experiments were approved by the Animal Care and Use Committee of Iwate University.

Blood analysis. Plasma concentrations of total and HDL cholesterol and triglyceride were enzymatically determined using commercial kits (Cholesterol E-Test Wako, HDL cholesterol E-Test Wako and Triglyceride E-Test Wako, Wako Pure Chemical Industries) respectively. Plasma concentrations of insulin and adiponectin were measured using ELISA kits (Levis mouse insulin kit, Shibayagi, Gunma, Japan, and the mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo), respectively.

Liver lipid analysis. Extraction of lipids from liver was performed by the method of Folch et al., and the cholesterol and triglyceride contents were measured with the commercial kits mentioned above.

Quantitative analysis of gene expression. Adiponectin and TNF-α mRNA levels in adipose tissues were analyzed by real-time PCR. Total RNA was extracted from epididymal and perirenal adipose tissues using an RNeasy lipid tissue mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Total RNA samples (500 ng) were converted to cDNA by reverse transcription using a PrimeScript™ RT reagent kit (Takara Bio, Ohtsu, Japan). Real-time PCR was performed with the SYBR® Premix EX Taq™ (Takara Bio) in a LightCycler (Roche diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Real-time PCR was run in duplicate for individual samples. The amount of gene expression was quantified relative to β-actin expression. The relative concentration of DNA was analyzed by the fit point method, LightCycler Software (Ver. 3.5). Primer sequences used for adiponectin, TNF-α, and β-actin were as follows: adiponectin forward primer, 5'-CAG GCA TCC CAG GAC ATC-3', and adiponectin reverse primer, 5'-TCT CAC CCT TAG GAC CAA GAA G-3', TNF-α forward primer, 5'-CTG TAG CCC ACG TCG TAG C-3' and 'TNF-α reverse primer, 5'-TTG AGA TCC ATG CCG TTG-3'. Beta-actin forward primer, 5'-CCA ACC GTG AAA AGA TGA TGA CC-3', and β-actin reverse primer, 5'-ACC AGA GGC ATA CAG GGA CA-3'.

Statistical analysis. Analysis of variance was done on the experimental data, and the differences between means were considered to be significant at p < 0.05 by Student’s t test. These analyses were performed using InStat ver. 2.03 (GraphPad Software, San Diego, CA).

Results

Compositions of milled grain and PMP

The dry weight of lipid and total dietary fiber in the milled grain was about 2-fold higher than that of Japanese proso-millet (Table 1), although the protein content was similar.27,29) The contents of lipid, ash, and dietary fiber in PMP were also about 3-fold higher than those of Japanese proso-millet, although the protein content was similar. The amino acid composition of PMP (Table 3) was similar to that of Korean foxtail-millet.23) The glycine and alanine contents in the milled grain and PMP were 2- or 4 fold higher than casein, whereas valine, methionine, and tyrosine were lower. The lysine and histidine content was markedly lower, and similar to those of Japanese proso-millet. The amino acid composition of Korean proso-millet was similar to that of Japanese proso-millet, with the exception of cystine, methionine, and tyrosine, which were higher in the Japanese proso-millet.19,21,27,29)
under high-fat feeding conditions in adipose tissues between the two diet groups (Table 4). There were no significant differences in gene expression of adiponectin or TNF-α/C6 between the two dietary groups (Fig. 1B and C). There were also no significant differences in insulin or adiponectin levels between these two dietary groups (Fig. 1A). There were no significant differences in cholesterol content in the liver (Table 4). No significant changes in plasma levels of glucose, total cholesterol, and triglyceride were not significantly altered between the two dietary groups (Table 4). No significant changes in plasma glucose concentrations during the study period of 3 weeks (Table 4) or the area under the curve (AUC) value at OGTT were observed (data not shown) but, the cholesterol content in the liver (3.17 ± 0.49 mg/g) was reduced significantly by ingestion of PMP relative to a casein diet (4.15 ± 0.36 mg/g; p < 0.01). Triglyceride levels were not influenced (Table 4).

**Effects of dietary PMP on body-weight gain, tissue weight, plasma levels of glucose and lipids, and liver lipid contents**

Body-weight gain, food intake, tissue weights, and plasma levels of glucose, total cholesterol, and triglyceride were not significantly altered between the two dietary groups (Table 4). No significant changes in plasma glucose concentrations during the study period of 3 weeks (Table 4) or the area under the curve (AUC) value at OGTT were observed (data not shown) but, the cholesterol content in the liver (3.17 ± 0.49 mg/g) was reduced significantly by ingestion of PMP relative to a casein diet (4.15 ± 0.36 mg/g; p < 0.01). Triglyceride levels were not influenced (Table 4).

