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Royal jelly improves hyperglycemia in obese/diabetic KK-Ay mice

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ABSTRACT. The study examined whether royal jelly (RJ) can prevent obesity and ameliorate hyperglycemia in type 2 diabetes. This study utilized obese/diabetic KK-Ay mice. RJ (10 mg/kg) was administered by oral gavage. Body weight, plasma glucose and insulin levels were measured. mRNA and protein levels were determined using quantitative reverse transcription polymerase chain reaction and western blotting, respectively. Four weeks of RJ administration improved hyperglycemia and partially suppressed body weight gain, although the latter effect did not reach statistical significance. In addition, RJ administration did not improve insulin resistance. RJ administration suppressed the mRNA expression of glucose-6-phosphatase (*G6Pase*), a key enzyme of gluconeogenesis, in the liver. Simultaneously, RJ administration induced adiponectin (*AdipoQ*) expression in abdominal fat, adiponectin receptor-1 (*AdipoR1*) expression in the liver and phosphorylated AMP-activated protein kinase (pAMPK) expression, which suppressed G6Pase levels in the livers of KK-Ay mice. pAMPK levels were also increased in skeletal muscle, but glucose transporter-4 (Glut4) translocation was not increased in the RJ supplementation group. The improvement in hyperglycemia due to long-term RJ administration may be because of the suppression of G6Pase expression through the upregulation of *AdipoQ* and *AdipoR1* mRNA and pAMPK protein expressions.

KEY WORDS: diabetes, hyperglycemia, KK-Ay mice, royal jelly

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Type 2 diabetes is caused by environmental and behavioral factors, such as a sedentary lifestyle and dietary intake [32]. Obesity is associated with the development of type 2 diabetes mellitus [9], and genetic predisposition and obesity are the major risk factors for this disease [14]. Interactions between these two factors possibly contribute to type 2 diabetes onset. Obesity is associated with an increased risk of developing insulin resistance [7]. A major cause of type 2 diabetes is considered to be impaired insulin action in the adipose tissue, liver and skeletal muscle. Overt hyperglycemia develops when β-cells cannot completely compensate for decreased insulin sensitivity [6].

Currently, pharmacotherapy and lifestyle modification are used for ameliorating and regulating type 2 diabetes in patients with obesity. Food restriction and exercise training are effective for preventing or treating type 2 diabetes in obese diabetes-prone rats [13, 20]. Royal jelly (RJ) supplementation has several pharmacological effects. RJ supplementation significantly decreased the mean body weight [15], mean fasting blood glucose and mean glycosylated hemoglobin (HbA1c) levels and elevated the mean insulin concentration in female patients with diabetes [16]. Shidfar *et al*. (2015) reported that RJ intake tended to reduce serum glucose and insulin levels and homeostatic model assessment-insulin resistance values compared with those by placebo intake, although differences between the two groups were not statistically significant [19]. Moreover, in the RJ intake group, total antioxidant capacity in serum was significantly increased in male and female patients [19]. RJ has potent ability to improve hyperinsulinemia and insulin resistance in fructose drinking rats [31]. These reports suggest that RJ supplementation ameliorates hyperglycemia and insulin resistance associated with type 2 diabetes; however, the molecular mechanisms involved are unclear. Because direct investigation of molecular mechanisms for improving type 2 diabetes associated with RJ supplementation in humans is difficult, using inbred animal models is essential for such investigations [2].

Here, we examined the effects of RJ administration on obese/diabetic KK-Ay mice and the mechanisms by which RJ administration improves hyperglycemia. The results suggested that adiponectin (*AdipoQ*) and adiponectin receptor-1 (*AdipoR1*) mRNA expression is enhanced, which can result in phosphorylated AMP-activated protein kinase (pAMPK) activation. Further,

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activated pAMPK suppresses the expression of glucose-6-phosphatase (*G6Pase*), which functions in the final step of glucose production via gluconeogenesis, thereby ameliorating hyperglycemia in KK-Ay mice. We demonstrated that peroxisome proliferator-activated receptor-α (*Pparα*) and peroxisome proliferator-activated receptor-γ coactivator-1α (*Pgc-1α*) are activated, which may be associated with body weight reduction in RJ-supplemented KK-Ay mice.

MATERIALS AND METHODS

Animals

Female KK-Ay mice were purchased at 7 weeks of age from CLEA Japan Inc. (Tokyo, Japan). All mice were maintained under specific pathogen-free condition in the animal facility of Kyoto Sangyo University as described previously [17]. Mice had free access to tap water and standard laboratory chow (MF, Oriental Yeast Co., Tokyo, Japan). The Institutional Animal Care and Use Committee of Kyoto Sangyo University approved the protocols for animal care and experimentation.

Animal groups and treatments

KK-Ay mice were divided into two groups. The RJ group was orally administered 10 mg/kg body weight RJ in 1/15 M phosphate buffer, pH 7.2, and the vehicle-treated group was orally administered 1/15 M phosphate buffer, pH 7.2. Animals were treated for 4 weeks (5 days/week) before being sacrificed. RJ was purchased from Beehive Co., Ltd. (Nagoya, Japan).

