

Royal Jelly Ameliorates Insulin Resistance in Fructose-Drinking Rats

Yoshito ZAMAMI,^a Shingo TAKATORI,^b Mitsuhiro GODA,^a Toshihiro KOYAMA,^a Yukiko IWATANI,^a Xin JIN,^a Shima TAKAI-DOI,^c and Hiromu KAWASAKI^{*,a}

^aDepartment of Clinical Pharmaceutical Science, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University; 1-1-1 Tsushima-naka, Okayama 700-8530, Japan; ^bPharmacology Department, Discovery Research Laboratories, Nippon Shinyaku Co., Ltd.; 14 Nishinosho-Monguchi-cho, Minami-ku, Kyoto 601-8550, Japan; and ^cDepartment of Research and Development, Yamada Apiculture Center, Inc.; 194 Ichiba, Kagamino-cho, Tomatagun, Okayama 708-0393, Japan.

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Royal jelly (RJ) is known to contain excellent nutrition and a variety of biological activities. The present study was designed to investigate the effects of RJ on insulin resistance (hyperinsulinemia) in fructose-drinking rats (FDR; insulin resistance animal model). Male Wistar rats (6 weeks old) received 15% fructose solution in drinking water for 8 weeks. FDR showed significant increases in plasma levels of insulin and triglyceride, Homeostasis Model Assessment ratio (HOMA-R, an index of insulin resistance), and systolic blood pressure, but not blood glucose levels, when compared with control rats. RJ (100, 300 mg/kg, *p.o.*) treatment for 8 weeks significantly decreased the plasma levels of insulin and triglyceride, HOMA-R, without affecting blood glucose or total cholesterol levels and tended to lower systolic blood pressure. In isolated and perfused mesenteric vascular beds of FDR, RJ treatment resulted in a significant reduction in sympathetic nerve-mediated vasoconstrictor response to periarterial nerve stimulation (PNS) and tended to increase the calcitonin gene-related peptide (CGRP) nerve-mediated vasodilator response to PNS, compared with those in untreated FDR. However, RJ treatment did not significantly affect norepinephrine-induced vasoconstriction or CGRP-induced vasodilation. These results suggest that RJ could be an effective functional food to prevent insulin resistance associated with the development of hypertension.

Key words royal jelly; fructose-drinking rat; insulin resistance; periarterial nerve function

Royal jelly (RJ) produced by honey bees is known to contain three major nutrients including amino acids, vitamins and minerals.¹⁾ Additionally, RJ has various biological activities such as a hypotensive effect, insulin-like action and antitumor activity.^{2–4)} Therefore, it is possible that RJ may have some effects on insulin resistance, which is considered to be a cause of various lifestyle-related diseases. Many clinical studies have shown a possible relationship between hypertension and insulin resistance, since patients with type 2 (non-insulin dependent) diabetes mellitus frequently have insulin resistance associated with hypertension.^{5–7)}

Chronic administration of fructose to rats has been reported to cause insulin resistance, which is characterized by increased serum insulin and euglycemia.⁸⁾ Especially, a hyperinsulinemic state associated with hypertension was more prominently induced by fructose drinking than by fructose feeding.⁹⁾ Previously, we reported that rats with chronic hyperinsulinemia, which was induced by giving a 15% fructose solution in drinking water for 10 weeks (fructose-drinking rats, FDR), showed deficient neuronal regulation of vascular tone, and this leads to hypertension.^{10,11)} Recently, we demonstrated that chronic administration of RJ prevented development of insulin resistance in Ohtsuka Long-Evans Fatty (OLETF) rats, which is a spontaneous type 2 diabetic model.¹²⁾

Thus, in the present study, we investigated the effects of RJ on the insulin resistance in FDR. We also examined whether RJ prevents hypertension and altered vascular responsiveness induced by insulin resistance using isolated mesenteric vascular beds of rats.

