Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats

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Resveratrol (RSV; 3,5,4′-trihydroxystilbene), a naturally occurring phytoalexin found in juice and red wines, has been reported to exert a variety of pharmacological effects. It has been shown to possess anticancer (18), anti-inflammatory (17), and antiplatelet properties (6). In purified or synthetic form, RSV has been shown to reduce the synthesis of lipids in rat liver (4), to inhibit the synthesis of eicosanoids in rat leukocytes (23), to interfere in arachidonate metabolism (36), to inhibit platelet activation and aggregation (5), to inhibit the activity of some protein kinases (19), to exert a strong inhibitory effect on reactive oxygen species produced by human polymorphonuclear leukocytes (39), and to have cardioprotective effects against ischemia-reperfusion injuries in rat hearts (15). RSV is believed to be the active ingredient in red wines that might provide answer to the “French paradox”, i.e., the apparent compatibility of a high-fat diet with a low incidence of coronary atherosclerosis (42). The beneficial effects of RSV on the pathogenesis of atherosclerosis have been attributed to its antioxidation and free radical scavenging properties, and in many experimental systems, RSV has been found to be a better antioxidant and free radical scavenger than α-tocopherol, a natural antioxidant of the human body (8–10).

DM is a metabolic disorder that is rapidly reaching epidemic proportions, and the World Health Organization has predicted that by 2025, 300 million people will be affected worldwide (3, 24). DM eventually leads to diseases of the coronary arteries and the cerebrovascular system, renal failure, blindness, neurological complications, and premature death (14, 29). Two forms of DM, type 1 and type 2, have been identified. Type 1 DM is primarily due to the autoimmune-mediated destruction of pancreatic β-cells of the islets, resulting in absolute insulin deficiency. People with type 1 DM must rely on exogenous insulin to prevent the development of ketoacidosis for survival. The incidence of type 1 DM is low relative to type 2 DM, which accounts for more than 90% of the DM cases globally. Type 2 DM is characterized by a failure of normal insulin levels to stimulate glucose uptake by tissue cells. People with type 2 DM are not dependent on exogenous insulin but may eventually become dependent because the pancreatic islet β-cells fail to compensate for insulin resistance.

In the present study, we show that pure RSV possesses significant hypoglycemic and hypolipidemic effects in STZ-DM and STZ-nicotinamide DM rats over a 14-day experimental period. RSV administration to these rats results in a dose-dependent lowering of the plasma glucose and lipid concentrations. RSV ameliorated the common DM symptoms
of decreased body weight, polyphagia, and polydipsia. RSV significantly decreased insulin secretion and delayed the onset of insulin resistance in STZ-nicotinamide DM rats. The mechanism underlying the insulinomimetic activity of RSV was explored and is discussed in this report.

MATERIALS AND METHODS

Materials. Synthetic RSV was purchased from Sigma Chemical (St Louis, MO). 2-Deoxy-D-[14C]glucose ([14C]2-DG; specific activity 302 mCi/mmol) and uridine diphospho-2-deoxy-D-[14C]glucose (UDP-[14C]2-DG; specific activity 325 mCi/mmol) were from Amer sham Biosciences (Buckinghamshire, UK).

Animals and cells. Male Sprague-Dawley (SD) rats ranging from 8–10 wk old were obtained from the National Laboratory Animal Center, Taipei, Taiwan. STZ-induced diabetic (STZ-DM) rats were used as the severe insulin-deficient diabetic model. STZ is widely used as a strong inducer of diabetes in the experimental animals (37). STZ selectively destroys the pancreatic cells that secrete insulin, inhibits synthases and the release of insulin, and produces DM (12). STZ-induced hyperglycemia has been described as a useful experimental model to study the activity of hypoglycemic agents (26).