Oral glucose tolerance test (OGTT)

OGTT was carried out by injecting glucose (2 g/kg in a 2.8 M solution) in overnight-fasted mice at 4 weeks after the long-term RJ administration. Blood samples were obtained from the tail veins at 0 (fasting), 30, 60, 90 and 120 min. Blood glucose levels were determined directly using the glucose oxidase method with Glutest Neo test strips (Sanwa Chemical Co., Nagoya, Japan). The area under the curve (AUC) was calculated according to the trapezoid rule from the glucose measurements at each time and is expressed as mg/d*l* x min. The blood samples were collected from tail veins using heparinized capillary tubes at 0 (fasting), 15 and 30 min, and then centrifuged to obtain plasma as described previously [17]. Plasma insulin levels were determined using an ELISA kit that detects mouse insulin (Shibayagi Co., Ltd., Shibukawa, Japan).

Insulin tolerance test (ITT)

The insulin tolerance test (ITT) was carried out by injecting human insulin (0.5 U/kg, Humulin R, Eli Lilly, Indianapolis, IN., U.S.A.) intraperitoneally into mice fasted overnight [26] after 3-week RJ administration. Blood glucose levels at 0 (fasting), 30, 60, 90 and 120 min were measured directly as described above.

Sample collection

Two days after the end of the RJ administration period, mice were anesthetized after overnight fasting. Blood was collected from the caudal vena cava and centrifuged to collect serum. Sera were stored at −80°C. The liver, skeletal muscle, mesenteric fat pad and retroperitoneal fat pad were removed and stored at −80°C. Plasma membrane fractions were prepared from skeletal muscles in KK-Ay mice as described by Nishiumi & Ashida [12].

Metabolic assays

Body weights and abdominal fat weights were determined. Food intake was measured for 7 days after 3-week RJ administration. As described previously [17], the serum levels of total cholesterol (TCHO), triglycerides (TGs) and non-esterified fatty acids (NEFAs) were determined using T-Cho-E, TG-E and NEFA-C test kits, respectively, (Wako Pure Chemical Industries, Osaka, Japan).

Quantitative real-time PCR (RT-qPCR)

Tissues were freshly homogenized using ISOGEN II reagent (Wako Pure Chemical industries Ltd.), and RNA was obtained from each tissue (liver, fat and muscle) using ethanol precipitation methods. RT-qPCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan), and a calibration curve method was used to analyze the data. The cDNA sequences were acquired from the genome database of the United States National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome). Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and Amplify-3 (http://engels.genetics.wisc.edu/amplify/) computer software. The relative expression levels were compared by normalization to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Takara Bio Inc., Otsu, Japan) as described previously [17].

Western blot

Western blot analysis was performed as described by Towbin *et al*. [24] with slight modifications. Tissue homogenates were centrifuged, and the supernatants were subjected to electrophoresis in 10% or 15% SDS-PAGE. The proteins separated in the gel were transferred electrophoretically to a polyvinyl difluoride (PVDF) membrane sheet (Immobilon-P, Millipore Co., Billerica, MA, U.S.A.), which was blocked with 5% nonfat dry milk/0.1% Tween 20 in PBS. After washing, the membrane was incubated with antibodies against AMPK (#2063, Cell Signaling Tech (CST) Japan, K.K., Tokyo, Japan) or pAMPK (Thr 172) (#4188, CST Japan,

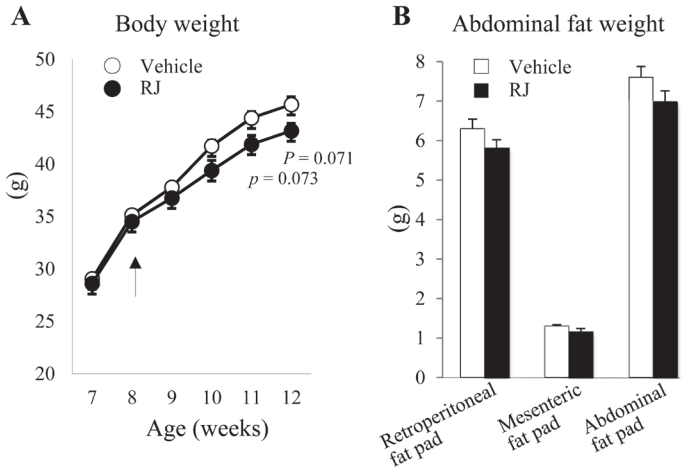

Fig. 1. Effects of long-term RJ administration on body weight and adipose tissues in KK-Ay mice. A: Body weight was measured in KK-Ay mice who were orally administered 10-mg/kg RJ in phosphate buffer or phosphate buffer alone (vehicle) for 4 weeks (n=8). RJ administration was initiated at 8 weeks of age (arrow). Adjusted *p* value was given at 11 and 12 weeks old. B: Mice fasted overnight were sacrificed 2 days after the end of the administration period, and the retroperitoneal fat pad and mesenteric fat pad were measured (n=8). Data are presented as the mean ± SEM.

