Neurodegeneration in Streptozotocin-Induced Diabetic Rats Is Attenuated by Treatment with Resveratrol

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Key Words

Diabetic encephalopathy · Resveratrol · Astrocyte · Neurodegeneration

Abstract

Aim: Diabetes mellitus-associated hyperglycemia and oxidative stress have been shown to have detrimental effects on the brain and may lead to impairment of cognitive functions. Resveratrol (Rsv), a polyphenolic antioxidant, has been shown to have moderate hypoglycemic and prominent hypolipidemic effects in diabetic rats. In the present study, we examined if Rsv improves the diabetic encephalopathy and explored its possible underlying mechanisms. **Methods:** Male SD rats were treated with streptozotocin (65 mg/kg), and the diabetic rats were orally fed with Rsv (0.75 mg/kg, every 8 h) or normal saline for 4 weeks. Animals were then sacrificed and the brain tissues (hippocampus) processed for biochemical and histological studies. **Results:** Neurodegeneration and astrocytic activation were noted in the hippocampus of the diabetic rats. Tumor necrosis factor- α , IL-6 transcripts and nuclear factor- κ B expression were increased in the brain. In addition, neuropathic alterations in the hippocampus were evident in diabetic rats, including increased blood vessel permeability and VEGF expression, decreased mitochondrial number and AMP-activated protein kinase

activity. In Rsv-treated diabetic rats, the aforementioned abnormalities were all attenuated. **Conclusion:** These observations suggest that Rsv significantly attenuated neurodegeneration and astrocytic activation in the hippocampus of diabetic rats. Our results suggested that Rsv could potentially be a new therapeutic agent for diabetic encephalopathy and neurodegeneration.

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Introduction

Increasing evidence has indicated that uncontrolled diabetes mellitus (DM) can lead to severe complications of peripheral and central nervous systems [1, 2]. Epidemiologic studies suggested that diabetes is a risk factor for brain degeneration and cerebrovascular disease [3]. Brain autopsy studies have shown widespread angiopathy and degenerative abnormalities in the type1 DM patients [4]. Animal studies have also reported cognitive deficits in both type 1 and type 2 DM, particularly in the areas of learning and memory [5, 6]. The pathological brain damages seen in DM patients have been termed diabetic encephalopathy [7].

The development of diabetic encephalopathy is correlated with persistent hyperglycemia and generation of re-

active oxygen species, which cause cerebral angiopathy as well as abnormalities of neurons and glials in the brain tissue [8–10]. Astrocytes, the main type of glial cells, are thought to be critical for the maintenance of water and electrolyte balance, blood-brain barrier (BBB) function, and modulation of immune and inflammatory responses in the brain [11–16]. Under brain injury conditions, astrocytes were shown to be activated and secreted inflammatory cytokines to regulate the inflammatory reactions in the brain. Mitogen-associated protein kinases (MAPK) were also shown to be activated as part of the stress responses [17–19].

Resveratrol (Rsv), a polyphenol phytoalexin (*trans*-3,4,5-trihydroxystilbene), is abundantly present in a wide variety of plant species [20, 21]. Rsv has been reported to possess a host of biological effects due probably to its antioxidant nature [22–24] and its effect on energy metabolism. Studies with non-neural organs such as rat heart and kidney have demonstrated the ability of Rsv to reduce ischemia/reperfusion injuries [25, 26]. A recent study showed that Rsv possesses neuroprotective effect in injuries due to acute focal cerebral ischemia [27]. Tsai et al. [28] demonstrated that treatment with Rsv protects against oxidative stress generated during the brain ischemic insult. It has also been reported that chronic administration of Rsv to young adult rats significantly decreased neuronal damage caused by systemic injection of kainic acid [29]. The mechanism underlying the protective effect of Rsv in neural insults has been suggested to be related to its reactive oxygen species scavenging activity and/or its effect on the improvement of neural energy homeostasis by the activation of AMP-activated protein kinase (AMPK) signaling [30, 31]. Rsv has also been shown to ameliorate the impaired cognition found in rats following intracerebroventricular streptozotocin (STZ)-induced spatial memory deficit [32, 33].

