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ORIGINAL

Resveratrol prevents streptozotocin-induced diabetes by inhibiting the apoptosis of pancreatic β-cell and the cleavage of poly(ADP-ribose) polymerase

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Abstract. Resveratrol (3,5,4′-trihydroxystilbene; RSV) is one kind of polyphenolic phytoalexin that has many effects on metabolic diseases. This study aimed to evaluate the protective effect of RSV pretreatment on β-cell. Male Sprague Dawley rats weighing 200-230 g were divided into 4 groups: (1) RSV; (2) streptozotocin (STZ, 70 mg/kg, intraperitoneally); (3) STZ after 7 days pretreatment with RSV; and (4) STZ pretreated with nicotinamide. Fasting glucose concentration was measured and an intraperitoneal glucose tolerance test was performed 72 h after STZ injection to determine the diabetic condition. The pancreas was removed 3, 6, 36, and 48 h after STZ injection. STZ induced diabetes in all rats not given RSV pretreatment, whereas none of the RSV-pretreated rats developed diabetes. Pretreatment with RSV inhibited apoptosis and reduced the activation of caspase-3 and poly(ADP-ribose) polymerase (PARP). However, expression of the total length PARP was not affected by pretreatment. Our findings suggest that RSV protects β-cells from STZ simultaneously with inhibiting the activation of PARP.

Key words: Resveratrol, Pancreatic β-cell, Poly(ADP-ribose) polymerase, Streptozotocin

STREPTOZOTOCIN (STZ) is used widely in experimental animal models of type 1 diabetes. STZ is a β-cell-specific toxin that induces irreversible and rapid apoptosis of pancreatic β-cells. The generation of essential downstream molecules of reactive oxygen species and activation of poly(ADP-ribose) polymerase (PARP) is involved in STZ-induced apoptosis of β-cells [1]. PARP-null mice do not develop diabetes after treatment with STZ [1]. Activation of PARP is involved in multiple diabetic complications such as diabetic neuropathy, nephropathy, and retinopathy [2]. Several natural substances, including antioxidants and PARP inhibitors, have been reported to protect against the initiation of diabetes [3-5].

Resveratrol (3,5,4′-trihydroxystilbene; RSV), a type of polyphenolic phytoalexin, is contained abundantly in red wine [6]. Many studies have shown that RSV has a wide variety of benefits in preventing illnesses such as cardiovascular disease and ischemic injuries, cancer, and in prolonging the lifespan of various organisms [7-10]. One report revealed that RSV has antihyperglycemic and antihypertriglyceridemic effects in diabetic rats [11]. Although the beneficial effects of RSV on glucose homeostasis have been reported, it is unknown whether RSV has a protective effect against β-cell-specific cytotoxic agents.

The aim of this study was to evaluate whether RSV can prevent the destruction of pancreatic β-cells by STZ. We were also interested in the mechanisms responsible for this protective effect of RSV on β-cells.

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Materials and Methods

Animals and treatment management

Male Sprague Dawley (SD) rats (Orient Bio Inc., Kyungido, Korea), 7 weeks of age and weighing 200

to 230 g, were housed in an animal room controlled at 23 ± 2°C and 55 ± 5% room humidity, under a 12 h light/12 h dark cycle and given standard rat chow and tap water *ad libitum*. Animals were divided into 4 groups: (1) STZ injection (STZ, n = 5); (2) STZ injection after pretreatment with RSV (RSV+STZ, n = 10); (3) RSV treatment alone (RSV, n = 9); and (4) STZ injection after pretreatment with nicotinamide (NA) (NA+STZ, n = 5). RSV (LG Life Science Ltd, Seoul, Korea) was resolved in 100% ethanol to make a stock solution. Stock solution was dissolved in distilled water and given intraperitoneally from 8 weeks of age at dose of 15 mg/kg twice a day (total 30 mg/kg/day) for one week and then 1 h before STZ injection. NA (300 mg/kg, Sigma-Aldrich Co., St. Louis, MO) was injected intraperitoneally 15 min before STZ injection. STZ (70 mg/kg, Sigma-Aldrich Co.) was injected intraperitoneally after 12 h fast. Blood glucose concentration was measured in blood obtained *via* tail vein laceration using glucometer (Accu-Check, Roche Diagnostics, Indianapolis, IN). Diabetes was defined as blood glucose concentration > 300 mg/dL at 3 days after STZ injection. An intraperitoneal glucose tolerance test (IPGTT, 2 g/kg) was performed at 72 h after STZ injection after 12 h fast. Animals were sacrificed at 3, 6, 12, 24, 48, and 72 h after STZ injection, and the pancreas was fixed in neutral buffered formalin and processed into paraffin blocks. Serum (fasting) was obtained at 72 h and stored at –80°C until insulin concentration was measured using rat insulin radioimmunoassay kit (Linco Research Inc., St. Charles, MO). Animal experiments and protocol were approved by the Institutional Animal Care and Use Committees of Yonsei University, College of Medicine.