Table 1 Comparison of body weight, food intake, liver weight, adiposity index and serum adiponectin in KK-Ay mice after 4 weeks of treatment with vehicle or royal jelly

	Vehicle (n=8)	Royal jelly (n=8)
Food intake (g/day)	5.43 ± 0.06	5.00 ± 0.18
Liver weight (g)	1.58 ± 0.069	1.50 ± 0.086
Adiposity index (%)		
Retroperitoneal fat	14.59 ± 0.39	14.09 ± 0.29
Mesenteric fat	3.02 ± 0.056	2.80 ± 0.17
Abdominal fat	17.61 ± 0.43	16.90 ± 0.44
Adiponectin (ng/m*l*)	2,542.68 ± 473.81	2,816.04 ± 238.98

Body weight and adiposity index were measured on the day the rats were sacrificed. Adiponectin levels were measured in the fasting state on the day the rats were sacrificed. Data are presented as the mean ± SEM.

K.K.) or glucose transporter-4 (Glut4) (#2213, CST Japan, K.K.) or β-Actin (#017-24573, Wako Pure Chemical industries Ltd.) in 5% nonfat dry milk/0.1% Tween 20 in PBS as described in detail previously [17]. Antigen-antibody complexes were detected using peroxidase conjugated secondary antibodies (SC-3837, Santa Cruz Biotechnology, Inc., Dallas, TX and 474-1806, Kirkegaard & Perry Laboratories, Inc., Washington, DC, U.S.A.). Reactive oxygen species (ROS) was detected as carbonylated proteins by immunoblotting assay kit (Shima Laboratories Inc., Tokyo, Japan). Bands were analyzed using a Molecular Imager ChemiDoc XRS+ (Bio-Rad Laboratories, Inc., Berkeley, CA, U.S.A.).

Statistical analysis

Data are presented as the mean ± SEM. The statistical significance of differences was evaluated using the Student unpaired *t* test (StatView, SAS Institute Japan Ltd., Tokyo, Japan) and two-way ANOVA (repeated measures) with Bonferroni post-hoc test (GraphPad Prism, GraphPad Software, Inc., San Diego, CA, U.S.A.). A value of P or adjusted $P<0.05$ was defined as statistically significant for *t* test and Bonferroni test, respectively.

RESULTS

Effects of RJ on body weight, abdominal fat pad weight and food intake

Body weight was lower in the RJ supplementation group than in the vehicle-treated group after 3 (P=0.073) and 4 weeks (P=0.071) of supplementation, although the difference was not statistically significant (Fig. 1A). No significant difference in food intake was observed between both groups (Table 1). The abdominal fat pad weight tended to be lower in the RJ supplementation group than in the vehicle-treated group, although the difference was not statistically significant (P=0.11; Fig. 1B). These results suggest that RJ has a suppressive effect on weight gain.

RJ improves hyperglycemia, but not insulin resistance

Blood glucose levels were significantly lower in the RJ supplementation group than in the vehicle-treated group at 30 (P=0.049), 60 (P=0.0036) and 90 min (P=0.024) after glucose loading (Fig. 2A). There was a significant difference in the glucose AUC between the RJ and vehicle-treated groups (P=0.027; Fig. 2B). This result clearly indicates that RJ supplementation can improve glucose intolerance in KK-Ay mice. Plasma insulin levels were not significantly different between the RJ supplementation and vehicle-treated groups (Fig. 2C), suggesting that RJ supplementation may not improve insulin sensitivity. Further, we performed ITTs to determine whether RJ supplementation improved insulin resistance in KK-Ay mice. As shown in Fig. 2D, the glucose-lowering effect was almost identical between the RJ supplementation and vehicle-treated groups. These results suggest that RJ has no effect on insulin sensitivity and insulin resistance.

Metabolic assays

The serum NEFA level was significantly lower in the RJ supplementation group (P=0.047) than in the vehicle-treated group