STZ-DM was induced by intravenous injection of STZ (60 mg/kg body wt, Sigma Chemical) after an overnight fast. Seven days after STZ injection, rats with a plasma glucose concentration of 20 mmol/l or greater and symptoms of polyuria, polyphagia, and polydipsia were considered to have moderate insulin-deficient diabetes. The fasting plasma insulin levels in STZ-DM rats were 1.32 ± 0.6 pmol/l (n = 24) and were markedly lower than that of the normal rat (166.2 ± 4.3 pmol/l, n = 8). The insulin levels of the STZ-DM rats were not significantly changed after the administration of RSV. STZ-nicotinamide DM rats were used as the moderate insulin-deficient diabetic model. Combined administration of STZ and nicotinamide to rats leads to the development of a diabetic syndrome that is characterized by moderate and stable hyperglycemia and reduced pancreatic insulin stores (30). This syndrome appears closely related to human type 2 DM with respect to plasma glucose regulation in response to insulin, and these diabetic animals could be useful in testing hypoglycemic agents with potential insulinotropic action (43a).

STZ-nicotinamide DM was induced (30) in overnight-fasted animals by a single intravenous injection of STZ (60 mg/kg body wt), and nicotinamide (120 mg/kg body wt; Sigma Chemical) was administered intraperitoneally 15 min after STZ. Hyperglycemia was confirmed by elevated plasma glucose levels that were observed on days 3 and 7 after drug injection. STZ-nicotinamide DM rats exhibited a plasma glucose concentration of 13.2 ± 0.8 mmol/l and a fasting plasma insulin level of 95.0 ± 0.6 pmol/l (n = 24). All studies were carried out with animals 2 wk after the induction of diabetes.

The HepG2 cell line was obtained from ATCC (Bethesda, MD), and was maintained in DMEM-F12 (1:1, vol/vol) supplemented with 5% fetal bovine serum (FBS) under 5% CO₂,95% air in a humidified incubator.

Acute animal studies. Healthy adult SD, STZ-DM, and STZ-nicotinamide DM rats were starved overnight and divided into groups (n = 8). For measurement of plasma glucose, animals were orally fed with RSV at 0.5 mg/kg body wt three times a day at 8-h intervals. For blood sugar measurement, animals were fasted overnight, and blood samples were collected on days 0, 2, 4, 6, 8, 10, 12, and 14, and glucose and triglyceride (TG) levels were determined. Parallel groups of animals (n = 8) were fed with or without RSV at the same dose and duration but were not fasted. These animals were used specifically for the measurements of body weight change and food and water consumption throughout the entire experimental period.

Determination of plasma glucose. Animals were fasted overnight and anesthetized by pentobarbital sodium (30 mg/kg body wt ip). Blood samples (0.1 ml) were collected from the femoral vein using a chilled syringe that contained 10 IU heparin. The samples were centrifuged at 13,000 rpm for 3 min, and aliquot (15 μl) of plasma was added to 1.5 ml of a Glucose Kit Reagent (Biosystems, Barcelona, Spain) and incubated at 37°C in a water bath (Yamato-BT-25, Tokyo, Japan) for 10 min. Plasma glucose was determined by a glucose analyzer (Quik-Lab; Ames Division, Miles Laboratories, Elkhart, IN).

Determination of plasma TG. For the determination of plasma TG, 10-ml aliquots of plasma were enzymatically hydrolyzed with a TG analyzing kit (Fuji Film, Fujitsu, Japan), and the glycerol-3-phosphate formed was reacted with NAD⁺ to generate NADH. NADH was reduced with nitrotetrazolium, and the formazan liberated was measured with Dri-Chem 3000 analyzer (Fuji Film).

Determination of plasma insulin. Plasma insulin levels were measured by enzyme immunoassay of 25-μl aliquots of plasma with a Rat Insulin ELISA kit (Mrecodia, Uppsala, Sweden). During incubation, insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies, which were bound to the plastic surface of the microtitration well. The bound conjugate was detected by reaction with 3′,3′,5,5′-tetramethylbenzide. The reaction was stopped by adding acid to give a colorimetric end point that was read spectrophotometrically.

