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RESEARCH ARTICLE

Resveratrol ameliorates diabetes-related metabolic changes via activation of AMP-activated protein kinase and its downstream targets in *db/db* mice

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Scope: This study investigated the effects of resveratrol (RV) on diabetes-related metabolic changes in a spontaneous model of type 2 diabetes, as well as activation of AMP-activated protein kinase (AMPK) and downstream targets.

Methods and results: C57BL/KsJ-*db*/*db* mice were fed a normal diet with RV (0.005% and 0.02%, w/w) or rosiglitazone (RG, 0.001%, w/w) for 6 weeks. Both doses of RV significantly decreased blood glucose, plasma free fatty acid, triglyceride, apo B/apo AI levels and increased plasma adiponectin levels. RV activated AMPK and downstream targets leading to decreased blood HbA1c levels, hepatic gluconeogenic enzyme activity, and hepatic glycogen, while plasma insulin levels, pancreatic insulin protein, and skeletal muscle GLUT4 protein were higher after RV supplementation. The high RV dose also significantly increased hepatic glycolytic gene expression and enzyme activity, along with skeletal muscle glycogen synthase protein expression, similar to RG. Furthermore, RV dose dependently decreased hepatic triglyceride content and phosphorylated I kappa B kinase (p-IKK) protein expression, while hepatic uncoupling protein (UCP) and skeletal muscle UCP expression were increased.

Conclusion: RV potentiates improving glycemic control, glucose uptake, and dyslipidemia, as well as protecting against pancreatic β-cell failure in a spontaneous type 2 diabetes model. Dietary RV has potential as an antidiabetic agent via activation of AMPK and its downstream targets.

Keywords:
AMPK / Glucose and lipid metabolism / PPARα / Resveratrol / Type 2 diabetes

1 Introduction

Type 2 diabetes is closely associated with abdominal obesity, dyslipidemia, and other chronic diseases, all of which characterize the metabolic syndrome [1]. Insulin resistance is central to the pathophysiology of the metabolic syndrome. Insulin resistance together with impaired insulin secretion from pancreatic β-cells results in inadequate control of hyperglycemia in type 2 diabetes [2]. Liver-specific inhibition of insulin signaling in mice leads to postprandial hyperglycemia and dyslipidemia [3]. Skeletal muscle is also a major contributor to the dysregulation of glucose homeostasis in type 2 diabetes, since skeletal muscle accounts for ~75% of whole body insulin-stimulated glucose uptake [4]. Therefore, enhancing glucose uptake in liver and skeletal muscle is a primary therapeutic strategy for treatment of metabolic syndrome and type 2 diabetes. And there is now rapidly growing interest in natural compounds, which can increase glucose uptake providing nonpharmacological ways to treat type 2 diabetes.

Resveratrol (RV, 3,4′,5-trihydroxystilbene) is a naturally occurring phenolic compound found in grapes, berries, and various other plants. RV is reported to confer a number of health benefits protecting against insulin resistance, obesity, metabolic syndrome, and type 2 diabetes [5]. RV can prevent

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Abbreviations: **GLUT**, glucose transporter; **G6Pase**, glucose-6-phosphatase; **IPGTT**, intraperitoneal glucose tolerance test; **p-ACC**, phosphorylated acetyl CoA carboxylase; **p-AMPK**, phosphorylated AMP-activated protein kinase; **PEPCK**, phosphoenolpyruvate carboxykinase; **PPARα**, peroxisome proliferator activated receptorα; **RV**, resveratrol; **UCP**, uncoupling protein

lipid accumulation in vitro by stimulating AMP-activated protein kinase (AMPK) phosphorylation in human HepG2 cells exposed to high glucose [6]. RV can also prevent hepatic lipid accumulation in vivo, in obese Zucker rats [7], and high-fat fed rodents [8, 9]. Furthermore, RV can enhance glucose uptake in skeletal muscle cells [10, 11], as well as liver, heart and fat [12, 13]. Evidence of the metabolic effects of RV on diabetes have predominately been established in animals with experimentally induced diabetes, using streptozotocin (STZ), nicotinamide/STZ, or long-term high-fat diet [14]. Recent studies in humans indicate RV or grapes extract can improve glycaemic control, insulin sensitivity, and reduced oxidative stress in type 2 diabetes patients [15, 16]. However, despite evidence of the health benefits of RV, the direct cellular targets remain a mystery.

RV appears to modulate multiple targets, including transcription factors, signaling proteins, and enzymes [17]. AMPK is an important cellular energy sensor and activation of the AMPK pathway, along with its downstream effectors modulate glucose and lipid metabolism [18]. RV can activate AMPK in hepatocytes, myocytes, and adipocytes. Furthermore, AMPK-deficient mice fed a high-fat diet are resistant to the metabolic effects of RV [19]. Studies also indicate RV is a selective activator of peroxisome proliferator activated receptorα (PPARα) [20]. RV appears to prevent metabolic dysregulation and inflammation in wild-type mice, but not in PPARα knockout mice [21]. RV also acts on pancreatic cells, and has been shown to protect against oxidative damage in the pancreatic β-cell line RIN-5F [22], as well as type 1 diabetic animals [23].

Few studies have assessed RV effects on *db*/*db* mice, which naturally develop diabetes [22, 24]. In *db*/*db* mice despite peripheral insulin resistance, initially glucose homeostasis can be maintained by increased insulin secretion, however, hyperglycemia develops when insulin secretion fails to compensate for insulin resistance, leading to β-cell dysfunction [25]. Hence, *db*/*db* mice provide a useful model to determine the effect RV on molecular targets and metabolic changes in type 2 diabetes.