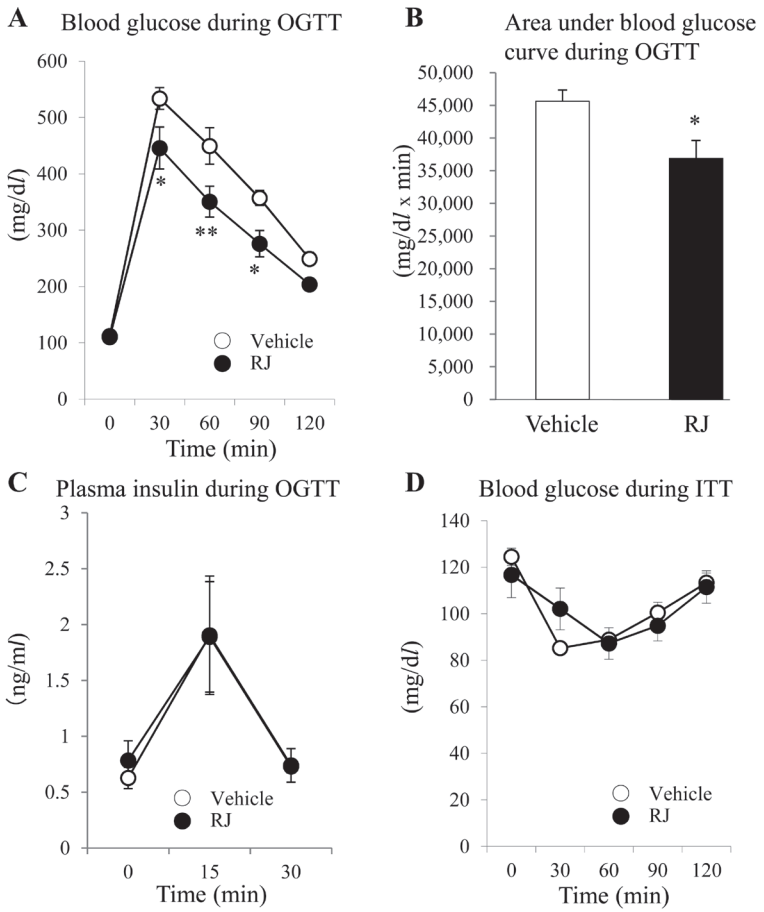

Fig. 2. Effects of long-term RJ administration on blood glucose levels, insulin levels and insulin resistance in KK-Ay mice. A: Blood glucose levels after 4 weeks of treatment with 10 mg/kg RJ (n=8) or vehicle (n=8) were measured during an oral glucose tolerance test (OGTT) in KK-Ay mice fasted overnight. B: The area under the curve (AUC) for blood glucose levels was calculated from the results of the OGTT at all sampling times. C: Insulin levels during the OGTT were determined in mice administered vehicle (n=8) or 10 mg/kg RJ (n=8) for 4 weeks. D: The insulin tolerance test (0.5 U insulin/kg body weight) was performed in overnight-fasted mice after 3 weeks of treatment with RJ (n=8) or vehicle (n=8). Data are presented as the mean ± SEM. **P*<0.05, ***P*<0.01 vs. vehicle.

(Fig. 3A), and there was no difference in serum TG and TCHO levels between the groups (Fig. 3B and 3C). Because insulin's ability to suppress hormone-sensitive lipase (HSL) is reduced in diabetic and obese states, NEFA levels (0.96 mEq/*l*) were slightly above normal (0.1–0.9 mEq/*l*) in the vehicle-treated animals. NEFA levels are believed to be decreased, because of HSL suppression by insulin. However, although RJ had no effect on insulin sensitivity and resistance in our study (Fig. 2C and 2D), the serum NEFA level was decreased in the RJ supplementation group. The reason for the improved serum NEFA level on RJ supplementation was further clarified.

Therefore, *Hsl* mRNA levels were measured. Relative *Hsl* mRNA expression levels in the liver and fat did not significantly differ between the groups (Fig. 3D and 3E), whereas its expression in skeletal muscle was significantly lower in the RJ supplementation group than in the vehicle-treated group (*P*=0.048; Fig. 3F). RJ supplementation may directly alter *Hsl* expression in skeletal muscle.

Effect of RJ on AdipoQ and AdipoR1 mRNA expressions

Although serum AdipoQ levels did not significantly differ between RJ supplementation and vehicle-treated groups (Table 1), we performed RT-qPCR to examine mRNA levels of *AdipoQ* and its receptors in tissues. *AdipoQ* mRNA levels were significantly higher in the abdominal fat in the RJ supplementation group than in the vehicle-treated group (*P*=0.043) (Fig. 4B); however, its expression in the liver and skeletal muscle did not significantly differ between the groups (Fig. 4A and 4C). Moreover, *AdipoR1* mRNA levels were significantly higher in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.00017; Fig. 4D); however, its expression in the abdominal fat and skeletal muscle did not significantly differ between the groups (Fig. 4E and 4F). RJ did not affect *AdipoR2* mRNA expression (Fig. 4G–4I).