MATERIALS AND METHODS

Animals Six week-old male Wistar rats were used in this study. They were given 15% fructose solution as drinking water *ad libitum* for 8 weeks. The control group was given tap water instead of 15% fructose solution. Three rats were housed in each cage (W 220×L 320×H 135 mm; Natsume Seisakusho, Tokyo, Japan), and given normal rat chow (Oriental Yeast, Tokyo, Japan). They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22±2 °C with 50±10% relative humidity and with a 12-h light/12-h dark cycle (lights on at 8:00 a.m.). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Every effort was made to minimize the number of animals used and their suffering.

Long-Term Administration of RJ RJ, which was enzymatically treated and supplied by Yamada Apiculture Center, Inc. (Okayama, Japan) (Lot No. 020605), was used in this study. RJ was diluted by adding distilled water and orally administered at doses of 100 mg/kg/d and 300 mg/kg/d for 8 weeks from 6 to 14 weeks of age. Each animal was lightly anesthetized with ether and orally administered RJ solution at a volume of 2 ml/kg once a day (09:00 to 10:00). The control group was orally administered the same volume of tap water once a day.

Measurements of Body Weight and Food and Liquid Intake Body weight was measured once a week, and food and liquid intakes were measured every 2 d from 6 to 14

* To whom correspondence should be addressed. e-mail: Kawasaki@pheasant.pharm.okayama-u.ac.jp

weeks of age.

Biochemical Analysis Under light ether anesthesia, blood samples were obtained 0.8 ml from the heart by cardiopuncture after 12-h fasting. A drop of the blood sample was immediately used to measure the plasma level of glucose with a glucose analyzer (ADVANTAGE; Boehringer Mannheim, Tokyo, Japan). Blood samples were centrifuged to obtain plasma samples, which was stored at -80°C until the measurements. Plasma insulin was measured by a double-antibody method using a solid-phase insulin monoclonal antibody and a guinea pig insulin antibody with an ELISA insulin kit (Morinaga Biochemistry Co., Kanagawa, Japan). Plasma cholesterol and triglyceride were enzymatically measured using commercially available kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The effects of RJ were expressed as a ratio of the value obtained from 14 week-old rats or the value from 6 week-old rats. The Homeostasis Model Assessment ratio (HOMA-R), which is an index of insulin resistance, was calculated using this formula: plasma glucose (mmol/l) \times insulin ($\mu\text{U/ml}$)/22.5.¹³⁾

Systolic Blood Pressure (SBP) Measurement SBP under the conscious state was measured with a tail-cuff plethysmograph (TK-370C, Unicom, Tokyo, Japan) from 6 to 14 weeks of age once a week. The average of five readings per animal was used.

Perfusion of Mesenteric Vascular Beds The animals in the 14 week-old control group, FDR without RJ administration group and FDR with RJ administration group were anesthetized with pentobarbital-Na (50 mg/kg, intraperitoneally) and mesenteric vascular beds were isolated and prepared for perfusion as described previously.¹⁴⁾ After removal of the entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric arteries were tied off. The isolated mesenteric vascular was placed in a water-jacketed organ bath maintained at 37°C , perfused with a modified (see below) Krebs solution at a constant flow rate of 5 ml/min with a peristaltic pump (model AC-2120, ATTO, Tokyo, Japan) and superfused with the same solution at a rate of 0.5 ml/min to prevent drying. The Krebs solution was bubbled with a mixture of 95% O_2 -5% CO_2 before passage through a warming coil maintained at 37°C . The modified Krebs solution was of the following composition (mM): NaCl 119.0, KCl 4.7, CaCl_2 2.4, MgSO_4 1.2, NaHCO_3 25.0, KH_2PO_4 1.2, disodium EDTA 0.03 and dextrose 11.1 (pH 7.4). Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T, Nihon Koden, Tokyo, Japan) and recorded using a pen recorder (model U-228, Nihon Denki Kagaku, Tokyo, Japan).