The aim of this study was to first establish the effects of dietary RV on diabetes-related metabolic changes, due to spontaneous type 2 diabetes in *db*/*db* mice, compared to the antidiabetic drug RG. Second, we investigated whether activation of AMPK and its downstream effectors are related to the effect of dietary RV in liver and skeletal muscle of *db*/*db* mice.

2 Materials and methods

2.1 Animals

Four-week-old male C57BL/KsJ-*db*/*db* mice (Jackson Laboratories, Bar Harbor, ME) were randomly divided into four groups of ten mice each. The mice were fed an AIN-76 semisynthetic diet with or without 0.001% (w/w) RG (rosiglitazone, Avandia, GlaxoSmithKlein, UK), 0.005% (w/w) RV (Sigma Chemical, MO), or 0.02% (w/w) RV for 6 weeks. All experimental diets were prepared every week and stored in the dark at −4°C. Body weight and blood glucose level were measured every 1–2 weeks. At the end of the experimental period, all mice were anesthetized with ether after a 12-h fast. Blood was taken from the inferior vena cava for the determination of glucose, plasma lipid, and hormone concentrations. The liver, skeletal muscle, and pancreas were removed, rinsed with physiological saline, weighed, immediately frozen in liquid nitrogen, and stored at −70°C until analysis. Studies were performed under protocols for animal studies approved by the Ethics Committee at Kyungpook National University.

2.2 Blood glucose, glycosylated hemoglobin, and intraperitoneal glucose tolerance test (IPGTT)

The blood glucose concentration was measured every 2 weeks with whole blood obtained from the tail veins after withholding food for 12 h using a glucose analyzer, GlucDr supersensor (Allmedicus, Korea). The blood glycosylated hemoglobin (HbA1c) concentration was measured with an analyzer (MicormatTM I Hemoglobin A1c Test, Bio-Rad, CA). The IPGTT was performed at the fifth week. After a 12-h fast, mice were injected intraperitoneally with glucose (0.5 g/kg body weight). Blood glucose levels were determined from the tail vein at 0, 30, 60, and 120 min after the glucose injection. The cumulative changes in blood glucose responses were quantified based on the incremental area under the curve (AUC).

2.3 Plasma insulin, and glucagon, adiponectin, leptin, and resistin levels

Plasma insulin (insulin ELISA kit; Crystal Chem, Grove, IL), glucagon (glucagon ELISA kit; Wako, Japan), adiponectin (adiponectin ELISA kit; R&D systems, Minneapolis, MN), leptin (leptin RIA kit; Linco Research, St. Charles, IL), and resistin (resistin RIA kit; Diagnostic Systems Laboratories, Webster, TX) levels were measured using radioimmunometric assays.

2.4 Analyses of plasma and hepatic lipids

Enzymatic assays for plasma total-cholesterol, HDL-cholesterol, and triglycerides were performed using enzymatic kits (Asan Pharm, Korea). Apolipoprotein AI (apo AI) and apolipoprotein B (apo B) levels were also measured using enzymatic kits (Eiken, Japan). Plasma free fatty acid (FFA) was also measured using an enzymatic kit (Wako, Japan). Non-HDL-cholesterol was calculated as (total cholesterol) − (HDL-cholesterol) − (triglyceride/5). The hepatic lipids were extracted as described previously [26], hepatic cholesterol and triglyceride concentrations were conducted using the same enzymatic kit used for plasma analyses.

2.5 Immunohisochemistry analysis of pancreas

For immunohistochemistry, the islet was sectioned, fixed in 1% hydrogen peroxide, and washed in 0.01 M citrate buffer (pH 6.0). These sections were treated with blocking reagent (Ultra Tech HRP) to prevent nonspecific binding, and incubated with monoclonal antibodies against insulin (Santa Cruz Biotech, Inc., Santa Cruz, CA). Antibody reactivity was detected using HRP-conjugated biotin–streptavidin complexes and developed with diaminobenzidine tetrahydrochloride (DAB) as the substrate.

2.6 Glucose regulating enzyme activities and glycogen content in liver

Liver was separated to cytosolic and microsomal fractions as previously described [27]. The cytosolic fraction was used for the measurement of glucokinase (GK) and phosphoenolpyruvate carboxykinase (PEPCK) activity. The resulting microsomal fraction was used for the measurement of glucose-6-phosphatase (G6Pase). And the hepatic glycogen concentration was determined as described previously [26].

2.7 RNA isolation and mRNA expression analysis

The liver was homogenized in Trizol reagent (Invitrogen Life Technologies, Grand Island, NY), and total RNA was isolated according to the manufacturer's specifications. The total RNA was reverse-transcribed into cDNA using the RevertAid^{TM} First strand cDNA kit (Fermentas, Burlington, CA). The RNA expression was quantified by real-time quantitative PCR using ABsolute^{TM} QPCR SYBR Green Mixes (ABgene, Surrey, UK) and the SDS7000 sequence-detection system (Applied Biosystems, Foster City, CA). Primers were designed to detect PPARα, PPARγ, PK, and SREBP1c. The amplification was performed as follows: 10 min at 90°C, 15 s at 95°C, and 60 s at 60°C for a total of 40 cycles. The cycle threshold (Ct) values were normalized using GAPDH. Relative gene expression was calculated with the 2^{-Ct} method.