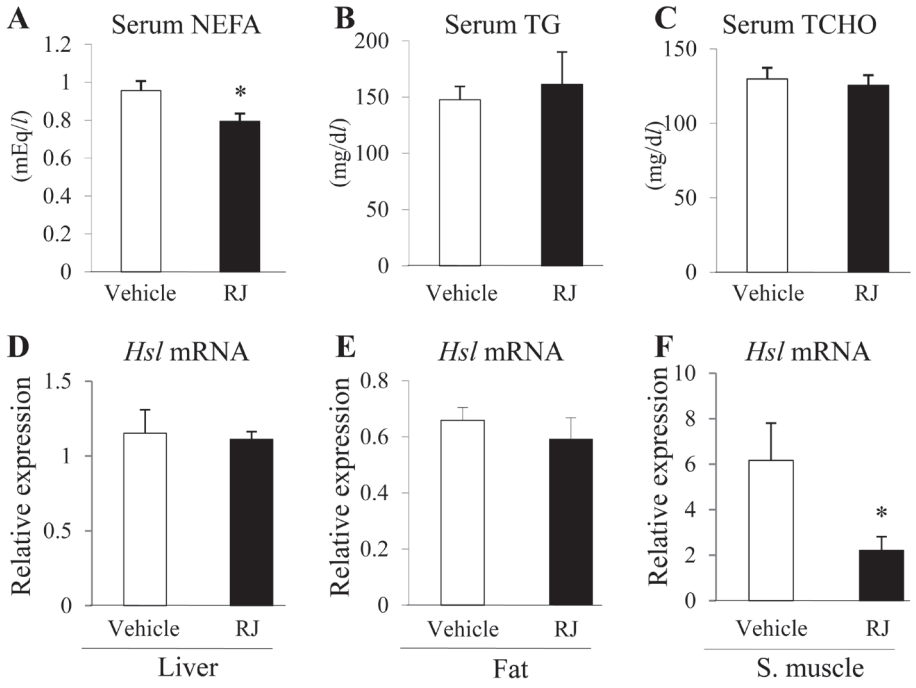

Fig. 3. Effects of long-term RJ administration on the serum NEFA, TG and TCHO levels and *Hsl* mRNA expression in KK-Ay mice. Mice fasted overnight were sacrificed 2 days after the end of the RJ administration period (n=8) or vehicle (n=8), and sera were collected for measuring serum levels of A: NEFAs, B: TGs and C: TCHO. D–F: RNA from the liver, fat and skeletal muscle was extracted to measure the *Hsl* mRNA expression levels between the RJ supplementation (n=8) and vehicle (n=8) groups. Data are presented as the mean ± SEM. **P*<0.05 vs. vehicle.

Suppression of gluconeogenic gene expression

AdipoQ reportedly suppresses genes involved in gluconeogenesis by activating AMPK [28]. Therefore, we measured AMPK phosphorylation at Thr-172 (pAMPK) using western blotting. pAMPK levels were significantly higher in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.0035; Fig. 5A). To test the contribution of increased pAMPK expression to the expression of genes involved in gluconeogenesis, we performed RT-qPCR. *G6Pase* mRNA levels were significantly lower in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.028; Fig. 5B). However, phosphoenolpyruvate carboxykinase (*Pck1*) mRNA levels did not significantly differ between the groups (Fig. 5C). These results suggest that AMPK activation results in suppressed G6Pase expression selectively in KK-Ay mice.

Furthermore, pAMPK levels were significantly higher in the muscle in the RJ supplementation group than in the vehicle-treated group (*P*=0.0076; Fig. 5D). However, Glut4 translocation did not significantly differ between the groups in the muscle membranes (Fig. 5E), suggesting that AMPK activation in muscle does not always result in increased Glut4 translocation. Therefore, hyperglycemia amelioration using RJ may be because of suppressed *G6Pase* expression as a gatekeeper of glucose production, but not because of the enhanced incorporation of glucose into the muscle caused by AMPK activation.

Enhancement of Ppar expression

Because Ppars including Pgc-1α control the expression of genes involved in lipid metabolism and adipogenesis, we compared the expression of these genes using RT-qPCR. The mRNA expression levels of *Pparα*, which is a major activator of fatty acid oxidation pathways and a protector against hepatic inflammation, were significantly higher in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.0021; Fig. 6A), suggesting that *Pparα* activation enhances lipolysis and contributes to the decline in body weight gain following RJ supplementation. Tumor necrosis factor-α (*Tnf-α*) mRNA expression levels were significantly lower in fat pads in the RJ supplementation group than in the vehicle-treated group (*P*=0.0099; Fig. 6B). *Pgc-1α* mRNA expression levels were significantly higher in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.0038; Fig. 6C), suggesting improved lipid utilization.

Pparγ is a master regulator of adipogenesis. We found that *Pparγ1* mRNA expression levels were significantly lower in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.016; Fig. 6D), whereas those of *Pparγ2* were significantly higher in fat pads in the RJ supplementation group than in the vehicle-treated group (*P*=0.015; Fig. 6E). These results suggest reduced lipid storage and gluconeogenesis in the liver and enhanced lipid metabolism and glucose homeostasis in fat pads on RJ supplementation.