In STZ-induced diabetic rats, it was shown that treatment with Rsv significantly reduced malondialdehyde, xanthine oxidase and nitric oxide production and increased glutathione levels in the brain [34]. However, little is known about whether Rsv can improve diabetic encephalopathy and, if this is the case, the nature of the underlying signals. In the present study, we investigated if Rsv attenuates DM-induced neurodegeneration, and explored its possible underlying mechanisms, particularly, the possible involvement of astrocytic activation. Because the hippocampus has been reported to be susceptible to hyperglycemic damage [35], we focused our study on the possible pathological changes of hippocampus in STZ-induced diabetic rats.

Materials and Methods

Animals and Reagents

Male Sprague-Dawley (SD) rats ranging from 8 to 10 weeks of age were obtained from the National Laboratory Animal Center, Taiwan. Rats were kept in an animal house at $22 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ relative humidity and maintained under a 12-hour light-dark cycle with food and water ad libitum. All experimental protocols were approved by the institutional Animal Ethic Committee, Chang Gung University. All efforts were made to minimize animal suffering and reduce the numbers of animal used. STZ, Rsv, pentobarbital, Evans blue, and Fluoro-Jade C were purchased from Sigma (St. Louis, Mo., USA). Enzymatic diagnostic kits were purchased from Randox (Crumlin Co., Antrim, UK). Plasma glucose test films were purchased from Fuji (Fujitsu, Japan). Nuclear and cytoplasmic extraction reagents were purchased from Pierce (Rockford, Ill., USA). Mouse monoclonal anti-GAPDH and glial fibrillary acidic protein (GFAP) antibodies were obtained from Millipore (Billerica, Mass., USA). Rabbit polyclonal anti-NF- κB antibodies were obtained from Upstate (Lake Placid, N.Y., USA). Rabbit polyclonal anti-ERK1/2, P38 and phospho-ERK1/2 (P-ERK1/2), phospho-p38 (P-p38) antibodies were obtained from Cell Signaling (Boston, Mass., USA). Rabbit polyclonal anti-AMPK, phospho-AMPK- α_1 and Lamin B₁ antibodies were obtained from Abcam (Cambridge, UK).

Experimental Protocols

DM was induced by a single injection of STZ (65 mg/kg BW) through the femoral vein to overnight-fasted rats as previously described [24, 36]. The age-matched normal rats received an equivalent volume of normal saline. One week after STZ injection, the blood samples were collected through the tail vein and the plasma glucose level was measured by plasma glucose test films and enzymatic diagnostic kits. Rats with plasma glucose levels ≥ 300 mg/dl and symptoms of polyuria, polyphagia and polydipsia were considered to be diabetic and used in the present study. STZ-DM rats were randomly divided into two groups ($n = 16$ in each group) and were treated without or with Rsv. Rsv was administered through gastric intubation at 0.75 mg/kg BW 3 times a day at 8-hour intervals for 4 weeks. Rsv was also administered in normal rats to evaluate its possible effects on normal brain. The age-matched normal rats and untreated DM rats were fed equal volumes of normal saline. Overnight fasted rats were anesthetized by pentobarbital sodium (65 mg/kg BW i.p.), blood samples (0.5 ml) were collected from the femoral vein using a chilled syringe containing 10 IU of heparin. The blood sample was centrifuged at 13,000 rpm, and the plasma was isolated and stored at -80°C for further use.

Plasma Biochemistry

Plasma glucose, triglyceride, cholesterol, fructosamine were measured using respective Enzymatic Diagnostic Kits. All the data were obtained from two independent measurements each with triplicate incubations.