2.8 Western blot analysis

Liver and skeletal muscle protein was extracted with lysis buffer and quantified using the Bradford method. Total protein (80∼100 μg) was electrophoresed on to 10% SDS polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Milipore), blocked, and probed with rabbit anti-p-AMPK (phosphorylated AMP-activated protein kinase, 1:2000; Cell Signaling Technology), rabbit anti-PPARα (1:1000; Santa Cruz Biotech), rabbit anti-p-ACC (phosphorylated acetyl CoA carboxylase, 1:2000; Cell Signaling Technology), mouse anti-p-IKK (phosphorylated I kappa B kinase, 1:1000; Santa Cruz Biotech), rabbit anti-GLUT4 (glucose transporter 4, 1:1000; Santa Cruz Biotech), mouse antiglycogen synthase (1:1000; Santa Cruz Biotech), goat anti-UCP2 (uncoupling protein 2, 1:1000; Santa Cruz Biotech), and goat anti-UCP3 (1:1000; Santa Cruz Biotech), respectively. The immunoreactive antigen was then recognized by using a horseradish peroxidase-labeled anti-rabbit or anti-goat IgG (1:2000; Amersham Biosciences) and an enhanced ECL kit from Pierce Biotechnology (Rockford, IL). The immunoreactive bands were quantified using a Bio Image Whole Band Analyzer (50S; BI System Co.).

2.9 Statistical analysis

The parameter values were all expressed as the mean ± standard error of the mean (SEM). Significant differences among the groups were determined using one-way analysis of variance (ANOVA) in SPSS (SPSS Inc.). Any significant between group differences identified at each time-point were analyzed further using Duncan's multiple-range post-hoc test. Results were considered statistically significant at $p < 0.05$.

3 Results

3.1 RV lowered body weight gain and increased plasma adiponectin level in *db*/*db* mice

Initial body weight of *db*/*db* mice exhibited approximately the same values in all four groups, and the body weight in control mice was gradually increased until the end of experimental period (Table 1). RG-0.001 feeding induced a significant increase of body weight gain from the second week in *db*/*db* mice, whereas RV-0.005 significantly lowered body weight compared to the control group at 6 weeks on the experimental diet. RV-0.02 also tended to lower body weight compared to the control group.

Similar to body weight gain, RV-0.005 significantly decreased plasma leptin level compared to the RG group (Table 1). Plasma adiponectin level was significantly higher in the two RV groups compared to the control group and there were no significant differences in plasma resistin level between the groups (Table 1).

3.2 RV lowered fasting blood glucose, HbA1c levels and increased insulin secretion

Initial fasting blood glucose level was not different among the groups (Fig. 1A). RG significantly reduced hyperglycemia in the *db*/*db* mice from the second week until the sixth week. Supplementation of RV-0.005 or RV-0.02 also tended to suppress increases in fasting blood glucose levels from the second week on these diets. At the end of the experimental period, after 6 weeks, RV-0.005 and RV-0.02 both significantly lowered fasting blood glucose levels compared to the control

Table 1. Effects of RV supplementation on weekly body weight changes and plasma adipokine levels in C57BL/KsJ-*db*/*db* mice fed a normal diet for 6 weeks

	C	RG-0.001	RV-0.005	RV-0.02
Body weight (g)				
0 week	27.72 ± 0.57	26.94 ± 0.58	26.45 ± 0.74	27.34 ± 0.62
1 week	30.67 ± 0.81[a)]	31.43 ± 0.80[b)]	29.09 ± 1.00[a)]	29.96 ± 0.79[a)]
2 week	33.10 ± 1.03[a)]	37.23 ± 0.55[b)]	34.94 ± 0.98[a)]	33.70 ± 0.57[a)]
3 week	33.17 ± 1.70[a)]	40.76 ± 0.67[b)]	34.93 ± 1.01[a)]	35.06 ± 0.55[a)]
4 week	32.47 ± 1.77[a)]	42.85 ± 0.84[b)]	34.14 ± 1.34[a)]	35.53 ± 0.53[a)]
5 week	36.18 ± 1.52[a)]	44.66 ± 1.08[b)]	34.62 ± 1.90[a)]	36.24 ± 0.79[a)]
6 week	38.54 ± 1.32[a)]	43.76 ± 1.23[c)]	32.72 ± 2.46[b)]	34.58 ± 1.61[a)b)]
Glucagon (ng/mL)	0.71 ± 0.02	0.70 ± 0.04	0.70 ± 0.05	0.69 ± 0.02
Adiponectin (ng/mL)	43.18 ± 0.75[a)]	46.45 ± 2.58[a)b)]	55.12 ± 1.87[b)]	53.72 ± 1.54[b)]
Leptin (ng/mL)	9.94 ± 1.42[a)b)]	13.37 ± 1.00[a)]	7.46 ± 1.36[b)]	9.72 ± 1.55[a)b)]
Resistin (ng/mL)	11.23 ± 2.29	9.23 ± 0.98	8.27 ± 0.52	10.24 ± 0.90

Mean ± SE values of ten mice per group.
[a, b, c)]Means in the same row not sharing a common superscript are significantly different among the groups at $p < 0.05$.
C, control diet; RG-0.001, 0.001% RG-supplemented diet; RV-0.005, 0.005% RV-supplemented diet; RV-0.02, 0.02% RV-supplemented diet.

db/*db* mice. Moreover, blood HbA1c levels were significantly lower in the RG-0.001 and RV-0.02 groups. However, RV-0.005 only tended to lower HbA1c levels compared to the control group (Fig. 1B). The IPGTT revealed blood glucose levels were significantly lower in the RG-0.001 and RV-0.02 groups than in the control group at 30 and 120 min after glucose load (Fig. 1C). Moreover, RG-supplemented *db*/*db* mice exhibited a significant decrease in AUC compared to the control group, also RV-0.02 tended to lower AUC (Fig. 1D). In contrast, the blood glucose level during the IPGTT did not differ between the RV-0.005 and control groups.