Measurement of glucose uptake. Glucose uptake was determined by the rate of [14C]2-DG uptake (28). Animals were killed by cervical dislocation, and the soleus muscle was quickly excised with a pair of scissors, dissected free of any adjoining connective tissue, blotted, and divided into long longitudinal strips (25–35 mg/strip). Muscle strips were placed in 3 ml of Krebs-Ringer bicarbonate buffer (KRB) (pH 7.4) containing 1 mmol/l glucose and 1% fatty acid-free bovine serum albumin (BSA) and aerated with 5% CO₂ in O₂ at 37°C for 30 min. The muscle tissue was further incubated for 30 min with RSV at specified concentrations and then pulsed with 50 μl of KRB containing [14C]2-DG (302 mCi/mmol; 1 μCi/ml) for 5 min in a shaking water bath under aeration. Glucose uptake was terminated by quick blotting followed by dissolving the muscle strips in 0.5 ml of 0.5 N NaOH for 45 min and neutralization with 0.5 ml of 0.5 N HCl. After centrifugation, 0.5 μl of the supernatant were mixed with 1 ml of aqueous counting scintillant, and the radioactivity was determined using a β-counter (Beckman LS-6000). Non-specific uptake of [14C]2-DG was assessed by incubating muscle strips with 20 μmol/l cytochalasin B (Sigma Chemical) to block cross-membrane transportation before the addition of [14C]2-DG. The non-specific value was subtracted from the experimental. Specific 2-DG uptake was expressed as the percent increase of [14C]2-DG uptake by soleus muscle without RSV treatment.
were collagenase under sterile conditions at 4°C (11). Cells thus obtained were prepared by perfusion of isolated liver with 0.01% type I using a centrifugation, 800 g, 5 min, and the adipocytes were filtered and washing three times in KRB. The packed cells were adjusted to a suitable dilution (10⁷ to 10⁸ cells/ml) for experimentation. After preincubation for 30 min, cells were incubated with RSV at the specified concentrations for 30 min, 50 μl of KRB containing 2-DG (1 μCi/ml) was added, and incubation was for 5 min at 37°C in the shaking water bath under aeration. Reactions were terminated by quick blotting and dissolving of tissues in 0.5 ml of 0.5 N NaOH for shaking water bath under aeration. Reactions were terminated by quick blotting and dissolving of tissues in 0.5 ml of 0.5 N NaOH for 45 min before neutralization with 0.5 ml of 0.5 N HCl. After centrifugation, 800 μl of supernatant were mixed with 1 ml of aqueous counting scintillant, and the radioactivity was determined using a β-counter (Beckman LS-6000).

**Measurement of glycogen synthesis in hepatocytes.** Hepatocytes were prepared by perfusion of isolated liver with 0.01% type I collagenase under sterile conditions at 4°C (11). Cells thus obtained were >90% viable, as judged by trypan blue exclusion. One to two milliliters of a cell suspension containing 40 to 80 mg of cell protein were incubated with specified concentrations of RSV for 30 min in KRB at 37°C. The cells were further incubated with UDP-[14C]2-DG (0.1 μCi/ml, 325 mCi/mmol) for 1 h under continuous shaking. Incorporation of UDP-[14C]2-DG into glycogen was determined by ethanol precipitation (7). The amount of UDP-[14C]2-DG incorporated into glycogen was expressed as nanomoles of UDP-[14C]2-DG per milligram of cell protein for 1 h or as the percent increase of UDP-[14C]2-DG uptake by hepatocytes without RSV preincubation.

**Statistical analysis.** Data are expressed as means ± SE. Repeated-measures analysis of variance was used to analyze the changes in plasma glucose and other parameters. The Dunnett range of post hoc comparisons was used to determine the source of significant differences where appropriate. A P value of ≤0.05 was considered to be significant.