Assessment of Neurodegeneration

Fluoro-Jade C staining and imaging analysis was performed as previously described [37]. Dried sections were dipped in 80% ethanol solution containing 1% sodium hydroxide for 5 min, 70% ethanol for 2 min and 0.06% potassium permanganate for 10 min. After rinsing with distilled water, sections were incubated with

0.0004% Fluoro-Jade C in 0.1% acetic acid for 20 min. Fluoro-Jade C staining was detected under a fluorescent microscope with excitation at 480 nm and emission at 525 nm. Images were acquired through a 20× objective, and Fluoro-Jade C-positive cells in the CA3 of hippocampus were counted with an image processing and analysis system (Q570IW, Leica, Germany).

Immunohistochemistry

Rats were anesthetized with pentobarbital sodium (65 mg/kg BW i.p.) and transcardially perfused with 0.9% saline followed by 4% ice-cold phosphate-buffered paraformaldehyde. The brain tissue was then removed and postfixed in 4% phosphate-buffered paraformaldehyde for 12 h, immersed sequentially in 20 and 30% sucrose solutions in 0.1 M phosphate buffer (pH 7.4) until the tissue sank. Twenty-five μ m coronal sections were cut using a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) at -2.5 to -4.5 mm from the bregma according to the atlas of rat brain [38] and stored at -20°C in cryoprotectant solution and were used for GFAP immunohistochemical staining and Fluoro-Jade C staining. For GFAP immunohistochemical staining, sections were incubated with 0.3% H_2O_2 for 30 min and placed in a blocking buffer containing 10% normal goat serum and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min at 37°C . The sections were then incubated with antibody against mouse monoclonal anti-GFAP (1:5,000) overnight at 4°C , followed by incubation with corresponding biotinylated secondary antibody (1:200, 1 h at 37°C) and avidin-biotin-peroxidase (1:200, 1 h at 37°C). Immunoreactivity was visualized with 0.05% DAB. Negative controls received the same treatment except that the primary antibodies was omitted and showed no specific staining.

Cell Count

Cell counting was performed in a double-blinded fashion. Six coronal sections (25 μ m thick at 250- μ m intervals) at the bregma level of -2.5 to -4.5 mm were carried out to identify the GFAP or Fluoro-Jade C-positive cells by immunohistochemistry. Positive cells number at CA1 and CA3 regions of hippocampus of each section were counted and estimated as previously described [39, 40]. In brief, the total cell number was estimated according to the formula: $[(S1 + S2)/2 + (S2 + S3)/2 + (S3 + S4)/2 + (S4 + S5)/2 + (S5 + S6)/2] \times 10$, where S1 to S6 represent, respectively, the cell number in section 1 to section 6. The coefficient 10 reflects the selection of one section (25 μ m thick) from 10 serials coronal sections (250- μ m intervals) for staining; 6 such sections were selected and stained with GFAP or Fluoro-Jade C in each animal.

Reverse Transcription-PCR

Total RNA was extracted using the guanidine isothiocyanate method according to Chomczynski and Sacchi [41]. 2 μ g of total RNA was reverse transcribed in a reaction mixture containing Moloney murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany), RNase Inhibitor rNasin[®] (Promega), dNTP master mix (Invitex, Berlin, Germany) and random hexamer primers (Promega). PCR was carried out in a reaction mixture containing Taq DNA polymerase (Promega), dNTP master mix (Invitex, Berlin, Germany) and the appropriate primers. Primers for rat tumor necrosis factor- α (TNF- α) mRNA were: forward, 5'-TCG AGT GAC AAG CCC GTA-3', reverse, 5'-CAG AGC AAT GAC TCC AAA GTA GAC-3'. Primers for rat IL-6 mRNA were forward, 5'-AAA ATC TGC TCT GGT CTT CTG-3',

reverse, 5'-GGT TTG CCG AGT AGA CCT CA-3'. β -Actin was used as internal control, the primers used were forward, 5'-TTC TAC AAT GAG CTG CGT GTG G-3', reverse, 5'-ATA CCC AGG AAG GAA GGCTGG AAG-3'. PCR products were separated electrophoretically on a 2% agarose gel. To ensure there was no genomic DNA contamination, RNA preparations were subjected to PCR amplification using primers for α -actin in the absence of reverse transcriptase. RNA samples used in the study were shown to generate no α -actin amplification product. Primers were designed using the Primer 3 software and synthesized by the Bioprotech Co. (Taipei, Taiwan).