Plasma insulin levels were increased by 81%, 36%, and 64% in the RG-0.001, RV-0.005, and RV-0.02 groups compared to the control group, respectively, but the effect of RV-0.005 was less than that of RG-0.001 or RV-0.02 (Fig. 1E). To investigate pancreatic insulin expression, we detected insulin by immunohistochemistry. The pancreas of control *db*/*db* mice showed loss of islet boundary definition and degeneration (Fig. 1F). In addition, there were less insulin-positive β-cells in control *db*/*db* mice compared to the RG-0.001 and RV groups. Mice supplemented with RV-0.02 showed an increased insulin-stained area with preservation of pancreatic β-cells compared to the RV-0.005 supplemented mice, which was consistent with blood glucose and plasma insulin levels. There were no significant differences in plasma glucagon levels among the groups (Table 1).

3.3 RV improved glucose homeostasis in liver and skeletal muscle

Glycolysis and gluconeogenesis enzyme gene expression and activity were measured in liver (Fig. 2A to E). The supplementation of RV-0.02 as well as RG-0.001 significantly increased hepatic GK activity and PK mRNA expression in *db*/*db* mice. Furthermore, *db*/*db* mice supplemented with RV-0.02 showed a significant decrease in G6Pase and PEPCK enzyme activity, as well as glycogen content compared to the control *db*/*db* mice. RV-0.005 tended to lower only hepatic PEPCK enzyme activity and glycogen content. However the hepatic GK enzyme activity, G6Pase enzyme activity, and PK mRNA expression of the RV-0.005 group were similar to the control group.

Skeletal muscle GLUT4 protein expression was higher in the *db*/*db* mice supplemented with RV-0.005, RV-0.02, or RG-0.001 compared to the control mice. In particular, RG-0.001 and RV-0.02 markedly increased glycogen synthase protein expression in skeletal muscle (Fig. 2F).

3.4 RV lowered plasma and hepatic lipid levels

The *db*/*db* mice supplemented with RV-0.005, RV-0.02, or RG-0.001 exhibited significantly lowered plasma FFA and triglyceride concentrations compared to the control group (Table 2). Plasma total-cholesterol and non-HDL-cholesterol levels were also lowered by RV-0.005, RV-0.02, and RG-0.001 supplementation, although these values were only statistically significant between the RV-0.005 and control groups (Table 2). No significant differences in plasma HDL-cholesterol or apo AI levels were observed between groups, while plasma apo B levels and the apo B/apo AI ratio were dose-dependently lowered in the RV-0.005 and RV-0.02 supplemented *db*/*db* mice (Table 2).

RV also dose-dependently lowered hepatic triglyceride levels compared to the control group (Fig. 3A). RG-0.001 significantly increased hepatic cholesterol content, whereas RV-0.005 and RV-0.02 had no significant effect on hepatic cholesterol levels compared to the control group (Fig. 3A).

3.5 RV activated p-AMPK and modulated downstream targets in liver and skeletal muscle

Hepatic p-AMPK protein expression was increased in the RV groups compared to the control group (Fig. 3B). In contrast