Perfusion Experimental Protocol After 30 minutes' perfusion, isolated mesenteric vascular beds with resting tone were subjected to periarterial nerve stimulation (PNS) (8, 12 Hz) and bolus injection of norepinephrine (NE) (5, 10 nmol), which induced an increase in perfusion pressure due to vasoconstriction. Thereafter, Krebs solution was switched to Krebs solution containing $5\ \mu\text{M}$ guanethidine and $7\ \mu\text{M}$ methoxamine to increase the perfusion pressure to about 100 mmHg levels. After elevated perfusion pressure stabilized, PNS (2, 4 Hz) and a bolus injection of rat CGRP

(50, 100 pmol) were applied to decrease the perfusion pressure due to vasodilation. PNS was performed using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and a supramaximal voltage (50 V) were applied for 30 s via an electronic stimulator (model SEN 3301; Nihon Bunko). NE was directly injected into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus Inc., Holliston, MA, U.S.A.). A volume of 100 μl was injected over a period of 12 s. At the end of each experiment, 100 μM papaverine was perfused to produce complete relaxation. Vasoconstrictions were expressed as the increase in perfusion pressure. Vasodilations were expressed as the percent perfusion pressure at maximum relaxation induced by papaverine.

Statistical Analysis The experimental results are presented as the mean \pm S.E.M. Statistical analyses were performed using one analysis of variance (ANOVA) followed by Tukey's test or two-way ANOVA, when appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of 8-Week Treatment with RJ on Body Weight and Food and Liquid Intake in FDR As shown in Table 1, food intake in the vehicle-treated FDR group was significantly lower than that in the control group, while liquid intake in the vehicle-treated FDR group was significantly higher than that in the control group. However, there was no significant difference in food or liquid intake between the vehicle-treated and RJ-treated FDR groups (Table 1).

As shown in Fig. 1, there was no significant difference in body weight between the control group and vehicle- or RJ-treated FDR (Fig. 1).

Table 1. Total Food Intake and Fluid Intake during the Experiment in Fructose-Drinking Rats (FDR)

	Total food intake (g)	Total fluid intake (ml)
Control	792 \pm 5	1003 \pm 12
FDR + vehicle	598 \pm 15*	1396 \pm 36**
FDR + RJ 100 mg/kg	647 \pm 17*	1541 \pm 42**
FDR + RJ 300 mg/kg	627 \pm 9*	1460 \pm 22**

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. Royal jelly (RJ) at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. Each value represents the mean \pm S.E.M. of 5–6 experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

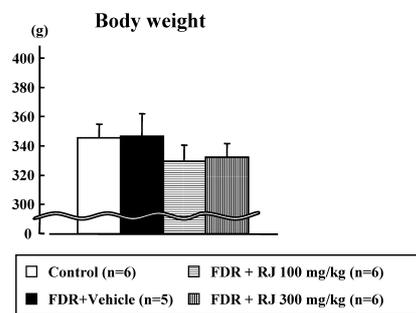


Fig. 1. Effect of 8-Week Treatment with Royal Jelly (RJ) on Body Weight in Fructose-Drinking Rats (FDR)

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. RJ at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. Each value represents the mean \pm S.E.M.

Effects of 8-Week Treatment with RJ on Plasma Levels of Insulin, Glucose and the HOMA Score in FDR Figure 2 shows changes in fasting blood glucose (A), fasting serum insulin level (B) and the index of insulin resistance (HOMA) (C) of FDR before and after 8-week treatment with RJ or the vehicle. The plasma glucose levels in the control, vehicle-treated, RJ 100 mg/kg-treated and RJ 300 mg/kg-treated FDR at 6 weeks of age before fructose-loading were 88 ± 5 mg/dl, 92 ± 6 mg/dl, 92 ± 4 mg/dl, and 98 ± 5 mg/dl, respectively. Plasma glucose levels at 14 weeks of age in the control and vehicle-treated FDR groups were significantly higher than those at 6 weeks of age. However, there was no significant difference in the plasma glucose levels between vehicle- and RJ-treated FDR (Fig. 2A).