Western Blotting

Four rats were selected randomly from each group, anesthetized with pentobarbital, and the hippocampus was procured and placed in ice-cold PBS. Samples were frozen in liquid nitrogen, homogenized, and the cytosolic and nuclear protein fractions were prepared using nuclear and cytoplasmic extraction reagent containing protease inhibitors. Cytosolic and nuclear proteins (30 μ g) were fractionated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes containing fractionated cytosolic proteins were blotted with anti-ERK1/2 (1:2,000), anti-P-ERK1/2 (1:1,000), anti-p38 (1:2,000), anti-P-p38 (1:1,000), anti-AMPK (1:1,000), anti-P-AMPK- α 1 (1:1,000), anti-VEGF (1:250) and anti-GAPDH (1:5,000) antibodies. Membranes containing fractionated nuclear proteins were blotted with anti-NF- κ B (1:1,000), and anti-Lamin B₁ (1:2,000) antibodies. Membranes were then blotted with horseradish peroxidase conjugated second antibody (1:5,000) and the immunoreactive protein bands were visualized by enhanced chemiluminescence. All Western blot experiments were carried out at least three times using protein preparations from three independently treated animal groups.

Evaluation of Blood-Brain Barrier Permeability with Evans Blue

Four rats were randomly selected from each group for BBB permeability assay at the end of RSV treatment. BBB permeability was evaluated using the Evans blue dye leakage method as previously described [42]. Briefly, Evans blue was dissolved in normal saline (20 mg/ml), sonicated for 3 min and filtered through a 5- μ m filter. Rats were anesthetized with pentobarbital (30 mg/kg BW i.p.), and the Evans blue solution was injected through the femoral vein at a dose of 20 mg/kg body weight. Sixty minutes after dye injection, the chest cavity was open and the left ventricle was cannulated. Each rat was perfused with PBS containing 1% paraformaldehyde (37°C , contained 10 IU/ml heparin) for 5 min at 150 mm Hg pressure to purge out the circulating dye. After perfusion, the bilateral hippocampus was removed immediately, rinsed in ice cold saline and the excessive saline was removed by adsorption with tissue paper. The tissues were weighed and homogenized in formamide (4:1 v/w). The homogenate was incubated at 60°C for 24 h and centrifuged at 70,000 rpm for 45 min at 4°C . The extractable Evans blue in the supernatant was quantitated by absorbance at 620 nm.

Electron Microscopy

Glutaraldehyde (3% in PBS)-fixed CA3 of the hippocampus was postfixed with 1% OsO_4 for 90 min, and dehydrated with graded ethanol. Samples were then embedded with Epon 812, and polymerized at 72°C for 48 h. Four ultrathin sections (50–70 nm)

Fig. 1. Effects of Rsv in the brain of normal SD rats. SD rats were fed with or without Rsv (0.75 mg/kg t.i.d.) for 4 weeks, and possible hippocampal neurodegeneration was evaluated by Fluoro-Jade C staining. Representative images are presented in **a**. Squares **ai** were magnified and are shown in **aii**, and squares in **aii** were further magnified and are shown in **aiii**. **b** Fluoro-Jade C-positive cells in the CA₃ field of the hippocampus were computed as described in 'Materials and Methods'. **c** Hippocampal astrocytic activation was

evaluated, and representative images of GFAP-positive cells in the hippocampus are shown. Squares marked with CA3 in **ci** were magnified and are shown in **cii**. Squares marked with CA1 in **ci** were magnified and are shown in **ciii**. Quantitation of the GFAP-positive cells in CA3 (**d**) and CA1 (**e**) areas of the hippocampus were performed as described in 'Materials and Methods'. NS = $p > 0.05$ between normal and normal + Rsv groups ($n = 8$).

were selected at every 1.0- μ m interval per rat ($n = 3$), stained with lead acetate and uranyl, and observed with a JEM1230 microscope (Tokyo, Japan). The images were captured with the Gatan image system (N.Y., USA). The number of mitochondria in the cell body of the neuron was computed.