**RESULTS**

Effect of RSV on the plasma glucose concentration in normal, STZ-DM, and STZ-nicotinamide DM rats. As shown in Fig. 1, a dose-dependent reduction of plasma glucose was observed in three animal groups after the oral RSV feeding at a dose range of 0.1–0.75 mg/kg body wt. Ninety minutes after RSV (0.5 mg/kg body wt) administration, a plasma glucose-lowering effect of 25.3 ± 4.2, 20.3 ± 3.3, and 33.5 ± 2.8% in STZ-DM, STZ-nicotinamide DM, and normal SD rats (n = 8), respectively, was observed. The maximal plasma glucose-lowering activity in all three animal groups was achieved at an RSV dose of 0.5 mg/kg body wt. An increase in dose to 0.75 mg RSV/kg body wt had no further effect. Thus the dose of 0.5 mg RSV/kg body wt was employed in the experiments presented. Ninety minutes after RSV treatment, the plasma glucose concentration was decreased from a basal level of 29.2 ± 2.8 to 21.1 ± 1.8 mmol/l in STZ-DM rats (n = 8), 13.3 ± 1.3 to 10.7 ± 1.2 mmol/l in STZ-nicotinamide DM rats (n = 8), and 4.2 ± 0.4 to 3.2 ± 0.2 mmol/l in normal SD rats (n = 8; Table 1). The hypoglycemic effects of RSV started to decline after 90 min, and by 5 h post-RSV feeding, the plasma glucose was returned to the original level (not shown).

Effect of RSV on the plasma glucose concentration in normal rat receiving an intravenous glucose challenge. The basal plasma glucose concentration in SD rats was 5.1 ± 2.2 mmol/l. Ninety minutes after RSV feeding (0.5 mg/kg body wt), it was decreased to 3.8 ± 0.5 mmol/l. The plasma glucose concentration in the vehicle control was 5.2 ± 0.3 mmol/l and was not significantly different from the basal level. Five minutes after intravenous glucose injection at 60 mg/kg body wt, the plasma glucose concentration was elevated to 17.3 ± 1.2 mmol/l in vehicle-treated rats and 12.3 ± 0.6 mmol/l in RSV-treated rats.

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<th>Table 1. Acute phase effect of RSV on plasma glucose concentrations in SD, STZ-DM, and STZ-nicotinamide DM rats</th>
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<td><strong>Blood Glucose Concentration, mmol/l</strong></td>
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Data are presented as means ± SE; n = 6 for each experimental group. RSV, resveratrol; SD, normal Sprague-Dawley; STZ, streptozotocin; DM, diabetes mellitus. Blood glucose was determined at 0, 30, 60, 90, and 120 min after RSV (0.5 mg/kg) administration. *P < 0.05; †P < 0.01 vs. respective vehicle-treated group.
The plasma glucose-lowering effect of RSV remained significant for 2 h after glucose injection compared with the vehicle-treated group.

Chronic administration of RSV ameliorates hyperglycemia in STZ-DM and STZ-nicotinamide DM rats. In this experiment, the plasma glucose levels of the untreated (vehicle) STZ-DM animals ranged from 29.4 ± 2.3 to 31.2 ± 2.2 mmol/l, whereas levels in STZ-nicotinamide DM animals ranged from 13.4 ± 2.1 to 13.6 ± 1.8 mmol/l. Fig. 3 shows that 14 days after the administration of RSV at 0.5 mg/kg body wt, plasma glucose levels were reduced by 22.3 ± 3.2 and 14.8 ± 3.3% in STZ-DM and STZ-nicotinamide DM rats, respectively (n = 8), compared with the untreated counterpart. In addition, in the STZ-nicotinamide DM rats, the plasma insulin concentration was significantly reduced by RSV treatment (Fig. 4).

RSV exhibits a hypolipidemic effect in STZ-DM and STZ-nicotinamide DM rats. RSV administration reduced the plasma TG levels in both STZ-DM and STZ-nicotinamide DM rats. Significant reduction of plasma TG in STZ-DM rats was observed on day 4 of RSV administration, and a 50.2 ± 3.2% reduction (n = 8) was observed on day 14 compared with untreated control animals (Fig. 5). In STZ-nicotinamide DM rats, significant reduction of plasma TG was observed on day 6 of RSV administration, and a 33.3 ± 2.2% reduction (n = 8) was observed on day 14 compared with that of the control animals (Fig. 5).