At 6 weeks of age before fructose-loading, serum insulin levels in the control, vehicle-treated, RJ 100 mg/kg-treated FDR and RJ 300 mg/kg-treated FDR were 0.27 ± 0.03 ng/ml, 0.26 ± 0.02 ng/ml, 0.34 ± 0.03 ng/ml, 0.29 ± 0.03 ng/ml, respectively. There was no significant difference between each group. Serum insulin levels in vehicle-treated FDR at 14 weeks of age after 15% fructose-loading were about two times higher than controls at 14 weeks of age and vehicle-treated FDR at 6 weeks of age before fructose-loading, indicating hyperinsulinemia in vehicle-treated FDR at 14 weeks of age. As shown in Fig. 2A, RJ treatment for 8 weeks induced a dose-dependent decrease in serum insulin levels. The plasma insulin level in the RJ 300 mg/kg-treated FDR group at 14 weeks of age was significantly lower than that in vehicle-treated FDR, and was similar to that in the control group (Fig. 2B).

As shown in Fig. 2C, the HOMA score (index of insulin resistance) in vehicle-treated FDR at 14 weeks of age was about 7 times higher than that in vehicle-treated FDR at 6 weeks of age before 15% fructose-loading and about 2 times higher than that in the control group at 14 weeks of age, indicating that insulin resistance had developed in FDR. RJ treatment for 8 weeks dose-dependently decreased the HOMA score. The HOMA score in RJ 300 mg/kg-treated FDR at 14 weeks of age was significantly lower than that in vehicle-

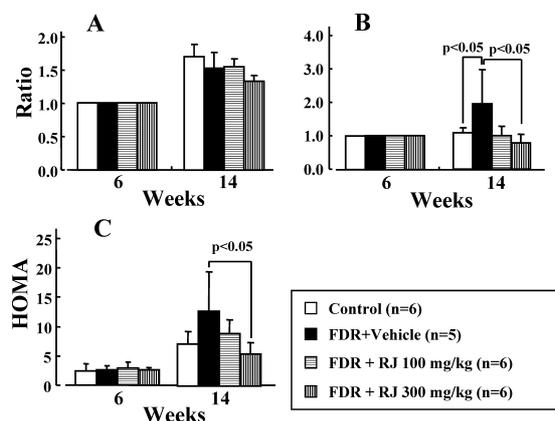


Fig. 2. Effect of 8-Week Treatment with Royal Jelly (RJ) on Fasting Blood Glucose (A), Fasting Serum Insulin Level (B) and the Index of Insulin Resistance (Plasma Glucose \times Insulin/22.5) (Homeostasis Model Assessment; HOMA) (C) in Fructose-Drinking Rats (FDR)

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. RJ at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. The vertical scale in A and B indicates the fold increase over the value in the 6 week-old rats. Each value represents the mean \pm S.E.M.

treated FDR, and was similar to that in the control group (Fig. 2C).

Effects of 8-Week Treatment with RJ on Plasma Levels of Triglycerides and Total Cholesterol in FDR Figure 3 shows the effects of 8-week treatment with RJ on fasting serum total cholesterol (A) and fasting serum triglyceride (B) in FDR. At 6 weeks of age before fructose-loading, the plasma levels of total cholesterol in the control, vehicle-treated FDR, RJ 100 mg/kg-treated FDR and RJ 300 mg/kg-treated FDR groups were 88 ± 5 mg/ml, 94 ± 10 mg/ml, 90 ± 8 mg/ml, 91 ± 7 mg/ml, respectively. There was no significant difference between each group. In RJ-treated FDR at 14 weeks of age, the plasma levels of total cholesterol were similar to those in control and vehicle-treated FDR. There was no significant difference in the plasma levels of total cholesterol among all groups (Fig. 3A).

At 6 weeks of age before fructose-loading, the plasma levels of triglycerides in the control, vehicle-treated FDR, RJ 100 mg/kg-treated FDR and RJ 300 mg/kg-treated FDR groups were 65 ± 2 mg/ml, 58 ± 8 mg/ml, 55 ± 3 mg/ml, 59 ± 4 mg/ml, respectively. There was no significant difference between each group.