Statistical Analysis

In table 1, data were expressed as mean $\pm$ SD. Other data were expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) was conducted for multiple-group comparisons and Tukey's post hoc analysis was used to evaluate the significance of the paired groups. In all analyses, $p < 0.05$ was considered to be significant.

Results

Effects of Rsv in the Normal SD Rats

To evaluate whether Rsv affects neurons of the hippocampus in normal SD rats, healthy, age-matched rats were fed with or without Rsv (0.75 mg/kg t.i.d.) for 4 weeks. Animals were sacrificed and the brain tissues were removed and serial coronal sections were prepared. Neurodegeneration in the hippocampus was evaluated with Fluoro-Jade C staining. As shown in figure 1a, b, Rsv treatment did not alter the Fluoro-Jade C staining pattern in the hippocampus of the healthy rats. Astrocyte activation in the hippocampus was also compared between normal rats fed with or without Rsv. The

Fig. 2. Rsv reduced the hippocampal neurodegeneration index in the diabetic rats. **a–i** Representative images of Fluoro-Jade C staining, squares in **a**, **c**, and **e** were magnified and are shown in **b**, **d**, and **f**, respectively. Squares in **b**, **d**, and **f** were magnified and are

shown in **g**, **h**, and **i**, respectively. **j** Fluoro-Jade C-positive cells in the CA3 field of the hippocampus were counted as described in 'Materials and Methods'. # $p < 0.01$ between normal and DM groups; * $p < 0.05$ between the DM and DM+Rsv groups ($n = 5$).

GFAP-positive cells in hippocampus were computed as described in 'Materials and Methods'. As shown in figure 1c–e, Rsv treatment did not affect the astrocytic activation in the CA1 and CA3 areas of hippocampus. The data suggested that Rsv administration exerted no obvious beneficial or adversary effect in normal rat brain.

Effects of Rsv on the Plasma Biochemical Parameters

Plasma glucose, triglyceride and fructosamine were measured as described, and the data are shown in table 1. Levels of plasma glucose, triglyceride and fructosamine were significantly elevated in diabetic rats compared with the age-matched normal rats. The levels of plasma triglyceride and fructosamine in Rsv-treated diabetic rats were significantly lower than that of the untreated diabetic rats. While the plasma glucose levels of the diabetic rats were moderately reduced by Rsv treatment.

Rsv Ameliorated the Neurodegeneration of Hippocampus in the Diabetic Rats

The metabolic disorders in the diabetic rats are hypothesized to lead to pathological changes in brain tissue, we therefore examined if there were signs of neurodegeneration in the hippocampus. The presence of degenerated

Table 1. Effects of Rsv on general metabolic parameters of the diabetic rats

	Normal (n = 16)	DM (n = 16)	DM+Rsv (n = 16)
Body weight, g	330±42	210±45 [‡]	224±39
24-Hour urine output, ml	24±6	148±16 [‡]	136±15
Plasma glucose, mg/dl	128±7.4	534±20.6 [‡]	489±11.38 [†]
Plasma triglyceride, mg/dl	40±6.9	213±58 [‡]	180±21.8 [†]
Plasma cholesterol, mg/dl	36±2.8	42±4.1	38±3.46
Plasma fructosamine mmol/l	0.45±0.037	2.1±0.22 [‡]	1.8±0.14 [†]

STZ-induced diabetic rats were fed with or without Rsv (0.75 mg/kg t.i.d.) for 4 weeks. Data are shown as mean ± SD. Student's t test and multiple comparisons with t test post hoc analysis of variance were used. [‡] $p < 0.01$ between normal and DM; [†] $p < 0.05$ between DM and DM+Rsv ($n = 16$).

neurons was examined by Fluoro-Jade C staining. As shown in figure 2, there were more positively stained neurons in the CA3 region of the hippocampus in diabetic rats ($3,235 \pm 502$) than in age-matched normal rats (142 ± 37). In Rsv-treated diabetic rats, the number was reduced ($2,111 \pm 386$, $p < 0.05$).