RSV ameliorates general diabetic symptoms in STZ-DM rats. STZ-DM rats were treated with or without RSV as described for 14 days, and body weight changes were recorded daily for each animal in each experimental group. Figure 6A shows that the average body weight of the untreated (vehicle) animals decreased with time throughout the entire experimental period. In contrast, the body weight of the RSV-treated animals was moderately increased (Fig. 6A).
also reduced water and food (Fig. 6, B and C) intakes compared with the untreated STZ-DM rats.

**Effect of RSV on glucose uptake in insulin-responsive tissues**

Soleus muscle strips, hepatocytes, and adipocytes prepared from STZ-DM rats were treated with or without RSV for 30 min as indicated. As shown in Fig. 7A, RSV pretreatment resulted in a dose-dependent stimulation of \([^{14}\text{C}]2\)-DG uptake by the muscle strips, and a twofold stimulation was observed at an RSV concentration of \(10^{-6}\) M. The glucose uptake by hepatocytes and adipocytes was stimulated by more than 2- and 2.5-fold, respectively, at these RSV concentrations (Fig. 7, B and C). To see whether RSV also promotes glucose uptake by insulin-sensitive human cells, confluent HepG2 cultures were incubated with specified concentrations of RSV for 30 min. After incubation, RSV was removed by medium replacement. \([^{14}\text{C}]2\)-DG was then added at 0.2 μCi/ml, and the uptake was allowed to proceed for 10 min. As shown in Fig. 8, RSV alone exerted a dose-dependent stimulation of glucose uptake. At the optimal effective dose of RSV, the glucose uptake was further
stimulated by the addition of insulin (0.17 μM). It appeared that RSV and insulin together exerted an additive stimulation. RSV promotes glycogen synthesis by hepatocytes. Hepatocytes isolated from STZ-DM rat were treated with or without different doses of RSV for 30 min. Glycogen synthesis was measured by the ethanol precipitable UDP-[14C]2-DG radioactivity. The incorporation of UDP-[14C]2-DG into glycogen by hepatocytes was promoted by RSV pretreatment, and at an RSV dose range of 1.0–10 μM, a nearly twofold stimulation of glycogen synthesis was observed (Fig. 9).

**DISCUSSION**

In the present study, we showed that RSV possesses hypoglycemic and hypolipidemic activities in both STZ-DM and STZ-nicotinamide DM rats. The STZ-DM rats in our hands were characterized by hyperglycemia (Fig. 3), hyperlipidemia (Fig. 5), very low to undetectable plasma insulin levels, polyphagia, polydipsia, and growth arrest (weight loss). Administration of RSV at 0.5 mg/kg body wt is associated with a moderate, yet persistent, improvement of the hyperglycemic and hyperlipidemic conditions over an experimental period of 14 days. The administration of RSV was also associated with a prominent improvement of the polyphagia and polydipsia conditions exhibited by STZ-DM rats. Most significantly, the administration of RSV alleviated the body weight loss of the STZ-DM rats (Fig. 6A).

The hypoglycemic and hypolipidemic effects of RSV administration seen in STZ-DM rats may be explained by a reduced food intake and/or a reduced digestion and absorption of the foods by the gastrointestinal system. This possibility can be ruled out by the acute phase experiment, which showed that the hypoglycemic effect was observed within 1 to 2 h after the first RSV administration (Table 1). The reduction in the food and water intake to nearly normal levels observed in RSV-treated STZ-DM was probably due to improved glucose and lipid metabolism. The most striking effect was that the administration of RSV reversed body weight loss to a moderate body weight gain in STZ-DM rats during the 14-day experimental period. The resumption of moderate body growth in STZ-DM rats by RSV treatment strongly suggests that sugar and lipid metabolism in these animals is improved.