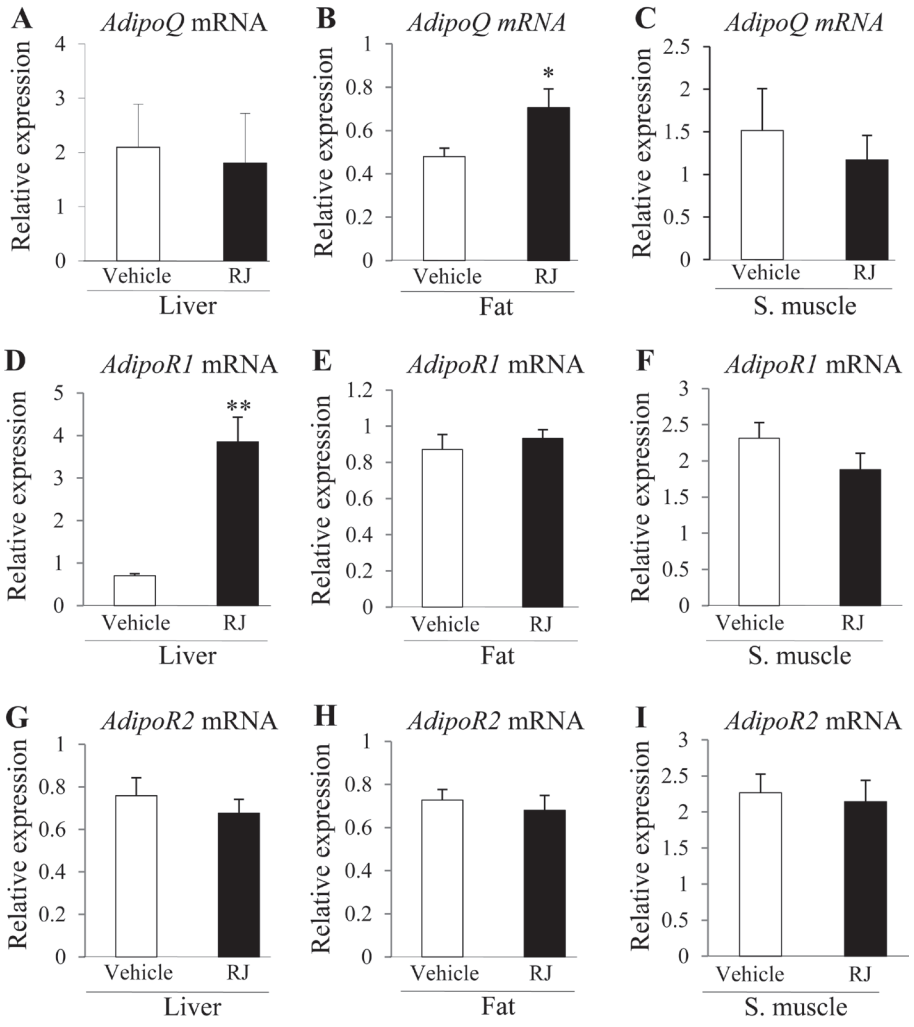

Fig. 4. Effects of long-term RJ administration on *AdipoQ*, *AdipoR1* and *AdipoR2* mRNA expressions in overnight-fasted KK-Ay mice. Mice fasted overnight were sacrificed 2 days after the end of the RJ administration period (n=8) or vehicle (n=8), and RNA was extracted from the liver, retroperitoneal fat and skeletal muscle. A–C: Relative mRNA expression levels of *AdipoQ*, D–F: *AdipoR1* and G–I: *AdipoR2* were quantified using quantitative reverse transcription-PCR and normalized to the mRNA levels of *Gapdh*. Data are presented as the mean ± SEM. **P*<0.05, ***P*<0.01 vs. vehicle.

Suppression of reactive oxygen species (ROS) production

Pgc-1α reportedly suppresses ROS production [21]. Therefore, we examined ROS by measuring carbonylated protein levels via western blotting. ROS levels were significantly lower in the liver in the RJ supplementation group than in the vehicle-treated group (*P*=0.023; Fig. 7), suggesting that metabolism in the liver is improved by decreased oxidative stress.

DISCUSSION

RJ supplementation significantly reduces the mean serum glycosylated hemoglobin and fasting blood glucose levels in females with type 2 diabetes [16], suggesting that RJ supplementation ameliorates the disease. However, the mechanisms involved are unknown. Here, we demonstrated that RJ supplementation reduced postprandial hyperglycemia and partially reduced body weight in obese/diabetic KK-Ay mice, although the latter effect did not reach statistical significance. We found that *AdipoQ* mRNA expression levels were significantly increased in the abdominal fat pad in the RJ supplementation group compared with those in the vehicle-treated group. Furthermore, we found that *AdipoR1* expression was significantly higher in the liver in the RJ supplementation group than in the vehicle-treated group. Further, we examined AMPK expression levels, because high expression of *AdipoQ* or *AdipoR1* promotes AMPK phosphorylation [29, 30]. Our result illustrated that pAMPK expression levels were enhanced in the liver and muscle in the RJ supplementation group. This suggests that enhanced *AdipoQ* and *AdipoR1* mRNA expression contributes to AMPK activation. Moreover, other reports revealed that AdipoQ directly stimulates AMPK phosphorylation in the liver and muscle [5, 28, 29].

AMPK upregulation reportedly results in suppressed mRNA expression of *G6Pase*, which is a key enzyme in the final step