Rat islets isolation

Islets were isolated from the male 8 weeks old SD rats, weighing 250 to 280 g by a collagenase digestion technique and discontinuous Ficoll density gradient centrifugation [12]. The main common bile duct was exposed and clamped at both ends. Ten milliliters of collagenase *P* (Roche Applied Science) solution (1 g/L, pH 7.8) was injected into the duct and the distended pancreas was removed, and incubated at 38°C for 15 min. The digested gland was shaken for 10 s and the digestion was stopped by Hank's solution (4°C) with 100 mL/L fetal calf serum (Gibco, BRL, USA). The tissue was filtered through a 600 μm screen, and then washed by Hank's solution twice. Islets were purified by centrifugation at 3000 r/min for 20 min on discontinuous Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradients. After several washes with Hank's solution, islets were suspended in RPMI-1640 medium (Gibco) containing 100 mL/L fetal calf serum (Gibco), 20 mmol/L HEPES (Sigma-Aldrich Co.), 100 kU/L of penicillin and 100 g/L of streptomycin at 37°C in a humidified atmosphere of 50 mL/L CO_2. Islets purity was assessed by dithizone (Sigma-Aldrich Co.) staining, and islets were counted and scored in size. An algorithm was used for the calculation of 150 μm diameter islet equivalent number.

Immunohistochemistry, immunofluorescence, and terminal deoxynucleotidyl transferase (TdT)-mediated UTP end-labeling (TUNEL) assays

Four-micrometer-thick sections of pancreas were deparaffinized in xylene and rehydrated with graded alcohol. Endogenous peroxidase activity was blocked by methanol containing 3% hydrogen peroxide for 15 min, and blocking solution with 10% goat serum was applied for 60 min. Slides were then incubated overnight at 4°C with primary antibodies including rabbit anti-PARP (1:200, Cell Signaling Technologies, Danvers, MA) or rabbit anti-cleaved PARP (1:100, Cell Signaling Technologies). After washing, biotinylated goat anti-rabbit immunoglobulin (1:100, Vector Laboratories, Burlingame, CA) and streptavidin–peroxidase conjugate (Dako, Kyoto, Japan) were added sequentially. Diaminobenzidine (DAB, Vector Laboratories) was used as chromogen, and counterstaining was performed using hematoxylin. To detect cleaved caspase-3, antigen retrieval was performed using citrate buffer. The primary antibody was rabbit anti-cleaved caspase-3 (1:500, Cell Signaling Technologies). After secondary antibody incubation, fluorescein isothiocyanate (FITC) (Vector Laboratories) was used for visualization. Nucleus was counterstained with propidium iodide (PI) (Vector Laboratories). For TUNEL assay, 4 μm sections of pancreas were analyzed using an apoptosis-specific staining kit (ApopTag, Chemicon International, Inc., Temecula, CA) according to the manufacturer's instructions.

Immunoblotting

After isolation of rat islets, RSV was pretreated at concentration of 30 μM for 12 hr and STZ was treated at concentration of 5 mM for 30 min. Immunoblotting was performed at 3 h, 6 h, 12 h, and 24 h after STZ treatment.