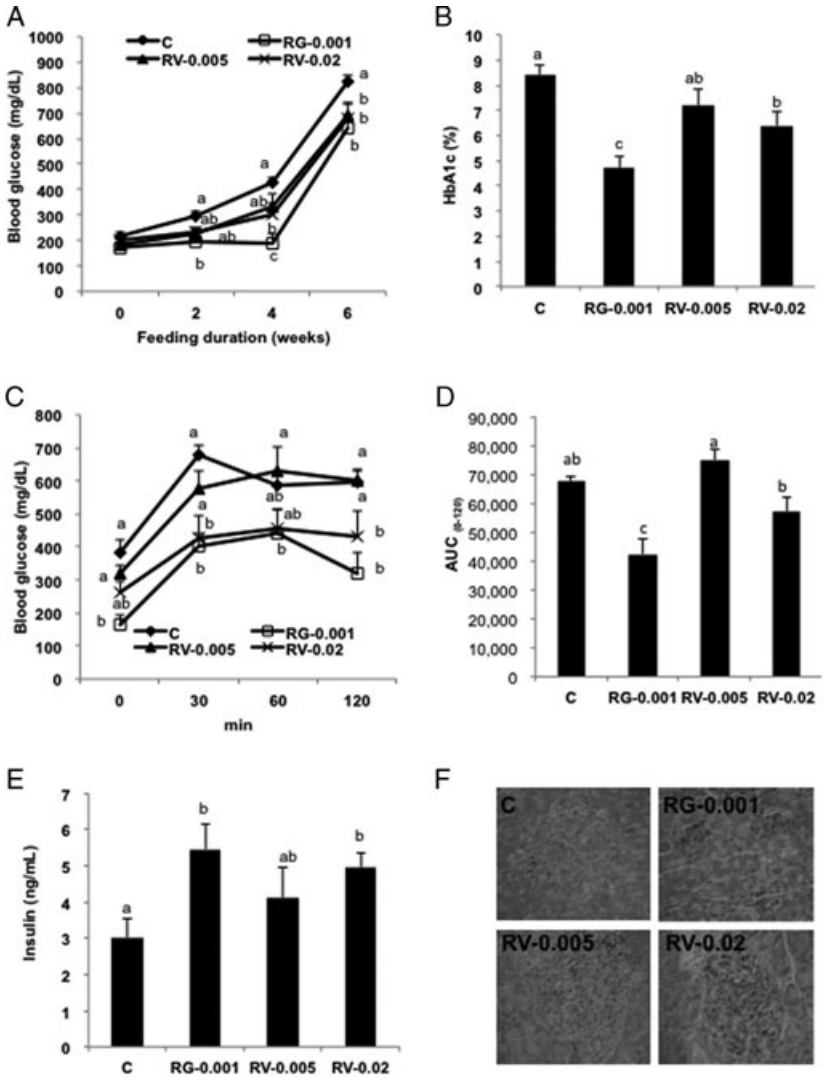

Figure 1. Effects of RV supplementation on blood glucose (A), blood HbA1c (B), IPGTT (C), AUC (D), plasma insulin (E), and immunohistochemistry staining for pancreatic insulin (F) in C57BL/KsJ-*db*/*db* mice fed a normal diet for 6 weeks. Data shown as mean ± SE. ^{abc}Means not sharing common superscript letters are significantly different between groups at $p < 0.05$. Original magnification 200 ×. C, control diet; RG-0.001, 0.001% RG-supplemented diet; RV-0.005, 0.005% RV-supplemented diet; RV-0.02, 0.02% RV-supplemented diet; IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve.

to hepatic p-AMPK expression, RV-0.02 resulted in much higher skeletal muscle p-AMPK protein expression compared to the rest of the groups. However, skeletal muscle and hepatic p-ACC protein expression was lower in the RV-0.02 group compared to the RV-0.005 group (Fig. 3D).

The *db*/*db* mice supplemented with RV-0.02 or RG-0.001 showed a significant increase in mRNA expression of hepatic SREBP1c (Fig. 2D). However, RV-0.005 and RV-0.02 resulted in an increase in hepatic UCP2 protein expression (Fig. 3B). Furthermore, hepatic PPARα protein expression of the RV-0.005 group was higher than that of the control group (Fig. 3B). Hepatic PPARα mRNA expression was also increased by 49% in both RV-0.005 and RV-0.02 supplemented *db*/*db* mice compared to the control group, although the difference was not statistically significant (Fig. 3C). Hepatic PPARγ mRNA expression was not changed after supplementation with RV-0.005 or RV-0.02, but was significantly increased by RG-0.001 (Fig. 3C).

Consistent with changes in liver, RV increased protein expression of UCP2 and UCP3 in skeletal muscle compared to the control group (Fig. 3D). In particular, skeletal muscle PPARα protein expression was higher in the RV-0.02 group compared to the control group (Fig. 3D). In addition, p-IKK protein expression was lower in both the liver and skeletal muscle of both RV groups (Fig. 3B and D).

4 Discussion

Evidence is accumulating that RV exerts multiple beneficial effects on the metabolic syndrome and type 2 diabetes [5, 14]. In the present study, we show RV at two dietary doses (0.005%, 0.02%) lowered fasting blood glucose levels with similar effectiveness as the antidiabetic drug RG, without increasing body weight gain. But 0.02% RV was necessary to significantly improve glucose tolerance and HbA1c levels,

