Food Sci. Biotechnol. 20(5): 1265-1270 (2011)
DOI 10.1007/s10068-011-0174-7

RESEARCH ARTICLE

Red Yeast Barley Reduces Plasma Glucose Levels and Activates AMPK Phosphorylation in *db/db* Mice

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Received: 5 March 2011 / Revised: 30 May 2011 / Accepted: 25 July 2011 / Published Online: 31 October 2011

Abstract Red yeast (*Monascus purpureus*) fermented over rice has a limited hypoglycemic activity. To enhance its glucose-lowering effect, we fermented red yeast over waxy barley, a hypoglycemic grain with high levels of fibers and β-glucans, and investigated the metabolic effects of red yeast barley (RYB) in high-fat-fed hyperglycemic *db/db* mice for 6 weeks. The fasting glucose levels were significantly reduced in the RYB group at 6 weeks by 25% ($p<0.05$), as was the glucose tolerance (–27% of area under the curve in RYB vs. controls, $p<0.05$). Plasma insulin levels and the expression of PPAR-γ were unaltered, however, the phosphorylation activation of hepatic AMP-activated protein kinase (AMPK) was increased significantly in RYB group compared with controls suggesting that hypoglycemic effect of RYB may be achieved by AMPK-dependent mechanism. RYB may be used as a hypoglycemic functional food modulating cellular AMPK activity.

Keywords: *Monascus purpureus*, red yeast, barley, glucose tolerance, AMP-activated protein kinase (AMPK)

Introduction

Whole-grain foods are nutrient enriched compared with refined-grain foods. Dietary fibers in whole-grain foods are indeed especially beneficial for the prevention of cardiovascular disease, reducing total cholesterol levels (1-4) and type 2 diabetes, while improving glucose tolerance and insulin sensitivity (5-7). These beneficial effects are related to the soluble fibers especially abundant in oatmeal, barley, and psyllium (8). In particular, a strain of barley called waxy barley is a low glycemic food due to high levels of soluble fibers but low levels of starch content compared with rice or wheat (9). The major soluble dietary fibers in barley strains, especially in waxy barley, are β-glucans, which are reported to have multiple beneficial hypoglycemic effects (10,11).

Red yeast rice is made by fermenting the red yeast, *Monascus purpureus*, over rice and it has been used as a common medicinal food in Korea, Japan, and China for centuries to prevent and treat metabolic disorders (12). Monascus yeast produces monacolin K, also known as lovastatin, capable of inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, thus it could consequently reduce plasma LDL-cholesterol levels (13). Moreover, studies have suggested that red yeast rice could have mild hypoglycemic effect by improving insulin sensitivity and glucose tolerance (14,15) and showed that an unidentified compound from fermentation may play a role directly on insulin sensitivity by increasing insulin-sensitizing adiponectin and plasma insulin levels (16), or improving insulin signaling in liver and muscle (17), or indirectly affect on insulin sensitivity by reducing plasma free fatty acid levels (18). However, a hypoglycemic effect of red yeast rice is limited (14,15). In order to enhance glucose-lowering effect, we fermented red yeast over waxy barley, which contains high levels of soluble fiber, mainly β-glucan (19). In the present study, the *db/db* mice, a mouse model of type 2 diabetes, were fed the high-fat diet containing 15%(w/w) of red yeast barley (RYB) for 6 weeks to investigate key hypoglycemic biomarkers. Plasma glucose and lipid levels, the insulin concentrations, the peroxisome proliferator-activated receptor (PPAR)-γ expression, and the phosphorylation activation of AMP-

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activated protein kinase (AMPK) were examined.

Materials and Methods

Animals Animal care and handling were performed according to protocols approved by the Animal Experimentation and Ethics Committee of Korea University (Protocol No. KUIACUC-20090420-4). Diabetic *db/db* mice, 7 males per group, aged 12 to 15 weeks were obtained from Oriental Bio (Seoul, Korea). After 1 week of quarantine, randomly assigned mice were put on 6-week test diets. Food intake, body weight, and insulin levels were monitored every 2 weeks (day 1, 14, 28, and 42), and the mice were kept in a temperature-controlled (25°C) specific-pathogen-free facility on a 12 h light/dark cycle. Food and water were given *ad libitum*. Blood samples were collected retro-orbitally after 8-10 h of fasting. After 6-week of feeding, mice were sacrificed and blood was collected by cardiac puncture and organs were collected according to the approved protocol and samples were stored at –20°C until analysis. If required, animals were sacrificed with CO_2.