Fig. 3. Rsv attenuated astrocytic activation in the hippocampus of the diabetic rats. **a** Distribution of GFAP-positive cells in various brain regions in normal, diabetic and Rsv-treated diabetic rats. Cor = Cortex; Hi = hippocampus; Th = thalamus. Sections were selected at bregma -3.8 . **b** Representative microphotographs from

control rats, diabetic rats, and Rsv-treated diabetic rats are shown. **c** Quantitation of the GFAP-positive cells in the CA₃ (**c**) and CA1 (**d**) areas of the hippocampus. ## $p < 0.01$ between normal and DM groups ($n = 5$); * $p < 0.05$ between DM and DM+Rsv groups ($n = 5$).

Rsv Attenuated Astrocytic Activation in Hippocampus of Diabetic Rats

Astrocytic activation was analyzed by counting GFAP-positive cells in the rat brain. The data showed that GFAP immunoreactive cells were located mainly in the hippocampus region (fig. 3a). A total of 6 sections per animal taken from serial coronal sections at bregma -2.5 to -4.5 were prepared for microscopy. GFAP-positive cells in the CA1 and CA3 regions of the hippocampus were counted and computed as described in 'Materials and Methods'. As shown in figure 3b–d, GFAP-positive cells were significantly increased in diabetic rats compared with the age-matched normal rats. In the normal rats, the GFAP-positive cells in CA3 and CA1 were 970 ± 123 and 397.5 ± 31.6 , respectively. In the diabetic rats, the positive cells were increased to $11,343.75 \pm 290.5$ and $7,188 \pm 318.6$, respectively. Furthermore, the GFAP-pos-

itive cells in Rsv-treated diabetic rats were reduced to $7,075 \pm 432.2$ and $4,089 \pm 147.6$ in CA3 and CA1, respectively.

Rsv Attenuated Inflammation in the Hippocampus of the Diabetic Rats

Astrocytic activation is implicated in the inflammatory reaction during brain injury. To examine if the Rsv treatment exerted an anti-inflammatory effect, we analyzed the relative abundance of some pro-inflammatory cytokines such as IL-6 and TNF- α . As shown in figure 4a–c, the IL-6 and TNF- α transcripts of the diabetic rats were elevated by about 1.5- and 2.2-fold, respectively, over the normal control. In the hippocampus of the Rsv-treated diabetic rats, the levels of IL-6 and TNF- α transcripts were lower than those of untreated rats. Figure 4d shows that NF- κ B expression was also elevated in the hip-

Fig. 4. Rsv attenuated inflammation in the hippocampus of the diabetic rats. **a** Relative abundance of TNF- α and IL-6 transcripts assessed by RT-PCR. **b, c** Bar graphs of the relative expression of TNF- α and IL-6, quantified by densitometry from **a** (n = 4). **d** Relative abundance of NF- κ B in the hippocampus analyzed by Western blotting. # p < 0.05 between normal and DM groups; * p < 0.05 between DM and DM+Rsv groups (n = 4).

Fig. 5. Rsv attenuated MAPK activation in the hippocampus of the diabetic rats. Western blot analysis of total and phosphorylated ERK1/2 (**a**) and p38 (**b**) in the hippocampus of the diabetic rats with and without Rsv treatment. # p < 0.05 between normal and DM groups; * p < 0.05 between DM and DM+Rsv groups (n = 4).

pocampus of the diabetic rats and was reverted back to a level slightly below that of the normal rats upon Rsv treatment.

Rsv Attenuated MAPK Activation in the Hippocampus of Diabetic Rats

Oxidative stress has been shown to activate the MAPK pathway and was correlated with astrocytic activation. In the present study, we showed that the phosphorylation levels of ERK1/2 and p38 were higher in the hippocampus of diabetic rats, and the level of phospho-JNK was not

changed (data not shown). In Rsv-treated diabetic rats, the phosphorylation levels of ERK1/2 and p38 were reduced (fig. 5a, b).