After fructose-loading, the plasma level of triglycerides in the vehicle-treated FDR group was significantly higher than that in the control group. The 8-week treatment with RJ caused a dose-dependent decrease in plasma levels of triglycerides. The plasma triglycerides level in RJ 300 mg/kg-treated FDR at 14 weeks of age was significantly lower than that in vehicle-treated FDR (Fig. 3B).

Effect of 8-Week Treatment with RJ on Systolic BP in FDR As shown in Fig. 4, 15% fructose-loading for 8 weeks induced a significant increase in systolic blood pressure. The systolic blood pressure in the RJ 300 mg/kg-treated FDR group tended to be lower than that in the vehicle-treated FDR group, but there was no significant difference between the RJ-treated and vehicle-treated FDR groups (Fig. 4).

Effect of 8-Week Treatment with RJ on Adrenergic Nerve-Mediated Vasoconstrictor Responses to PNS and Bolus Injection of Norepinephrine in FDR PNS at 8 and 12 Hz of the perfused mesenteric vascular beds with resting tone produced frequency-dependent vasoconstriction. In the same preparation with resting tone, bolus injection of norepinephrine caused a concentration-dependent vasoconstriction

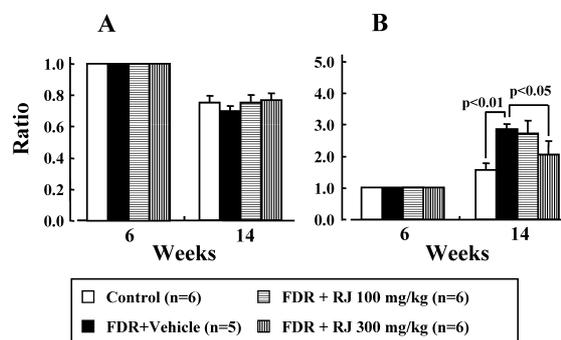


Fig. 3. Effect of 8-Week Treatment with Royal Jelly (RJ) on Fasting Serum Total Cholesterol (A) and Fasting Serum Triglyceride (B) in Fructose-Drinking Rats (FDR)

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. RJ at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. The vertical scale indicates the fold increase over the value in the 6 week-old rats. Each value represents the mean \pm S.E.M.

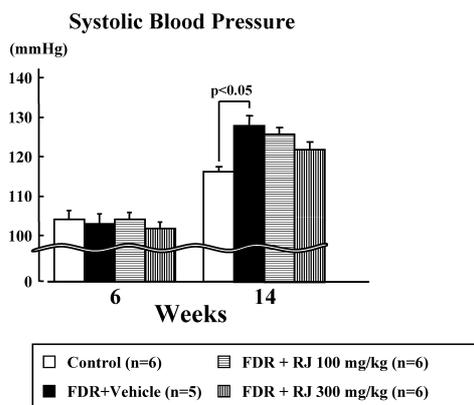


Fig. 4. Effect of 8-Week Treatment with Royal Jelly (RJ) on Systolic Blood Pressure in Fructose-Drinking Rats (FDR)

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. RJ at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. Each value represents the mean \pm S.E.M.

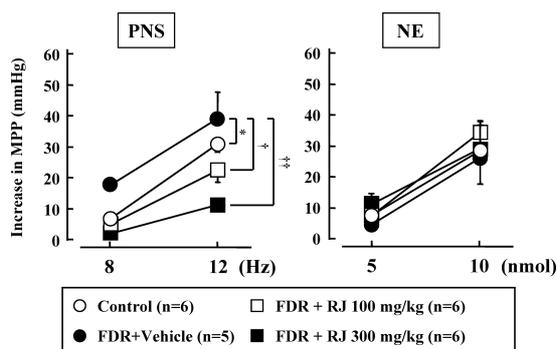


Fig. 5. Effect of 8-Week Treatment with Royal Jelly (RJ) on Vasoconstrictor Responses to Periarterial Nerve Stimulation (PNS) or Norepinephrine (NE) Injection in Perfused Mesenteric Vascular Beds with Resting Tone in Fructose-Drinking Rat (FDR)

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. RJ at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. Each value represents the mean \pm S.E.M. * $p < 0.05$ vs. control. † $p < 0.05$, †† $p < 0.01$ vs. FDR+vehicle. MPP, mean perfusion pressure.