Diets The control and test diets were formulated by Dyets Inc. (Dyets, Bethlehem, PA, USA). For the 6-week test diet period, the control group was continually fed the background diet consisting of modified AIN-76 diet containing 65% of the calories from fats, and the treatment group was fed a high-fat diet, in which 15%(w/w) of the corn starch was replaced with RYB. The waxy barley used was bred and cultivated at the National Institute of Crop Science, Rural Development Administration (Iksan, Korea). To prepare RYB, the strain *Monascus purpureus* (DSM 1603) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and was cultured as described in Wild *et al*. (20) with slight modifications. Briefly, it was cultured on a waxy barley medium consisting of an autoclaved mixture of 20 g ground waxy barley, 8 g sucrose, 2 g yeast extract, 0.1 g KH_2PO_4, 0.1 g $CaCl_2$, and 0.5 g $MgSO_4$ in 100 mL of water in petri dishes for 3 weeks at 30°C. The fermented barley was dried at 30°C and subsequently pulverized.

Analysis of β-glucan, dietary fiber, monacolin K, and citrinin β-Glucan was determined by an enzymatic method with a commercial kit that quantifies glucose released by β-glucosidase from β-glucan with the glucose oxidase/peroxidase system (Megazyme International, Bray, Ireland). Total, insoluble, and soluble dietary fibers were analyzed using the enzymatic-gravimetric American Organization of Analytical Chemists (AOAC) method using a total dietary assay kit (Sigma TDF-100A; Sigma-Aldrich, St. Louis, MO, USA). A HPLC system (1100 Series; Agilent, Santa Clara, CA, USA), UV detector, and C18 octadecyl saline (ODS) column (250×4.6-mm, 5-μm, OptimaPak; RStech, Daejeon, Korea) were used to analyze monacolin K and citrinin. RYB (80 mesh) was extracted with 75% ethanol for monacolin K and with 100% ethanol, respectively, for citrinin at 30°C on an ultrasonic bath and subsequently centrifuged. The supernatant was filtered, subjected to HPLC based on described methods for monacolin K (21) and citrinin (22). The levels in the samples were calculated from standard curves generated for purified monacolin K and citrinin (Sigma-Aldrich).

Blood lipid and glucose analysis and oral glucose tolerance test (OGTT) Blood samples were analyzed at 0 and 6 weeks of feeding. Plasma glucose, triglycerides (TG), total cholesterol, and HDL-cholesterol were quantified spectrophotometrically using a colorimetric enzymatic method with an Asan Pharmaceutical kit (Seoul, Korea). Plasma insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA; Shibayagi, Shibukawa, Japan). After 6-week of feeding period, the OGTT was performed by giving the mice 1.5 g/kg BW of glucose solution by gavage after sampling blood for determination of glucose at time 0. The glucose levels at 15, 30, 60, 90, and 120 min after glucose administration were measured with a glucometer (Roche, Mannheim, Germany). The integrated area under the glucose response curves (AUC) was calculated using the trapezoid method.

Reverse transcription (RT)-PCR Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from liver tissue, and cDNA was synthesized with oligo dT and Superscript II, as previously reported (23,24). The cDNA was used for RT-PCR with primers for the LDL receptor, CYP7A1, PPAR-γ, and HMG-CoA reductase (Bioneer, Seoul, Korea). β-Actin was used as a reference. The RT-PCR products were quantified by measuring the intensity of the target band on agarose gels using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). Primer sequences are shown in Table 1.