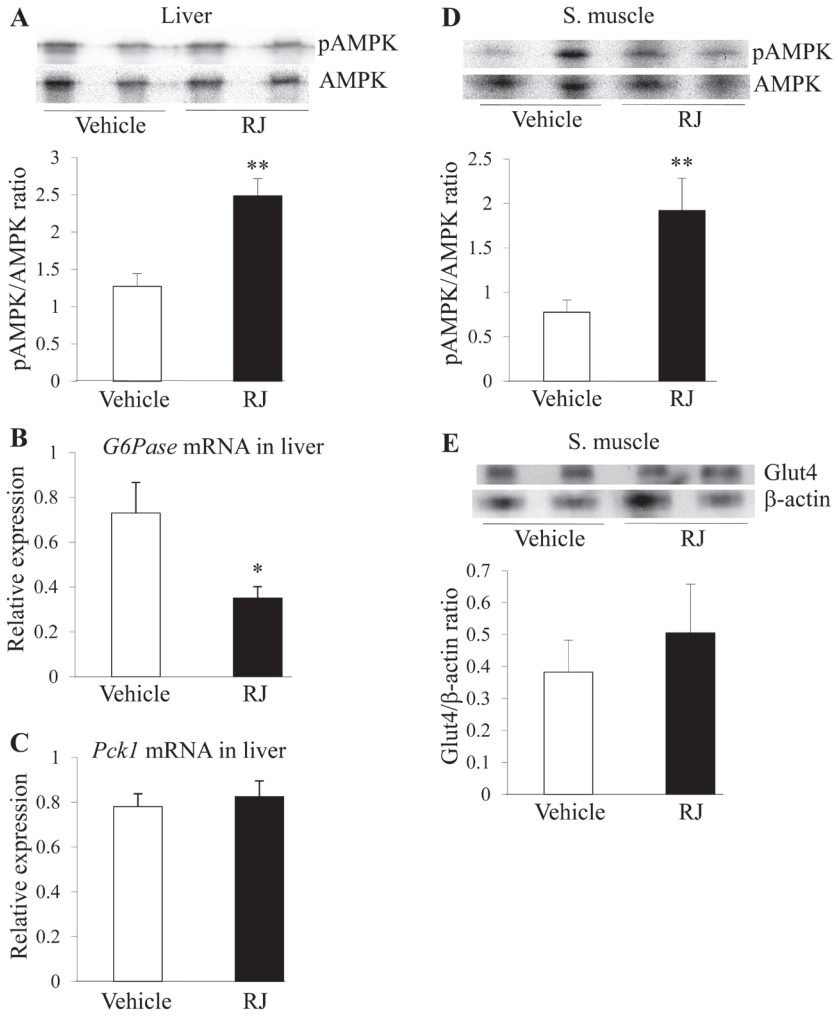

Fig. 5. Effects of long-term RJ administration on the protein contents of phosphorylated AMPK and Glut4 and *G6Pase* and *Pck1* mRNA expressions in KK-Ay mice. Mice fasted overnight were sacrificed 2 days after the end of the RJ administration period (n=6) or vehicle (n=6). A: Relative pAMPK levels in the liver were determined by western blotting. B and C: Relative mRNA expression levels of *G6Pase* (n=8) and *Pck1* (n=8) in the liver were quantified by quantitative reverse transcription-PCR. D: Relative pAMPK levels in skeletal muscle (supernatant of crude extract) (n=6) were determined by western blotting. E: Relative Glut4 levels in the plasma membrane fraction purified from skeletal muscle (n=6) were determined by western blotting. Data are presented as the mean ± SEM. **P*<0.05, ***P*<0.01 vs. vehicle.

in the production of glucose via gluconeogenesis [27]. We revealed that *G6Pase* mRNA expression was suppressed in the RJ supplementation group. Therefore, we could describe the mechanism that improved hyperglycemia, because of RJ administration as follows. RJ administration enhances *AdipoQ* mRNA expression, resulting in increased *AdipoR1* mRNA expression. Both *AdipoQ* and *AdipoR1* stimulate AMPK phosphorylation in the liver and muscle, and activated AMPK suppresses G6Pase expression levels. The higher *AdipoQ* expression caused by RJ administration may directly result in the suppressed gluconeogenic gene expression in mouse hepatocytes, as Miller *et al.* reported [11].

RJ supplementation significantly decreased the mean body weight in diabetic females [15] and improved lipoprotein metabolism in humans [4]. Tsuchida *et al.* reported that *Pparα* activation partially prevented adipocyte hypertrophy in KK-Ay mice [25]. *Pparα* expression protects mice against high fat-induced nonalcoholic fatty liver [1]. As shown in Fig. 6A, *Pparα* mRNA expression in the liver was significantly higher in the RJ supplementation group than in the vehicle-treated group among KK-Ay mice, suggesting that the enhanced *Pparα* expression may have contributed to the weight reduction observed in the RJ supplementation group. Because the absence of HSL prevented obesity and adipogenesis in obese mice [18], and inhibition of HSL improved lipid profiles and plasma glucose levels in mice with streptozotocin-induced diabetes [3], reduced HSL levels in muscle (Fig. 3F) may have contributed to decreased serum NEFA content and body weight in the RJ supplementation group.

Because *Pparα* protects against obesity-induced hepatic inflammation [22], the higher *Pparα* mRNA expression in the RJ supplementation group may have partially contributed to reduced *Tnf-α* mRNA expression (Fig. 6B).