pattern that mimicked the PNS-induced response. As shown in Fig. 5, vasoconstrictor responses to PNS at 8 and 12 Hz in preparations isolated from the vehicle-treated FDR group were significantly greater than those in preparations from the control group. The vasoconstrictor responses to PNS in preparations from the RJ 100 and 300 mg/kg-treated FDR groups were significantly smaller than those in preparations from the vehicle-treated FDR group. However, no significant difference in vasoconstrictor responses to norepinephrine injection was found among all of the groups (Fig. 5).

Effect of 8-Week Treatment with RJ on CGRPergic Nerve-Mediated Vasodilator Responses to PNS and Bolus Injection of CGRP in FDR To observe the vasodilator response, active tone was achieved by continuous perfusion of methoxamine (7 μ M; α_1 -adrenergic receptor agonist) in the presence of guanethidine (5 μ M; adrenergic neuron blocker), which was added to block adrenergic neurotransmission. In this preparation, PNS at 2 and 4 Hz caused frequency-dependent vasodilations. As shown in Fig. 6, the PNS (2 and 4 Hz)-induced vasodilator responses in preparations from vehicle-treated FDR tended to be smaller than those of the control group, but no significant difference was found between

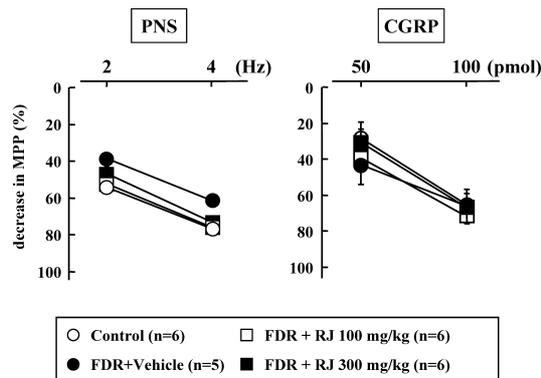


Fig. 6. Effect of 8-Week Treatment with Royal Jelly (RJ) on Vasodilator Responses to Periarterial Nerve Stimulation (PNS) or CGRP Injection in Perfused Mesenteric Vascular Beds with Active Tone in Fructose-Drinking Rats (FDR)

Control and FDR received normal water and 15% fructose solution as drinking water for 8 weeks, respectively. RJ at doses of 100 and 300 mg/kg was administered *p.o.* once a day for 8 weeks. Each value represents the mean \pm S.E.M. MPP, mean perfusion pressure.

the two groups. CGRPergic mediated-vasodilation in preparations from RJ-treated FDR groups was similar to that in preparations from the control group, but there was no significant difference between control and RJ-treated FDR groups (Fig. 6). In preparations from RJ-treated FDR groups (Fig. 6), vasodilator responses to CGRP injection were similar to those in preparations from control and vehicle-treated FDR groups.

DISCUSSION

In the present study, FDR given 15% fructose solution as their drinking water showed a marked increase in plasma levels of insulin (hyperinsulinemia) and triglycerides (hypertriglyceridemia) without a significant increase in blood glucose levels (euglycemia), when compared with the vehicle-treated control group. These findings are in good accordance with previous reports.^{10,11} Recently, we demonstrated that the treatment of FDR with an insulin-sensitizing drug, pioglitazone, normalized hyperinsulinemia in FDR without changing plasma glucose levels and abolished insulin resistance.¹¹ In addition to hyperinsulinemia, FDR developed hypertension, which has also been shown to be abolished by pioglitazone.¹¹ Taken together, it is very likely that FDR have insulin resistance and are a good animal model at insulin resistance.