The diabetic syndrome in rats administered STZ and nicotinamide is characterized by stable, moderate hyperglycemia; glucose intolerance; and altered but significant glucose-stimulated insulin secretion (30). Administration of RSV at 0.5 mg/kg body wt to STZ-nicotinamide DM rats resulted in a 14.3 ± 3.3% decline in the blood glucose levels (Fig. 3). It was of interest to note that RSV administration also caused a 20 to 40% reduction in the plasma insulin level (Fig. 4). The reduced plasma insulin level is probably secondary to the reduced plasma glucose level caused by RSV. However, it is also possible that insulin sensitivity is improved by RSV. This
insulin lowering effect may help delay the onset of insulin resistance in STZ-nicotinamide DM rats. Our result suggests that the hypoglycemic activity of RSV is not due to a stimulation of insulin secretion. Therefore, the RSV effect dose not seem to impose any additional load on pancreatic β-cells. Furthermore, because the metabolic effects of RSV observed in the present study do not rely on the presence of insulin, these observations are compatible with the assumption that the hypoglycemic effect is brought about by an additional pancreatic mechanism.

The most common lipid abnormalities in diabetes are hypertension and hypercholesterolemia (21, 32). Hypertriglyceridemia is also associated with the metabolic consequences of hypercoagulability, hyperinsulinemia, insulin resistance, and glucose intolerance (13). In our study, administration of RSV to STZ-DM and STZ-nicotinamide DM rats significantly improved some of these parameters. The observed hypolipidemic effect by RSV may be due to increased plasma lipid uptake by the liver and adipose tissue or by decreased hepatic cholestero genesis and fatty acid synthesis.

The hypoglycemic effect of RSV was moderate, and there was a mild, twofold stimulation of glucose uptake and glycogen synthesis in insulin-sensitive tissues. The hypolipidemic effect appeared to be more prominent where a reduction of more than 50% was observed. However, the overall 14-day effect of RSV administration in the amelioration of common DM symptoms is of great significance and suggests that mechanisms other than hypoglycemic and hypolipidemic may be involved.

Previous work by others has shown that monomeric and oligomeric forms of flavonoids exhibit hypoglycemic activity in animal models. The hypoglycemic activity of epicatechin was due to induction of pancreatic β-cell regeneration (22), and the hypoglycemic activity of catechin was due to inhibition of intestinal glucose absorption (41), whereas the hypoglycemic activity of epigallocatechin was due to the increase of hepatic glycogen synthesis (20, 45). The oligomeric flavonoid procianidins were found to lower blood glucose by an insulin-like effect on insulin-sensitive tissues and a delay in intestinal glucose absorption (35). Other studies have shown that wine flavonoids improve the altered oxidative stress associated with the diabetes (25). Epigallocatechin has been shown to exert its insulin-like effect on hepatocytes by changing the redox state that affects the functional states of some intracellular insulin-signaling mediators (45). RSV is a more powerful antioxidant than α-tocopherol in preventing lipid oxidation (10) and in scavenging reactive oxygen species (16). RSV also inhibits cyclooxygenase (31) and lipoxygenase (43) activities and thereby alters the production of inflammatory lipid mediators. Whether the RSV effects observed in the present study are mediated through its free radical scavenging and anti-inflammatory properties is presently not clear.

The present study also shows that at the maximal dose of RSV for the stimulation of glucose uptake by HepG2 cells, the addition of insulin resulted in a further stimulation, and the effects appeared to be additive (Fig. 8). Because RSV is a strong antioxidant, it is possible that RSV acts by changing the oxidative stress of the diabetic tissues and thus improves the functional states of the metabolic machinery of the cells. It is also possible that the improved cellular redox status helps maintain the normal function of the mediators involved in insulin signaling. In this way, the insulin-like effect of RSV could be insulin-related or insulin-unrelated. In our STZ-induced diabetic rats, the plasma insulin levels were not altered by RSV administration, suggesting that the RSV effect was not due to stimulation of insulin secretion by the residual β-cells. Taken together, our results strongly suggest that RSV does not use insulin-signaling mechanisms, at least in the HepG2 cells, for the stimulation on glucose uptake. The exact mechanism underlying the insulin-like activity of the RSV awaits further investigation.

REFERENCES

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