Immunoblotting analysis Proteins (30 or 40 μg) isolated from mouse tissues were subjected to SDS-PAGE (10%, w/w) and then transferred onto nitrocellulose membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20, the membranes were immunoblotted with polyclonal anti-AMPK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal anti-phospho-AMPKα (Santa Cruz Biotechnology), followed by anti-rabbit IgG-HRP or anti-mouse IgG-HRP (Santa Cruz Biotechnology). The proteins were visualized

Table 1. PCR primer sequences

Gene	Primer	Sequence (5'→3')	Size
Mouse			
LDL receptor	Forward	ACTCAGGCAGCAGGAACGAG	221 bp
	Reverse	GTCATTTTCACAGTCTACCT	
HMG-CoA R	Forward	AAGATCTTCAAGGAACGTGC	312 bp
	Reverse	AACTGTAGCTCACATCTGTG	
CYP7A1	Forward	CCTTGGACGTTTTCTCGCT	256 bp
	Reverse	GCGCTCTTTGATTTAGGAAG	
β-Actin	Forward	TGCTGTCCCTGTATGCCTCT	463 bp
	Reverse	AGGTCTTTACGGATGTCAACG	
PPAR-γ	Forward	CCATTCTGGCCCACCAA	313 bp
	Reverse	AATGCGAGTGGTCTTCCAT	

with an enhanced chemiluminescence (ECL) detection system (Anigen, Seoul, Korea).

Statistical analysis The results are expressed as the mean±standard error mean (SEM). Groups were compared using Student's *t*-test. A $p<0.05$ was considered significant.

Results and Discussion

Composition of RYB The total, insoluble, and soluble dietary fibers and β-glucan contents of RYB were 22.9±2.7, 6.9±1.3, 16.0±1.4, and 6.0±0.1%(w/w), respectively. The level of monacolin K was 0.03% (w/w, 3 mg/g) in RYB, which was slightly lower than the suggested levels for a cholesterol-reducing health claiming by the Korean Food & Drug Administration (0.05%, w/w), however, citrinin, a potential nephrotoxin byproduct formed during red yeast fermentation, was undetected in RYB.

Body weight, food intake, and insulin levels The body weight of the control *db/db* mice increased gradually during the 6-week feeding period, while the mice in the RYB group did not gain as much weight. After 6 weeks, the mean body weight of the RYB group was significantly lower than that of the control group (55.7±3.4 g vs. 63.1±2.6 g, respectively; $p<0.05$). Food intake did not differ between the 2 groups during the 6 weeks (Table 2). These suggest potential anti-obesogenic activities of RYB in mice of which mechanism should be confirmed in the future.

Metabolic parameters The baseline plasma glucose concentrations in the *db/db* mice were similar in both groups, whereas the levels in the RYB group at 6 weeks were significantly reduced by 38% compared with baseline and decreased by 25% compared with the controls at 6 weeks ($p<0.05$; Fig. 1A). The plasma triglyceride and total cholesterol levels were not different between control and RYB groups after 6 week (Fig. 1B, 1C). The HDL-cholesterol levels were increased in both groups at 6 weeks and the elevation was +36% in the RYB group compared with the levels at 0 week (Fig. 1D). The glucose-lowering effect of RYB found in our study was greater than the reported hypoglycemic effects of red yeast rice or even the effect of barley itself (19). Several studies have reported only mild hypoglycemic effects of red yeast rice (14,15,25). In addition, the results of the OGTT after 6-week of feeding showed that the RYB diet significantly improved the glucose tolerance compared with controls. The postprandial percent glucose concentrations were lower at 30 min and thereafter, and the AUC in the RYB group was significantly reduced by 27% (Fig. 2, $p<0.05$).