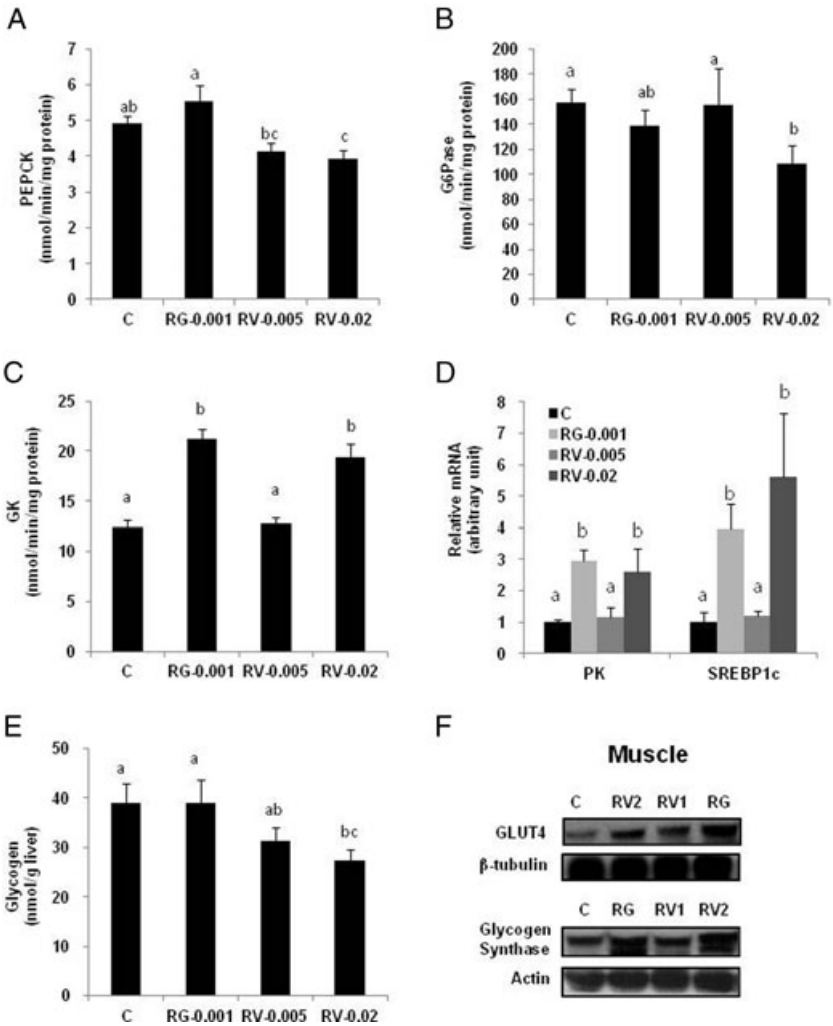

Figure 2. Effects of RV supplementation on the hepatic glucose regulating enzymes activities (A–C) and mRNA (D), hepatic glycogen content (E), skeletal muscle GLUT4 and glycogen synthase protein expression (F) in C57BL/KsJ-*db*/*db* mice fed a normal diet for 6 weeks. Data shown as mean ± SE. [abc]Means not sharing common superscript letters are significantly different between groups at $p < 0.05$. Note RV2, RV1, and RG was inadvertently loaded in reverse order detection of glycogen synthase. C, control diet; RG-0.001, RG, 0.001% RG-supplemented diet; RV-0.005, RV1, 0.005% RV-supplemented diet; RV-0.02, RV2, 0.02% RV-supplemented diet; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; GK, glucokinase; PK, pyruvate kinase; SREBP1c; sterol regulatory element binding protein 1c; GLUT4, glucose transporter 4.

similar to RG. Importantly, RV also prevented the deterioration of pancreatic β-cell function and loss of insulin secretion characteristic of natural type 2 diabetes progression in *db*/*db* mice. RV also ameriolated the diabetes-related metabolic changes via action on AMPK and downstream effectors in liver and skeletal muscle. Action of RV on multiple molecular targets resulted in increases in glycolytic activity, fatty acid oxidation, as well as decreases in gluconeogenesis and glycogen storage in liver. RV induced activation of AMPK in skeletal muscle resulted in similar modulation of downstream targets, as well as upregulation of GLUT4 protein responsible for glucose uptake.

4.1 Dose-dependent effects of RV on hyperglycemia and glucose tolerance

Evidence of the health benefits of RV is growing, however, whether the health benefits of RV on type 2 diabetes are dose dependent is still equivocal [14]. In the present study, we administrated RV doses equivalent to 7.08 mg/kg or 30 mg/kg body weight to *db*/*db* mice, which translates into 0.53 g/day or 2.25 g/day for a 75 kg male. Small milligram quantities of RV are typically consumed in the human diet, but animals studies suggest large doses of RV are safe and well tolerated [28]. The evidence from this study suggests higher doses are required for universal improvements in fasting glucose, HbA1c, and glucose tolerance in *db*/*db* mice. However, the lower dose of RV also modulates some cellular targets such as AMPK and metabolic enzyme activity. Furthermore, recent human trials indicate a dose of 30 mg/kg was sufficient to improve glucose tolerance [15], but it remains to be seen whether higher RV doses are more effective in type 2 diabetes patients [29].

4.2 RV induced activation of AMPK and downstream targets regulates glucose metabolism in *db*/*db* mice

AMPK is a central regulator of glucose homeostasis, therefore, is an important therapeutic target for the treatment of

Table 2. Effects of RV supplementation on the plasma lipids profile in C57BL/KsJ-*db*/*db* mice fed a normal diet for 6 weeks

	C	RG-0.001	RV-0.005	RV-0.02
Free fatty acid (mmol/L)	$1.22 \pm 0.11^{a)}$	$0.59 \pm 0.05^{b)}$	$0.81 \pm 0.05^{b)}$	$0.77 \pm 0.06^{b)}$
Triglycerides (mmol/L)	$1.80 \pm 0.28^{a)}$	$1.11 \pm 0.13^{b)}$	$0.95 \pm 0.08^{b)}$	$1.07 \pm 0.15^{b)}$
Total-cholesterol (mmol/L)	$4.6 \pm 0.3^{a)}$	$3.7 \pm 0.2^{a)b)}$	$3.0 \pm 0.3^{b)}$	$3.7 \pm 0.3^{a)b)}$
Non-HDL-cholesterol (mmol/L)	$3.75 \pm 0.31^{a)}$	$2.99 \pm 0.21^{a)b)}$	$2.24 \pm 0.22^{b)}$	$2.79 \pm 0.27^{a)b)}$
HDL-cholesterol (mmol/L)	0.85 ± 0.04	0.71 ± 0.08	0.76 ± 0.08	0.91 ± 0.09
Apo B (mmol/L)	$4.24 \pm 0.65^{a)}$	$4.77 \pm 1.07^{a)}$	$2.90 \pm 1.41^{a)b)}$	$1.47 \pm 0.25^{b)}$
Apo AI (mmol/L)	9.92 ± 0.36	9.70 ± 0.12	9.63 ± 0.27	9.73 ± 0.29
Apo B/apo AI	$0.43 \pm 0.06^{a)}$	$0.49 \pm 0.14^{a)}$	$0.30 \pm 0.05^{a)}$	$0.15 \pm 0.03^{b)}$