Recent studies revealed that RJ supplementation improves homeostatic model assessment-β-cell function levels in patients with diabetes [19] and ameliorates insulin resistance in fructose-drinking rats [31]; however, our result identified no difference in plasma

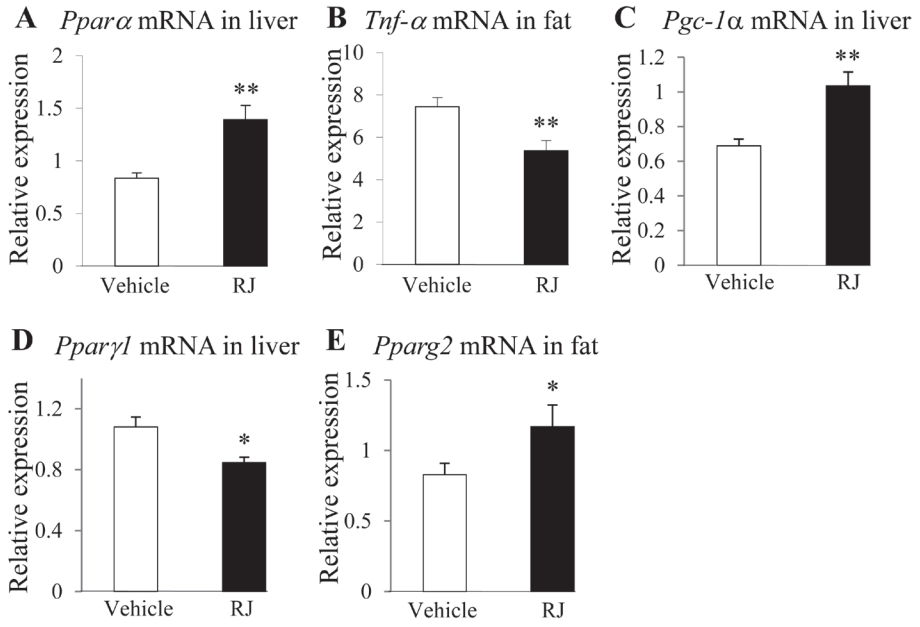

Fig. 6. Effects of long-term RJ administration on *Pparα*, *Pgc-1α*, *Pparγ1*, *Pparγ2* and *Tnf-α* mRNA levels in KK-Ay mice. Mice fasted overnight were sacrificed 2 days after the end of the RJ administration period (n=8) or vehicle (n=8). A: *Pparα* mRNA levels in the liver, B: *Tnf-α* mRNA levels in retroperitoneal fat, C: *Pgc-1α* mRNA levels in the liver, D: *Pparγ1* mRNA levels in the liver and E: *Pparγ2* mRNA levels in retroperitoneal fat. Data are presented as the mean ± SEM. **P*<0.05, ***P*<0.01 vs. vehicle.

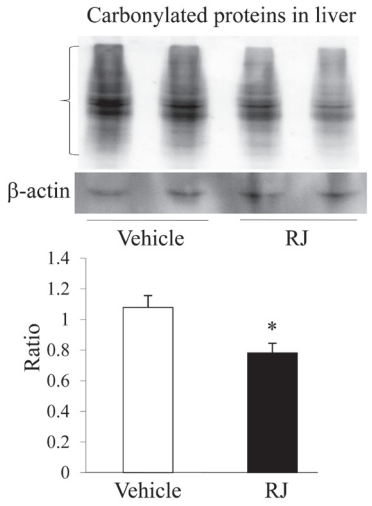

Fig. 7. Effects of long-term RJ administration on oxidized protein (carbonylated) contents evaluated by western blotting. Mice fasted overnight were sacrificed 2 days after the end of the RJ administration period (n=6) or vehicle (n=6). Relative expression levels of carbonylated proteins were normalized to the levels of β-actin. Data are presented as the mean ± SEM. **P*<0.05 vs. vehicle.

insulin levels or improvement in insulin resistance according to ITTs in RJ-treated mice. Expression levels of molecules involved in the insulin signal cascade, such as phosphorylated Akt, forkhead box O1, glycogen synthase and glycogen synthase kinase 3β, did not significantly differ between the RJ-treated and vehicle-treated mice (Suppl. Fig. 1). This suggests that RJ supplementation did not affect insulin resistance or insulin cascade activation in KK-Ay mice. Ameliorated hyperglycemia on RJ supplementation may depend on suppressed gluconeogenesis, but not on improved insulin action in KK-Ay mice. It is yet unclear why RJ does not improve insulin action in KK-Ay mice, although the finding may be because of species or strain differences.

Pgc-1α suppresses ROS production and protects neural cells against oxidative stress-induced death caused by the induction of several key ROS-detoxifying enzymes [8, 21]. We revealed that *Pgc-1α* mRNA expression was higher in the RJ supplementation group (Fig. 6C), whereas ROS production was lower in this group than in the vehicle-treated group (Fig. 7). These results suggest that RJ administration improves oxidative stress by enhancing *Pgc-1α* mRNA expression, thereby reducing ROS production. Recent studies illustrated that ROS production in response to physiological stimuli promotes insulin sensitivity and attenuates insulin resistance [10, 23]. However, decreased ROS production by RJ administration did not appear to affect insulin sensitivity and resistance in KK-Ay mice.

In conclusion, our study found that RJ administration improves hyperglycemia and partially reduces body weight in obese/diabetic KK-Ay mice. We proposed the mechanism by which RJ administration activates *AdipoQ* and *AdioR1* expression, after which AMPK is activated, resulting in suppressed *G6Pase* expression as a final gatekeeper of glucose production in the liver. Furthermore, enhanced *AdipoR1* expression on RJ administration promotes *Ppara* and *Pgc1a* expression, which improved lipid utilization, resulting in reduced body weight in KK-Ay mice (Suppl. Fig. 2).

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