**Effects of dietary PMP on plasma levels of HDL cholesterol, insulin, adiponectin and gene expression**

Intake of PMP significantly increased plasma HDL cholesterol concentration (46.3 ± 11.6 mg/dl) as compared to a casein diet (30.5 ± 12.6 mg/dl; p < 0.05) (Fig. 1A). There were no significant differences in insulin or adiponectin levels between these two dietary groups (Fig. 1B and C). There were also no significant differences in gene expression of adiponectin or TNF-α/C6 in adipose tissues between the two diet groups (Table 4).

**Experiment 2**

**Effects of dietary PMP on body-weight gain, tissue weight, plasma, and liver lipid concentrations in mice under high-fat feeding conditions**

As shown in Table 5, there was no significant difference in body-weight gain, food intake, or tissue weights. Plasma total cholesterol and triglyceride concentrations and liver lipid contents were also not significantly different between two dietary groups.

**Fig. 1. Effects of Dietary PMP on Plasma Levels of HDL Cholesterol, Insulin, and Adiponectin in Mice under Normal Feeding Conditions (Experiment 1).**

Panels show the changes in plasma levels of HDL cholesterol (A), insulin (B), and adiponectin (C). Values are means ± SD of 6–7 animals. *p < 0.05 vs. casein diet group.

**Table 4. Effects of Dietary PMP on Body-Weight Gain, Food Intake, Tissue Weights and Biochemical Measurements from Plasma and Liver in KK-Ay Mice (Experiment 1)***

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Casein</th>
<th>PMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (g/21 d)</td>
<td>10.5 ± 1.4</td>
<td>10.6 ± 1.6</td>
</tr>
<tr>
<td>Food intake (g/21 d)</td>
<td>97.2 ± 8.6</td>
<td>94.8 ± 4.2</td>
</tr>
<tr>
<td>Liver wt. (g/100 g body wt.)</td>
<td>4.70 ± 0.44</td>
<td>4.82 ± 0.28</td>
</tr>
<tr>
<td>Kidneys wt. (g/100 g body wt.)</td>
<td>1.45 ± 0.04</td>
<td>1.42 ± 0.09</td>
</tr>
<tr>
<td>Adipose tissue wt.:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymal (g/100 g body wt.)</td>
<td>3.31 ± 0.82</td>
<td>3.58 ± 0.59</td>
</tr>
<tr>
<td>Perirenal (g/100 g body wt.)</td>
<td>1.47 ± 0.37</td>
<td>1.47 ± 0.24</td>
</tr>
<tr>
<td>Plasma components (mg/dl):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>218 ± 65</td>
<td>205 ± 42</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>146 ± 47</td>
<td>157 ± 37</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>244 ± 82</td>
<td>217 ± 66</td>
</tr>
<tr>
<td>Liver lipids (mg/g):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4.15 ± 0.36</td>
<td>3.17 ± 0.49*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>11.4 ± 2.3</td>
<td>11.4 ± 5.4</td>
</tr>
<tr>
<td>Gene expressions in adipose tissues:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perirenal Adiponectin (% of control)</td>
<td>100 ± 86</td>
<td>103 ± 55</td>
</tr>
<tr>
<td>TNF-α (% of control)</td>
<td>100 ± 108</td>
<td>51.3 ± 36.2</td>
</tr>
<tr>
<td>Epididymal Adiponectin (% of control)</td>
<td>100 ± 55</td>
<td>117 ± 36</td>
</tr>
<tr>
<td>TNF-α (% of control)</td>
<td>100 ± 43</td>
<td>77.8 ± 15.3</td>
</tr>
</tbody>
</table>

*Values are means ± SD for 6–7 mice. For details, see "Materials and Methods."

*Within the same row but not sharing a common superscript means significantly different (p < 0.05).
was a tendency towards a decrease in AUC in the PMPF diet group (12.8 ± 4.2 g/dl/min) as compared with the casein and CF groups (18.6 ± 8.7 g/dl/min) (Table 5).

**Gene expression of adiponectin and TNF-α in adipose tissues**

In agreement with the results for plasma levels of adiponectin, by quantitative analysis of gene expressions, we also found that expression of adiponectin in both perirenal and epididymal adipose tissues of a PMPF diet group were significantly higher than those of mice given the CF diet (155 ± 43 vs. 100 ± 32; *p < 0.05 and 160 ± 43 vs. 100 ± 50; *p < 0.05; Fig. 3A and B respectively). In contrast, the expression of TNF-α in the PMPF diet group was significantly lower than that of mice given the CF diet (74 ± 19 vs. 100 ± 24; *p < 0.05 and 59 ± 26 vs. 100 ± 26; *p < 0.05; Fig. 3C and D respectively).

**Discussion**

Lifestyle factors that aggravate type 2 diabetes, cardiovascular diseases, and obesity are mainly diet and a sedentary lifestyle. Thus the role of diet should be emphasized in treatment of type 2 diabetes and dietary modifications that play a central preventive or protective role against type 2 diabetes should be investigated.