Mean ± SE values of 10 mice per group.
[a, b)]Means in the same row not sharing a common superscript letter are significantly different among groups at $p < 0.05$.
C, control diet; RG-0.001, 0.001% RG-supplemented diet; RV-0.005, 0.005% RV-supplemented diet; RV-0.02, 0.02% RV-supplemented diet.

type 2 diabetes [18]. The AMPK signaling pathway coordinates glucose metabolism via multiple downstream effectors involved in glucose uptake, glycolysis, gluconeogenesis, and glycogen synthesis. In the present study, hepatic p-AMPK activation was lower after RV-0.02 compared to RV-0.005 supplementation, while skeletal muscle p-AMPK activation was higher after RV-0.02 supplementation, consistent with previous reports of RV induced AMPK activation in streptomycin-induced diabetic mice and diet-induced obese mice [14]. Furthermore, we show here that several AMPK downstream effectors are modulated by RV supplementation in *db*/*db* mice.

In skeletal muscle, insulin and AMPK activation stimulate glucose uptake by increasing translocation of GLUT4 to the plasma membrane. Skeletal muscle insulin resistance is associated with reduced insulin stimulated GLUT4 translocation and insulin stimulated glycogen synthesis in type 2 diabetes [30]. In skeletal muscle of *db*/*db* mice, RV-0.005, RV-0.02, and RG-0.001 all increased GLUT4 protein expression,

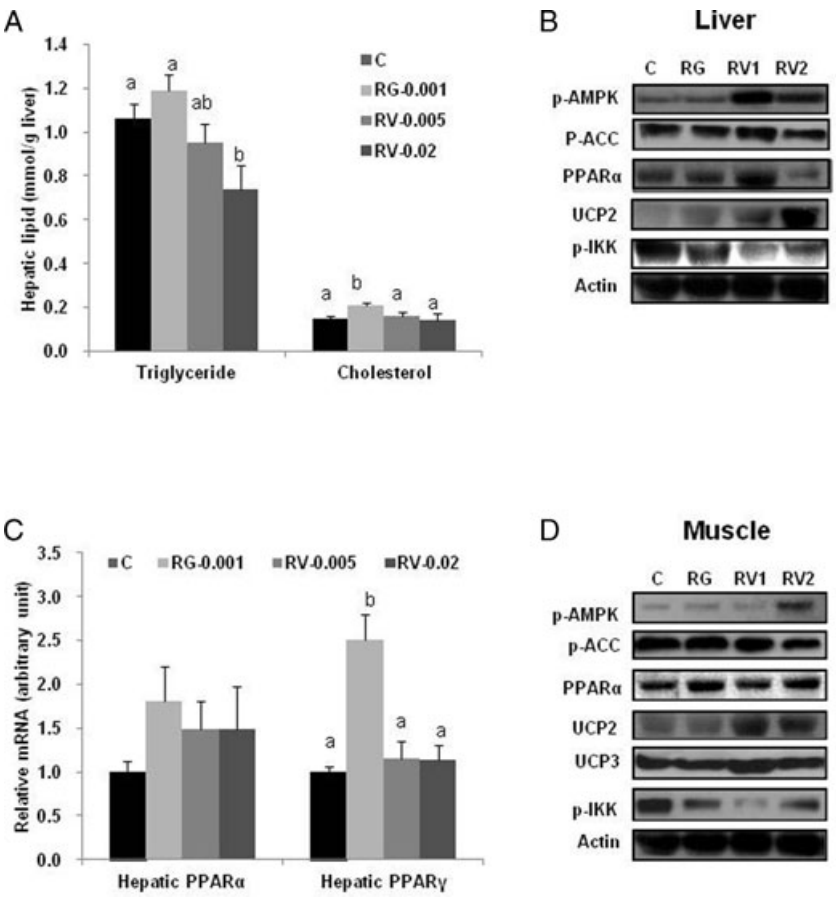

Figure 3. Effects of RV supplementation on hepatic lipid (A), p-AMPK, p-ACC, PPARα, UCP2, and p-IKK protein expression (B), PPARα and PPARγ mRNA expression (C) and skeletal muscle p-AMPK, p-ACC, PPARα, UCP2, UCP3, and p-IKK protein expression in C57BL/KsJ-*db*/*db* mice fed a normal diet for 6 weeks. Data shown as mean ± SE. [ab]Means not sharing a common superscript letters are significantly different between groups at $p < 0.05$. C, control diet; RG-0.001, RG, 0.001% RG-supplemented diet; RV-0.005, RV1, 0.005% RV-supplemented diet; RV-0.02, RV2, 0.02% RV-supplemented diet; p-AMPK, phosphorylated AMP-activated protein kinase; p-ACC, phosphorylated acetyl CoA carboxylase; PPARα, peroxisome proliferator activated receptorα; PPARγ, peroxisome proliferator activated receptorγ, UCP, uncoulpling protein; p-IKK, phosphorylated I kappa B kinase.

suggesting that RV-induced AMPK activation stimulates increased glucose uptake via GLUT4. The action of RV on GLUT4 is consistent with the decrease in blood glucose, HbA1c, and improvement in glucose tolerance in RV supplemented *db*/*db* mice. Glycogen synthase (GS) is also a target of AMPK, GS is a rate-limiting enzyme in glycogen synthesis. GS protein was increased by RV-0.02 or RG-0.001 supplementation compared to RV-0.005 supplementation [31]. However, insulin is a potent stimulator of glycogen synthesis in skeletal muscle, therefore, the progressive insulin secretion to cope with inherent peripheral insulin resistance in *db*/*db* mice may also stimulate skeletal muscle GS.