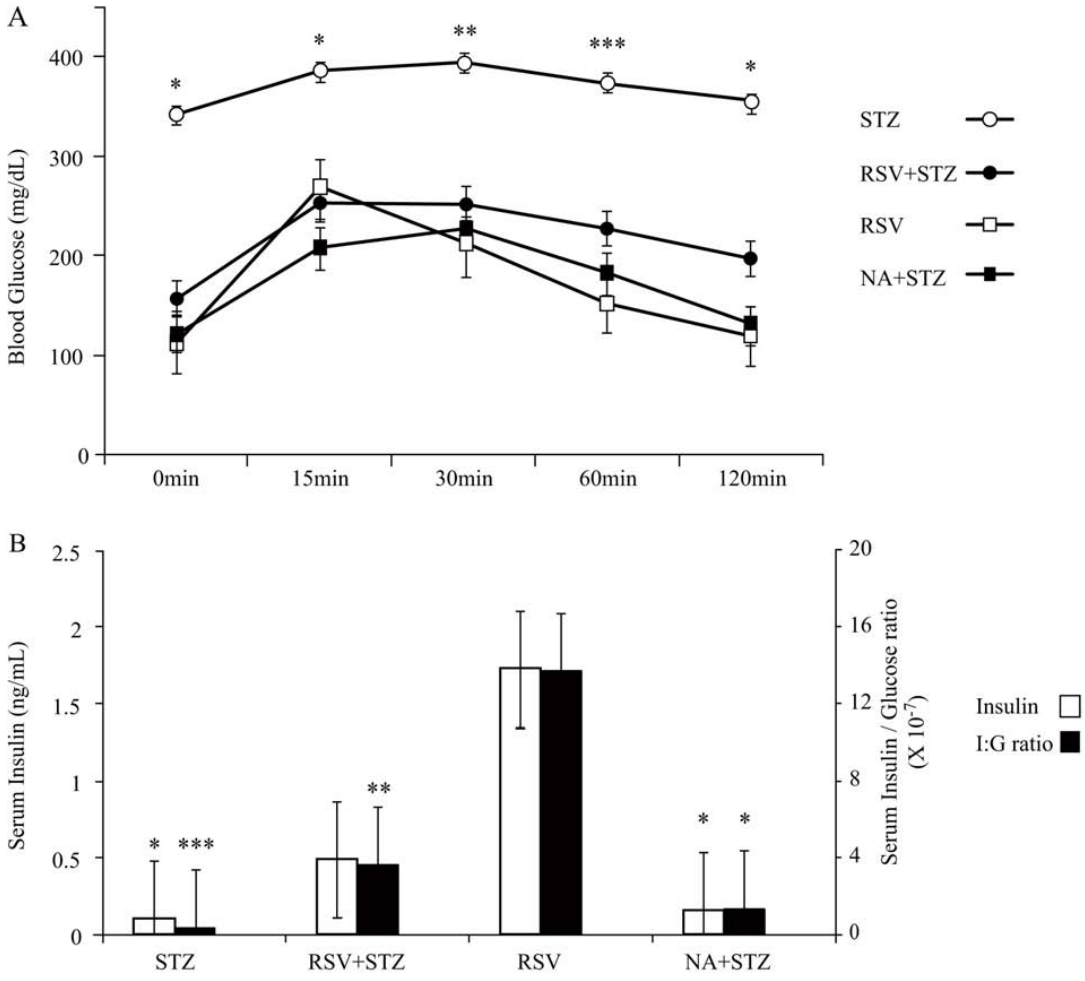

Fig. 1 A. Blood glucose level during the IPGTT (2 g/kg glucose) at 72 h after STZ injection. * $P < 0.05$ compared with all other groups. ** $P < 0.05$ compared with NA+STZ and RSV. *** $P < 0.05$ compared with RSV. B. Serum insulin level and insulin/fasting glucose ratio. * $P < 0.01$, ** $P < 0.05$ compared with RSV. *** $P < 0.01$ compared with all other groups. The bar indicates the standard error (Mann–Whitney U test).

Statistical analyses

Data were analyzed using a Mann–Whitney test. All statistical analyses were performed using SPSS (ver. 13.0 for Windows; SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant.

Results

RSV pretreatment prevents hyperglycemia in STZ-treated rats.

Before and during pretreatment with RSV, fasting glucose concentration did not differ significantly between the 4 groups (113 ± 7.2 mg/dL). STZ induced diabetes in all rats not pretreated, whereas diabetes did not develop in the rats pretreated with RSV or NA. In the IPGTT, the blood glucose levels were similar in the RSV+STZ, NA+STZ, and control RSV groups (Fig. 1A). The fasting serum insulin level was lower in the STZ group (0.10 ± 0.37 ng/mL) than in the RSV group (1.73 ± 0.37 ng/mL) (mean ± standard error) ($P < 0.01$) (Fig. 1B). Because blood glucose levels were higher in the STZ group than in the other groups, we calculated an insulin/glucose (I/G) ratio. The mean I/G ratio was lower in the STZ group than in all other groups ($P < 0.01$) (Fig. 1B).

RSV pretreatment inhibits pancreatic β-cell apoptosis.