Table 2. Food intake, body weight, and insulin concentrations in mice

	0 week	2 week	4 week	6 week
Body weight (g)				
Control	53.5±2.3[1)]	55.4±3.4	60.1±2.8	63.1±2.6
RYB[2)]	54.1±2.2	56.1±4.2	55.3±3.4	55.7±3.4*
Food intake (g/day)				
Control	6.5±0.4	6.8±0.5	7.0±0.5	7.0±0.7
RYB	6.7±0.3	7.0±0.4	6.8±0.4	7.2±0.5
Plasma insulin (μU/mL)				
Control	48.6±9.8	43.8±5.0	59.2±14.1	45.7±11.6
RYB	45.3±12.8	40.5±13.7	48.3±25.1	57.1±16.0

[1)]Mean±SEM; *$p<0.05$ compared with controls
[2)]Red yeast barley

Induction of AMPK activity The mRNA expression of key genes in hepatic cholesterol metabolism (HMG-CoA reductase, the LDL receptor, and CYP7A1) were marginally affected (Fig. 3), in line with the non-significant changes in the total cholesterol levels in *db/db* mice. We also examined the effect of the RYB-containing diet on the phosphorylation activation of AMPK in the mouse liver

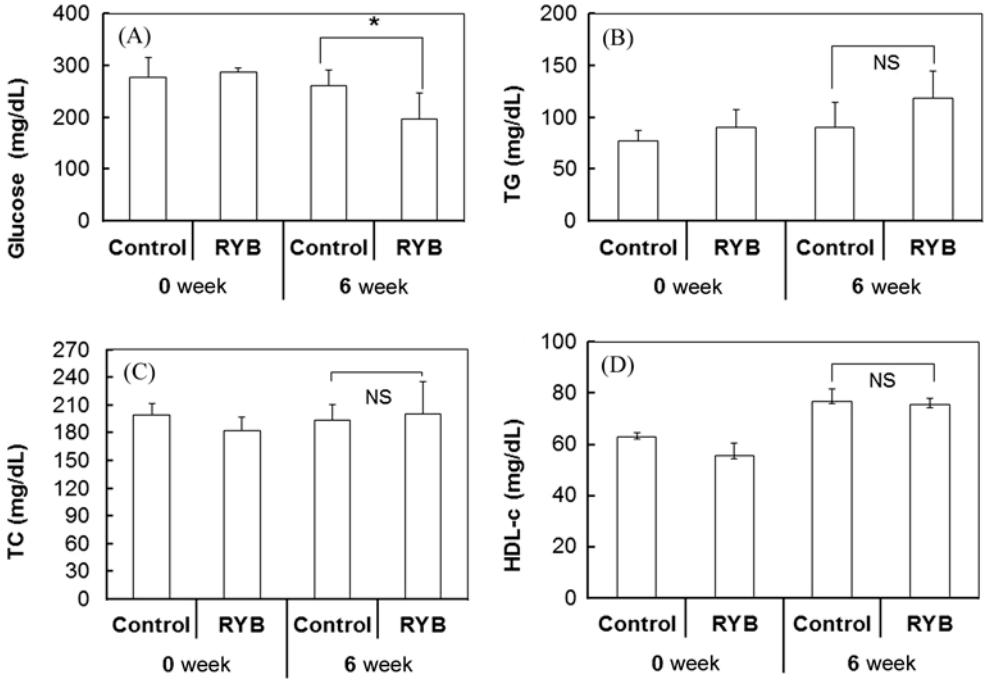

Fig. 1. Changes in plasma glucose (A), triglyceride (B), total cholesterol (C), and HDL-cholesterol concentrations (D) in *db/db* mice fed control or red yeast barley (RYB) diets for 6 weeks. Control group was fed the background diet consisting of modified AIN-76 diet containing 65% of the calories from fats, and the treatment group was fed a high-fat diet, in which 15%(w/w) of the corn starch was replaced with RYB. Error bars are SE; * Indicates $p<0.05$ between control and RYP group

with immunoblot analysis. In the liver, the ratio of p-AMPK to AMPK expression was increased significantly in the RYB group by up to 4.3-fold (Fig. 4).

Implication of the results Hyperglycemia is a major risk factor for type 2 diabetes and metabolic syndrome, which is one of the leading causes of mortality in western society. Accordingly, therapeutic and preventive interventions for hyperglycemia would have a significant impact on type 2 diabetes- and metabolic syndrome-related death. Glucose-lowering drugs are effective at improving hyperglycemia and insulin resistance, but have undesirable side effects and tolerance issues with long-term intake. Therefore, it is necessary for patients to undergo dietary modification using effective health functional foods that have both nutritional and medicinal benefits, to prevent and attenuate hyperglycemia. This study investigated the anti-diabetic effects of RYB in *db/db* mice.