In liver, AMPK activation inhibits hepatic glucose production by downregulating expression of gluconeogenic enzymes such as PEPCK and G6Pase, and stimulates glycolysis. Elevated hepatic glucose production is a major contributor to hyperglycemia in type 2 diabetes [32]. In liver, the hypoglycemic action of RV in *db*/*db* mice was related to a significant decrease in hepatic PEPCK and G6Pase activity. In parallel with the enhanced gluconeogenesis, hepatic glycogen content is also increased in *db*/*db* mice [33] and hepatic glycogen content is reported to be decreased in liver-specific PEPCK null mice [34]. In the current study, the supplementation of RV-0.02 also significantly lowered the hepatic glycogen content in *db*/*db* mice, indicating that these changes seemed to be related to the decreased hepatic gluconeogenic enzyme activity.

A similar effect on hepatic glycolytic enzyme activity and mRNA expression was observed in RV-0.02 or RG-supplemented *db*/*db* mice, where hepatic GK activity and PK mRNA expression were significantly increased in these groups. Taken together, these results indicate that the supplementation of RV in *db*/*db* mice protects against pancreatic β-cell failure and preserves insulin-stimulated glycolytic enzyme gene expression and activity, hence may help explain the RV induced improvements in glucose homeostasis.

4.3 RV-induced activation of AMPK and downstream targets regulates lipid metabolism in *db*/*db* mice

AMPK also plays a central role in the regulation of lipid metabolism via action on downstream targets involved in fatty acid synthesis and fatty acid oxidation [18]. Phosphorylation of ACC was reduced by RV supplementation in liver and skeletal muscle. AMPK induced inhibition of ACC, leads to increased transport of fatty acids into mitochondria and increased FA oxidation [18]. AMPK is also reported to target PPARα, a nuclear receptor protein, which functions as transcription factor regulating lipid metabolism genes [35]. PPARα mRNA levels were elevated by RV supplementation in liver alongside UCP2, which is transcriptionally regulated by PPARα. Activation of UCP2 in liver is reported to increase hepatic β-oxidation, leading to increases in energy expenditure, as well as decreases in lipid levels in blood and liver [36]. PPARα activators such as thiazolidinediones can increase hepatic UCP2 mRNA expression and hence can be used for the treatment of dyslipidemia [37]. AMPK was also recently reported to inhibit SREBP1 leading to decreased FA synthesis, but we found SREBP1 expression was increased by RV. However, insulin is also a powerful regulator of SREBP1 [38], and the increased insulin secretion in *db*/*db* mice, prior to β-cell deterioration may counteract any regulation by RV induced activation of AMPK.

Body weight gain and hepatic steatosis are reported to be unfavorable complications of RG [39]. Consistent with the RV induced activation of AMP, supplementation of two doses of RV, but not RG, led to a dose-dependent decrease in hepatic triglyceride content in *db*/*db* mice. Furthermore, RV-reduced plasma triglycerides, FFA, and the apo B/apo AI ratio, which is a stronger risk factor for cardiovascular disease development [40]. Taken together, the present findings suggest RV induced activation of AMPK, through the downstream effectors ACC, PPARα, and UCP2, promotes FA oxidation, while inhibiting FA synthesis. Therefore, lowering hepatic triglycerides, plasma triglycerides, and plasma FFA.

4.4 Adiponectin and other mediators of RV-induced diabetes-related metabolic changes in *db*/*db* mice

Recent studies suggest adiponectin can play a beneficial role in various metabolic diseases, especially diabetes. Circulating adiponectin is reported to correlate with insulin sensitivity in type 2 diabetes patients [41]. In the present study, plasma adiponectin levels were significantly higher in the RV-supplemented *db*/*db* mice, which was consistent with evidence that adiponectin also stimulates AMPK and PPARα activity in target tissues [42]. Furthermore, adiponectin also suppresses hepatic gluconeogenic gene expression [43] and promotes fatty acid oxidation in skeletal muscle [44]. It was beyond the scope of this study to determine whether RV regulates production and secretion of adiponectin from adipocytes. RV supplementation also lowered IKKβ protein levels in skeletal muscle and liver. IKKβ is an inhibitor of nuclear factor kappa-B kinase, which is activated by adipocytokines and during the immune response. IKKβ also inhibits upstream signaling proteins in the insulin signaling pathway, therefore can improve modulate glucose uptake [45]. A priority in future studies is to identify the direct molecular targets of RV, upstream of AMPK, as well as other molecular targets independent of AMPK activation [17]. Recently published findings indicate RV targets upstream regulators of AMPK, include cAMP-degrading phosphodiesterases, which activate CamKKβ an upstream regulator of AMPK [46].

In conclusion, we present evidence that RV activates AMPK and downstream targets involved in regulating diabetes-related metabolic changes in *db*/*db* mice, which naturally develop type 2 diabetes. The beneficial metabolic effects of RV are also partly related to increases in plasma adiponectin levels. Taken together, the present findings

suggest that RV may be as effective as RG for improving hyperglycemia, dyslipidemia, and glycemic control in type 2 diabetes without body weight gain and hepatic steatosis, which are reported to be unfavorable complications of RG.

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