In connection with the results of the present study, it has been reported that dietary soy protein elevated plasma adiponectin concentration and enhanced gene expression of adiponectin in adipose tissue, while plasma glucose and insulin concentrations were not
influenced in rats. It has also been found that when type 2 diabetic mice were given soy protein, although adiponectin concentrations did not change, plasma glucose concentrations decreased under conditions of restricted food intake after feeding of a high-fat diet. Further, in a study of cod and soy proteins, Lavigne et al. observed decreases in plasma glucose and insulin concentrations together with improvement of glucose clearance and insulin sensitivity as compared with casein. However, the studies on the effects of dietary proteins from plant sources on glycemic responses and on the levels and gene expression of adiponectin are not fully elucidated and are not yet unequivocal.

The findings of the present study are that feeding of PMP elevated plasma HDL cholesterol and adiponectin levels in mice under feeding conditions of high-fat (Fig. 2C and D). Especially, the levels adiponectin were about 2-fold higher than those for the CF diet (Fig. 2D).

In connection with relationship of adiponectin levels with HDL cholesterol, Zietz et al. reported that adiponectin represents an independent cardiovascular risk factor predicting serum HDL-cholesterol levels in type 2 diabetes. Further, it has been demonstrated that in subjects without type 2 diabetes, plasma adiponectin concentrations with obesity were strongly positively associated with HDL cholesterol concentrations. The relationship of plasma adiponectin levels with those of HDL cholesterol found in this study agree with these results. In addition, it is known that the ratio of plasma triglyceride to HDL cholesterol is an useful marker of insulin resistance. The ratio of mice fed a PMPF diet tended to be lower than in the CF diet group (Table 5).

Further, PMP diet brought about an effective reduction in the levels of glucose and insulin (Fig. 2A and B). These observations are also supported by findings that a rise in circulation level of adiponectin inhibits glucose production in liver.

It is well established that adiponectin accelerates insulin sensitivity and promotes lipid metabolism.

To determine whether PMP diet modulates gene expression of adiponectin and TNF-α in the same way as observed for plasma levels, we examined gene expression in adipose tissue, because it is accepted that TNF-α levels are negatively associated with insulin sensitivity. The results revealed that a PMP diet clearly up-regulates expression of adiponectin (Fig. 3A and B) and, in contrast to this, down-regulates that of TNF-α (Fig. 3C and D). In addition, it is known that the size of adipocytes influences the gene expression and secretions of adipocytokines such as adiponectin and TNF-α. But the sizes of adipocytes were not measured in this study. Hence, it is also necessary to examine the effects of a PMP diet on the sizes and differentiation of adipocytes. Thus, taking into account the favorable effects of PMP on plasma levels of glucose, HDL cholesterol, adiponectin, and insulin and on gene expression, we suggest that our results support the thesis that a PMP diet shows strong effectiveness against insulin sensitivity.

We were the first to report the beneficial effects of dietary protein concentrate of Korean foxtail-millet in genetically obese type 2 diabetic mice under the same feeding conditions as in the present study, finding that plasma levels of HDL cholesterol and adiponectin increased markedly, as compared with a casein diet group under both normal and high-fat dietary conditions, although plasma glucose levels were mostly the same value between the two groups, whereas concentrations of insulin decreased greatly. These results for Korean foxtail-millet were different from the observations reported here. The current results under normal dietary conditions exhibited no obvious effect on plasma levels of adiponectin or insulin (experiment 1; Fig. 1B and C). Further, although the present study found a significant reduction in glucose levels under high-fat feeding conditions (Fig. 2A), the levels were not influenced in mice fed a Korean foxtail millet diet with high fat. On the other hand, the present findings under high-fat diet conditions were similar to the results of our recent study with Japanese proso-millet, in which we found an increase in HDL cholesterol and adiponectin levels and a reduction in those of glucose (unpublished results).

The mechanism and components by which PMP exerts its beneficial effects on the levels of glucose, adiponectin, and insulin, and on gene expression, are unclear. It is known that amino acids play a role in insulin and glucose homeostasis, and that leucine and isoleucine modify glucose homeostasis, and insulin secretion. However, since the contents of these amino acids in PMP is similar to or less than that of casein (Table 3), it appears unlikely that the plasma glucose and insulin-lowering effects of PMP occur through the action of amino acids. Further studies are required to define this mechanism.

In conclusion, the feeding of PMP improved glycemic responses and plasma levels of adiponectin, HDL cholesterol, and insulin in obese genetically type 2 diabetic mice under high-fat feeding conditions. These results suggest that PMP is more effective in severe diseases such as type 2 diabetes with an insulin-resistant induced high fat diet. Considering the primary role of adiponectin and HDL cholesterol in improving or ameliorating type 2 diabetes, obesity, and cardiovascular disease, our findings imply that PMP plays a crucial role in improving type 2 diabetic treatments.

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