Activation of caspase-3, the main executor of apoptosis, was detected at 3 h and 6 h after STZ injection, whereas its activation was not observed in RSV-

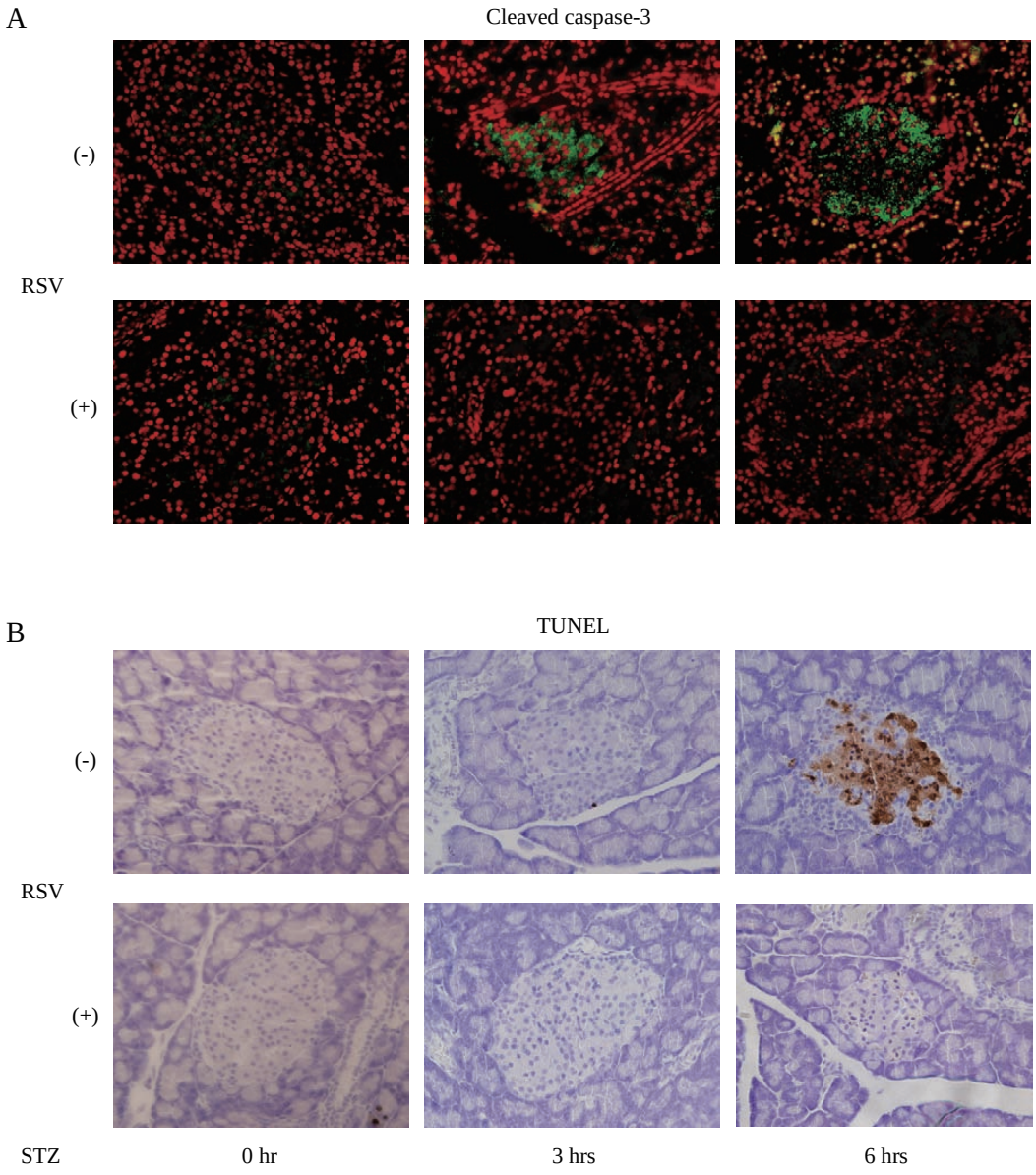

Fig. 2 Apoptosis of β–cells was inhibited by RSV pretreatment
A. Immunofluorescence staining for cleaved caspase-3. The nucleus was visualized with PI. B. TUNEL assay. Staining was performed at 3 and 6 h after STZ injection. All images were obtained at 400× magnification.

pretreated islets (Fig. 2A). The TUNEL assay showed that apoptosis of pancreatic β-cells was apparent at 6 h after STZ injection. RSV pretreatment inhibited β-cell apoptosis (Fig. 2B). At 24 h and 48 h after STZ injection, the number of TUNEL-positive cells and activation of caspase-3 were negligible (not shown). In Ki-67 staining, there was no difference between groups at 72 h after STZ injection (not shown).