BBB Function Was Preserved in Rsv-Treated Diabetic Rats

Astrocytes provide an important structural and functional component of the BBB, they play vital roles in maintaining homeostasis of the brain. In the present study, we found that the vascular permeability in the hippocampus of diabetic rats was significantly increased

Fig. 6. BBB function was preserved in the Rsv-treated diabetic rats. **a** Effects of Rsv treatment on the permeability of BBB in the hippocampus of diabetic rats. Evans blue leakage was used to evaluate BBB permeability as described in 'Materials and Methods'. **b** Relative expression of VEGF in the hippocampus of diabetic rats with and without Rsv treatment. Western blot analysis was performed as described in 'Materials and Methods'. [#] $p < 0.05$ between normal

and DM groups; ^{*} $p < 0.05$ between DM and DM+Rsv groups ($n = 4$). **c** Representative electron micrographs of the capillary in the hippocampus. Ultrathin sections of the hippocampus tissue were processed for electron microscopy as described in 'Materials and Methods'. The thickening of the capillary basement membrane in diabetic rats is prominent (indicated by asterisks). Et = Endothelial cell; Lu = lumen.

compared with the age-matched normal rats. In Rsv-treated diabetic rats, the BBB permeability was reduced to nearly that of the normal control (fig. 6a). Consistent with the increasing BBB permeability, the level of VEGF, an important regulator of capillary permeability and angiogenesis, was higher in the hippocampus of diabetic rats than that of the age-matched normal rats. Rsv treatment significantly down-regulated VEGF overexpression compared with the untreated diabetic rats (fig. 6b). Observation by electron microscopy found that the capillary basement membrane of the hippocampus was thickened in diabetic rats compared with the age-matched normal rats. Interestingly, the capillary basement membrane thickening was attenuated in Rsv-treated diabetic rats compared with the untreated diabetic rats by observations (fig. 6c).

Rsv Enhanced Mitochondrial Genesis in the Hippocampus of Diabetic Rats

Mitochondrial genesis was evaluated by the number of mitochondria in the cell body of neurons in the CA3 regions of the hippocampus. Ultrathin sections were prepared from each group and were mounted. Mitochondria of four random neurons in each grid were counted and the result is shown in figure 7a, b. The mean number of mitochondria in the cell body of the neuron was decreased in diabetic rats (12.6 ± 0.9) compared with the age-matched normal rats (31.3 ± 2.3). Treatment of the diabetic rats with Rsv moderately restored the number of mitochondria toward normal (19.4 ± 1.6). AMPK is a metabolic regulator that promotes insulin sensitivity and energy production. Its activity is correlated with phosphorylation at Thr172 of AMPK (P-AMPK) and Rsv has been shown to activate AMPK in

Fig. 7. Rsv enhanced mitochondrial genesis in the hippocampus of diabetic rats. **a** Representative electron micrographs of mitochondrial distribution in the neurons of the hippocampus. Nu = Nucleus; arrows indicate mitochondria. **b** Mitochondrial numbers in the cell body of the neuron were counted as described in ‘Materials

and Methods’. **c** Levels of AMPK phosphorylation analyzed by Western blotting and quantitated by densitometry. ^{##} $p < 0.01$ between normal and DM groups; ^{*} $p < 0.05$ between DM and DM+Rsv groups ($n = 4$).

cultured cells [31]. In the present study, we showed that P-AMPK level was reduced in the diabetic rats compared with the age-matched normal rats and was restored toward normal in Rsv-treated diabetic rats (fig. 7c).

Discussion

In the present study, we showed the presence of numerous degenerative neurons in the hippocampus of STZ-induced diabetic rats, and the predominant fields of neurodegeneration were located within CA3 area, as evidenced by Fluoro-Jade C staining.