Red yeast rice is known to lower blood cholesterol levels but its glucose-lowering activity is mild (14,15,25). In the present study, RYB, red yeast fermented over waxy barley, showed different metabolic effects compared with conventional red yeast rice showing strong hypoglycemic effect with marginal lipid lowering activity. This may be, at least in part, due to high fiber contents in waxy barley and relatively low level of monacolin K production during fermentation compared with conventional red yeast rice. In addition, it appeared that red yeast fermentation

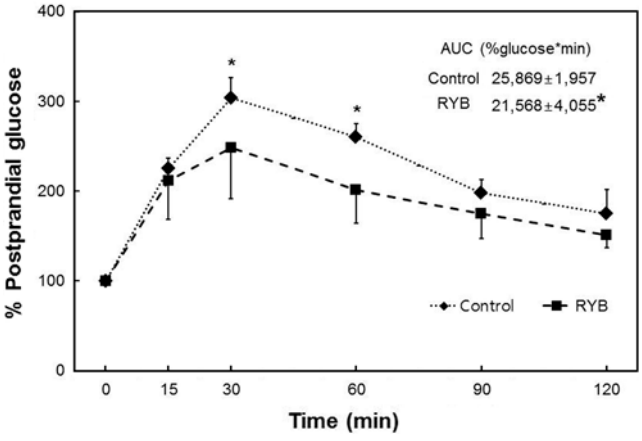

Fig. 2. Oral glucose tolerance test of *db/db* mice. Blood samples were collected from the tail, and the glucose concentration was measured with a glucometer. *$p<0.05$ compared with controls

successfully reinforced hypoglycemic effect of waxy barley to have apparent hypoglycemic effects.

Hypoglycemic mechanism of RYB We examined potential mechanism of hypoglycemic effects of RYB. The well known hypoglycemic mechanisms include: insulin secretagogue activity, which increases insulin release from pancreatic islets; the reduction of hepatic glucose production with AMPK activation; a mechanism by PPAR-γ agonists (thiazolidinediones), which enhances insulin sensitivity.

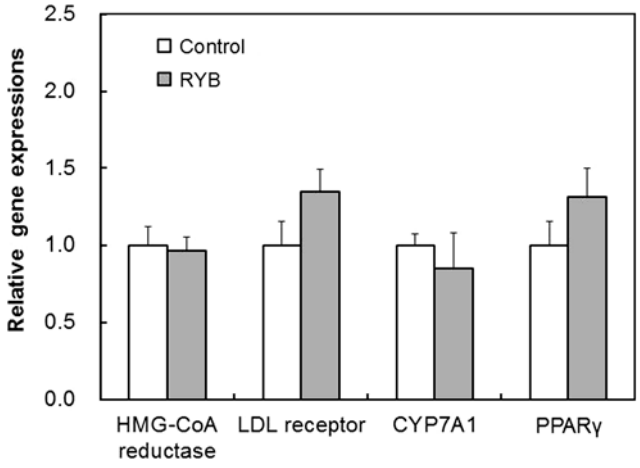

Fig. 3. Relative mRNA expression levels in the livers of *db/db* mice fed the control diet (AIN-76) and experimental diet containing 15% RYB. Each value represents mean±SE (n= 3). CYP7A1, HMG-CoA reductase, LDL receptor, and PPAR-γ mRNA levels in each sample were normalized using β-actin as a reference.