RSV does not inhibit PARP expression but inhibits the cleavage of PARP.

Expression of PARP increased in both the STZ group and RSV+STZ group and became prominent at 6 h after STZ injection (Fig. 3A). Its expression decreased after then and disappeared at 48 h (not shown). In contrast, expression of cleaved PARP differed markedly between the 2 groups. The expression of cleaved PARP was more prominent in STZ group than in RSV+STZ

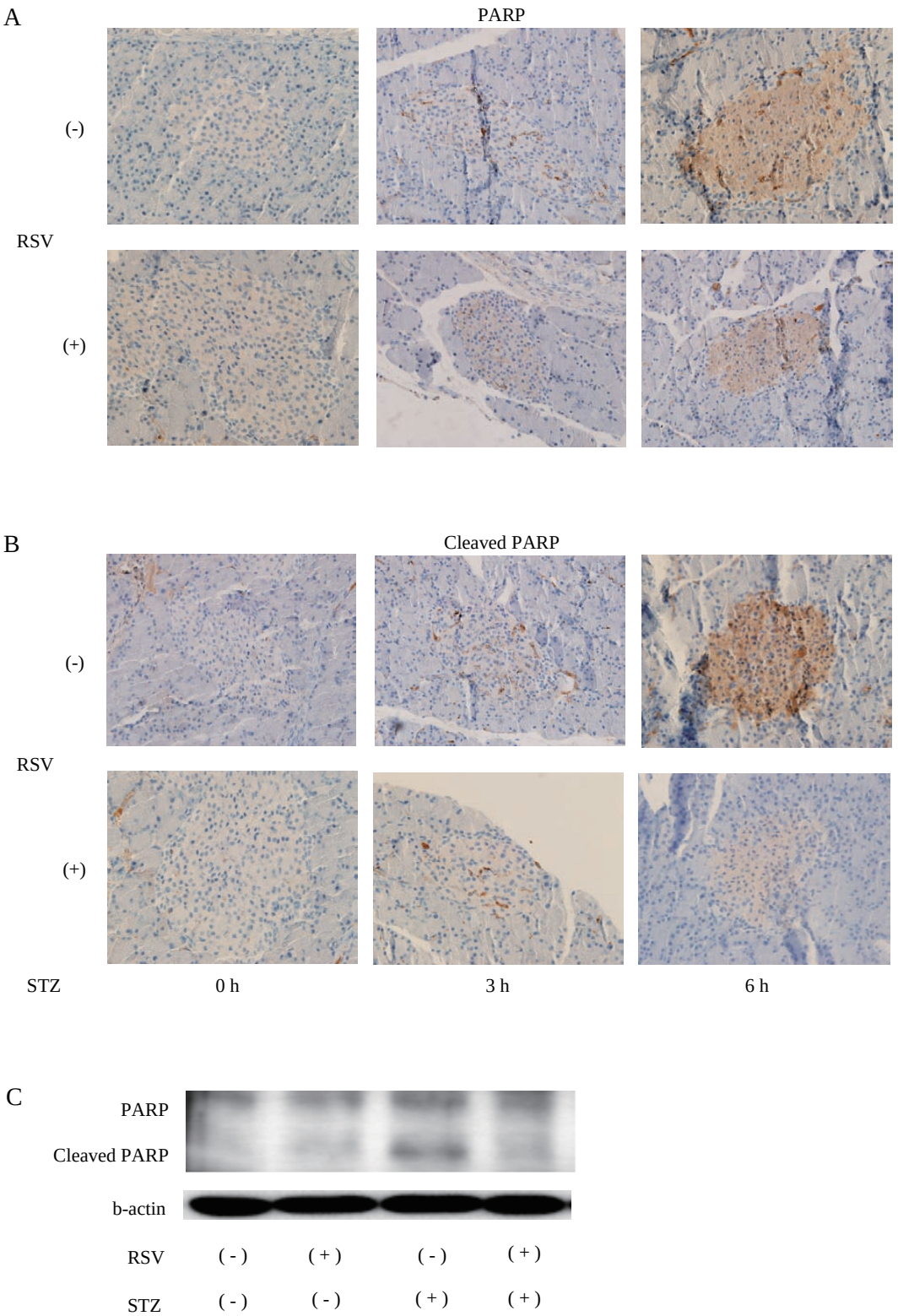

Fig. 3 Increased PARP expression and activation of PARP
A and B. Immunohistochemical staining for PARP and cleaved PARP. Staining was performed at 3 and 6 h after STZ injection. All images were obtained by 400× magnification. C. Western blotting for PARP and cleaved PARP. Images were obtained at 3 h after STZ treatment with or without pretreatment of RSV for 12 h.