It should be noted that 8-week treatment of FDR with RJ eliminated hyperinsulinemia and hypertriglyceridemia without changing the plasma glucose level, strongly suggesting that RJ has a potent ability to improve hyperinsulinemia and insulin resistance in FDR. Insulin resistance has been shown to be associated with changes in levels of oxidative stress.^{14–16} Furthermore, recent reports suggests that RJ has protective effects against oxidative stress-induced biologic dysfunction.^{17,18} Thus, it is likely that RJ ameliorates insulin resistance *via* antioxidant effect. Additionally, the RJ-treated FDR showed decreased SBP, while the non-treated FDR showed maintained hypertension. Therefore, the present findings imply that RJ may be effective to hypertension associated with insulin resistance.

The vasoconstrictor response to PNS in the rat mesenteric artery is mediated by activation of sympathetic adrenergic nerves, since a neurotoxin (tetrodotoxin), an adrenergic neuron blocker (guanethidine), an adrenergic neurotoxin (6-hydroxydopamine) and an α_1 -adrenoceptor antagonist (prazosin) abolished the response.^{19,20} The present study demonstrated that the PNS-induced pressor response in the mesenteric artery of FDR with insulin resistance was significantly greater than that of control rats. However, FDR showed an unchanged pressor response to exogenously applied noradrenaline, which is mediated by the postsynaptic α_1 -adrenoceptor. Therefore, it is very likely that the neurotransmitter noradrenaline release from sympathetic nerve terminals is augmented in the chronic hyperinsulinemic state. Our previous *in vivo* studies indicated that chronic hyperinsulinemia produced by fructose drinking resulted in potentiation of sympathetic nerve-mediated vasoconstrictor responses.^{10,11} Thus, it appears that the augmented pressor response in FDR mainly results from chronic hyperinsulinemia, which increases the sympathetic nerve activity and vasoreactivity of the blood vessels. Very important findings from the present study include the facts that the treatment of FDR with RJ for 8 weeks abolished the hyperinsulinemic state, resulting in significant attenuation of augmented vasoconstriction in response to PNS in the hyperinsulinemic state. Takatori *et al.*¹¹ reported that pioglitazone treatment ameliorated augmented adrenergic nerve-mediated vasoconstriction in FDR. Taken together, it is likely that RJ has the ability to improve altered adrenergic nerve function by inhibiting insulin resistance development.

The vasodilator response to PNS has been shown to be mediated by the CGRP-containing vasodilator nerve, since the response is blocked by CGRP (8–37), a CGRP receptor antagonist, and capsaicin, which causes CGRP depletion in CGRPergic nerves.^{21,22} Bolus injections of CGRP also induced concentration-dependent vasodilation, which has been shown to be mediated by postsynaptic CGRP receptors.²³ The present study showed that the vasodilator response to PNS in the mesenteric artery of FDR tends to be suppressed when compared with control rats. Our previous *in vivo* study showed that chronic hyperinsulinemia induced by fructose drinking resulted in a marked reduction of the vasodilatation mediated by CGRPergic nerve stimulation.^{10,11} The present findings that the CGRPergic nerve-mediated, but not exogenous CGRP-induced, vasodilator responses in FDR were smaller than those in control rats suggest that CGRPergic nerve activity, probably the transmitter (CGRP) release, is decreased in the mesenteric artery of FDR. In the present study, treatment of FDR with RJ restored the blunted CGRPergic nerve-mediated vasodilator response without affecting the exogenous CGRP-induced response. Previous *in vitro* studies reported that sympathetic adrenergic nerves and CGRPergic nerves reciprocally control the vascular tone of mesenteric resistance arteries in the rat.²⁴ The PNS-induced vasoconstriction in mesenteric arteries is considered to result from activation of both sympathetic nerves and CGRPergic

nerves, and to be attenuated by the simultaneous activation of CGRPergic nerves. Therefore, it is more likely that the restored vasoconstriction in response to PNS observed in the RJ-treated FDR results in part from the improved vasodilatation mediated by CGRPergic nerves.

In conclusion, the present study suggests that RJ is a food with health-promoting benefits, containing bioactive substances that improve not only insulin resistance, but also hypertension *via* indirectly vascular control dysfunction regulated by adrenergic and CGRPergic nerves in the hyperinsulinemic state. Additionally, the present study suggests that daily RJ intake would be effective to prevent the development of insulin resistance and hypertension.

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