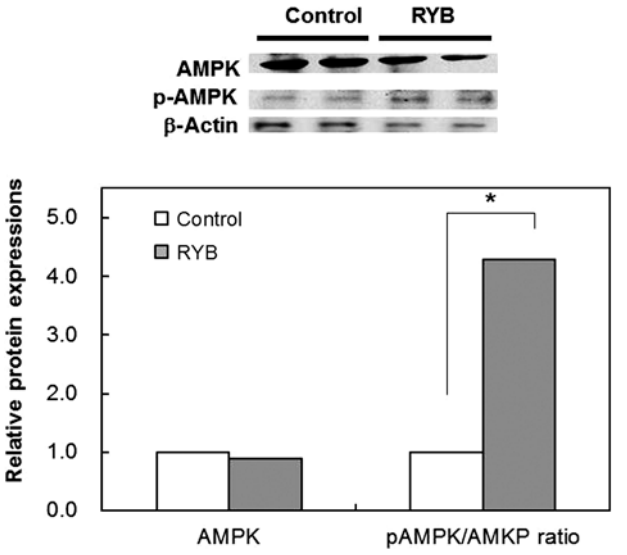

Fig. 4. Effect of RYB on the phosphorylation activation of hepatic AMPK in the livers of *db/db* mice. Expression of AMPK protein in each sample was normalized using α-tubulin as a reference. Relative band densities were determined using Gel-Pro analyzer.

First, no significant changes in the plasma insulin levels in the 2 groups were observed during the experimental period (Table 2, 5.9±1.4 ng/dL for controls vs. 5.7±1.6 ng/dL for RYB). This suggests that the RYB did not affect insulin secretion from pancreatic β-cells. Secondly, the expression of the PPAR-γ gene in adipose tissue was increased only marginally (Fig. 3). The data show that the improved glucose tolerance by RYB may not be due to increased insulin sensitivity with PPAR-γ activation in peripheral tissues, such as adipose tissue. Third, we also examined the effect of the RYB-containing diet on the phosphorylation activation of AMPK in the mouse liver with immunoblot analysis. In the liver, the ratio of p-AMPK to AMPK expression was increased significantly in the RYB group by up to 4.3-fold (Fig. 4). These suggest that the hypoglycemic effects and improved glucose tolerance of RYB are due to phosphorylation activation of hepatic AMPK. The results showed that the RYB lowered plasma glucose levels and improved glucose tolerance. Primary hypoglycemic mechanism of RYB was derived from the activation of AMPK that could result decreased hepatic glucose production. These results suggest that the RYB may be useful as a hypoglycemic functional food.

The enzyme AMPK is a major cellular sensor and a master regulator of metabolic homeostasis that induces multiple cellular effects. AMPK consists of a catalytic α-subunit and 2 regulatory (β and γ) subunits (26). In response to cellular signaling, AMPK is activated by the phosphorylation of Thr172 in the α-subunit, which facilitates the binding of AMP to the γ-subunit leading to the allosteric activation of AMPK. Active form of AMPK also protects Thr172 from dephosphorylation, thereby maintaining the enzyme in the activated state. Especially in liver, AMPK controls glucose homeostasis mainly through the inhibition of gluconeogenic gene expression thus reduting hepatic glucose production. AMPK activation is mediated by both 5-aminoimidazol-4carboxamide-1-γ-D-ribofuranoside (AICAR) and metformin, which downregulates phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6 phosphatase (G6Pase), 2 key genes in hepatic gluconeogenesis, thereby ameliorating hyperglycemia (27,28). In addition, AMPK enhances glucose tolerance by reducing hepatic lipogenesis. AMPK phosphorylates and inactivates acetyl-CoA carboxylase 1 (29) and malonyl-CoA decarboxylase (30), leading to the inhibition of *de novo* fatty acid synthesis by reducing the level of malonyl-CoA, which is both a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation. These cause improved glucose tolerance in both liver and muscle by reducing the ectopic accumulation of lipids in liver and muscle, contributing to the reduction of fasting triglyceride levels.

Concluding remark Our data showed that RYB reduced plasma glucose levels in *db/db* mice. The hypoglycemic effects of RYB may be due, at least in part, to phosphorylation activation of hepatic AMPK in RYB and delayed intestinal glucose uptake caused by the dietary fiber in waxy barley. The effects on humans and the molecular target of AMPK activation with RYB should be confirmed in the future.

Acknowledgments This work was supported by a grant from the Korean Health 21 R&D Project, Ministry of

Health and Welfare, Republic of Korea (A050376) and the support of 'Forest Science & Technology Projects (Project No. S120909L130110)' provided by Korea Forest Service.

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