group, indicating that RSV inhibited the cleavage of PARP (Fig. 3B and 3C). The ratio of stained cells for cleaved PARP to total islets significantly differed between the 2 groups at 6 h after STZ injection (0.72 ± 0.04 *vs*. 0.12 ± 0.02; $p<0.001$, Mann–Whitney test). At 3 h, 12 h, 24 h, and 48 h after STZ injection, the difference in the number of stained cells for cleaved PARP were minimized. In isolated islets, the expression of cleaved PARP was prominently increased at 3 h after STZ treatment. The expression of cleaved PARP was negligible at the other time in isolated islets, including 6 h, 12 h, and 24 h after STZ treatment. The pretreatment of RSV prevented the expression of cleaved PARP at all time after STZ treatment, even at 3 h after STZ treatment.

Discussions and Conclusions

PARP activation plays a role in development of diabetes induced by STZ [13], which is used in animal models of type 1 diabetes [14]. In the damaged pancreatic β-cell, PARP activation occurs through cleavage, and cleaved PARP repairs the damaged DNA with nicotinamide adenine dinucleotide$^+$ (NAD^+). When the amount of NAD^+ is exhausted, pancreatic β-cells are destroyed by apoptosis. NA provides sufficient NADH and acts as a weak PARP inhibitor, and is known to inhibit STZ-induced diabetes [15]. In our study, RSV inhibited the cleavage of PARP but did not reduce the expression of full-length PARP, indicating that RSV inhibits PARP cleavage.

RSV and NA have opposite effects as an activator and an inhibitor, respectively, of SIRT1 [16]. SIRT1 is an NAD^+-dependent deacetylase that combines cleaved NAD^+ and the deacetylated protein substrates to form NA, the deacetylated protein, and 2′-O-acetyl-ADP-ribose [17]. NA replacement modulates the catalytic activity and nicotinamide sensitivity of SIRT1, leading to the inhibition of SIRT1. Considering the analogous protective effect on pancreatic β-cells and the opposite effect on SIRT1, the protective effect of RSV on pancreatic β-cells may result from modulation of both PARP and SIRT1 (NAD^+), in contrast to the effect of NA, which results mostly from inhibition of PARP.

The activation of caspase-3 occurred at 3 h after STZ injection, and TUNEL-positive apoptotic cells appeared at 6 h. These results are similar to those reported by a previous study [18]. The expression of cleaved PARP was observed at 6 h after STZ injection in SD rats and at 3 h after STZ treatment in isolated islets. The different time in detecting the cleavage of PARP might be resulted from the diverse underlying conditions between *in vivo* and *in vitro*. In general, *in vitro* condition requires less time than that *in vivo* to achieve the effect of treated chemicals. In our study, pretreatment with RSV blocked the activation of caspase-3 and the cleavage of PARP. Due to the limitation of parallel observational experiment, the direct cause-effect relation between apoptosis and cleavage of PARP was not proved in this study. Further study modifying the cleavage of PARP would be needed to identify the direct protective mechanism of RSV in islets. However, as Kolb H. *et al*. reported that the cleavage of PARP is an essential downstream molecule for STZ induced apoptosis of pancreatic β-cell [1], the protective effect of RSV on pancreatic β-cell could be resulted from the inhibition of cleavage of PARP. We performed an *in vitro* study using INS-1E and MIN6 pancreatic insulinoma cell lines to investigate whether RSV has a similar protective effect on these cells. Interestingly, RSV alone activated PARP in these cells (not shown). In another study using human breast cell lines, RSV promotes the activation of caspase-3 and cleavage of PARP, which are followed by apoptosis [19]. This suggests that different mechanisms control the action of RSV according to the characteristics of the cells and underlying conditions. In *in vitro* study with human articular chondrocytes, Shakibaei M. *et al*. reported that RSV inhibited the cleavage of PARP *via* caspase-dependent pathway [20]. The underlying condition affecting the activity of caspase pathway could influence on the different role of RSV in the caspase-dependent cleavage of PARP.

In conclusion, RSV had a significant protective effect in pancreatic β-cells. Considering the approved role of RSV as a PARP inhibitor, it may be useful as a new therapeutic drug in the evaluation and management of diabetes.

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