Diabetes-related neurodegeneration was believed to be multicausal and hyperglycemia has been suggested to play important roles [43]. Hyperglycemia has been shown

to cause inflammation and oxidative stress in the brain. Evidence has accumulated indicating that inflammatory mediators, including TNF- α , cellular adherence molecules and C-reactive peptide were increased in the brain of both type 1 and type 2 DM [44–46]. These inflammatory factors could be derived from peripheral blood or be locally generated. In this regard, activation of astrocytes and microglia has been shown to elaborate inflammatory cytokines in the brain [47, 48]. In the present study, we showed that GFAP-positive astrocytes were significantly increased in the CA1 and CA3 areas of hippocampus in the diabetic rats, implying that astrocytes were activated under chronic hyperglycemic conditions and could thus affect the neuronal metabolism. Presumably, DM-induced astrocytes activation might be positively correlated with the severity of neurodegeneration in the hippocampus. This assumption appeared to be consistent with a

recent report by Tekkök et al. [49] which showed that CA1 of the hippocampus exhibited higher sensitivity to abnormal glucose metabolism in STZ-induced diabetic rats.

Moreover, under chronic hyperglycemia, endogenous TNF- α production was shown to be elevated in microvascular and neural tissues, which may cause increased microvascular permeability, hypercoagulability and nerve damage, and thus initiate and promote the development of characteristic diabetic microangiopathic polyneuropathy and encephalopathy [50, 51]. Our findings that TNF- α and IL-6 transcripts were upregulated, together with an increased translocation into the nucleus of NF- κ B, strongly suggested that the inflammatory cascade was initiated in the hippocampus of the diabetic rats. Interestingly, treatment of diabetic rats with Rsv significantly repressed TNF- α and IL-6 transcripts and the nuclear translocation of NF- κ B, suggesting an anti-inflammatory effect of Rsv in the brain. The anti-inflammatory effect of Rsv has been studied in many other organs, including liver, heart, lung and kidneys [52–56].

MAPK signal transduction has been thought to be associated with brain inflammation and gliosis in neurological disorders [57]. The activation of MAPK signaling is responsible for down-stream activation of inflammatory cytokines [57, 58]. Our findings showed that the levels of P-ERK1/2 and P-p38 in the hippocampus were higher in diabetic rats compared with age-matched normal rats. And in Rsv-treated diabetic rats, the levels of P-ERK1/2 and P-p38 were reduced. It was thus suggested that inhibition of the MAPK pathway could play an important role in the anti-inflammatory effect of Rsv on diabetic encephalopathy.

It has been proposed that structural damage and alteration to the permeability of the BBB are involved in diabetic encephalopathy [59, 60]. In the present study, we showed that 1 month after DM induction, permeability of the BBB was significantly increased, the basement membrane of the capillary was conspicuously thickened and the expression of VEGF in the hippocampus was elevated compared with age-matched normal rats. Numerous studies have suggested that VEGF is a potent factor causing increased vascular permeability [61, 62]. It could alter the expression and arrangement of tight junction proteins, such as ZO-1 and occludin, leading to increased BBB permeability [63, 64]. Interestingly, treatment of diabetic rats with Rsv reduced the permeability of the BBB in the hippocampus.

Diabetes and the accompanying hyperglycemia may induce oxidative stress by increasing ROS production or

impairing antioxidant defenses [65]. Mitochondria are the principal source of cellular ROS production, and impairment of mitochondrial function has been reported in diabetes [66, 67]. Rsv has been shown to improve energy expenditure through promotion of mitochondrial biogenesis and antioxidant activity [68]. Our study was the first to demonstrate that the number of mitochondria in the neurons in the CA3 area of the hippocampus was significantly decreased in diabetic rats compared with age-matched normal rats. Consistently, P-AMPK was also lower in the hippocampus of diabetic rats than that of age-matched normal rats. AMPK acts as an energy sensor of the cell and is a key regulator of mitochondrial biogenesis [69]. These results suggested that the aberrant energy utilization or metabolic abnormality occurred in the brain of diabetic rats. Importantly, Rsv treatment improved the mitochondrial biogenesis and elevated P-AMPK level in the hippocampus of diabetic rats. The improved energy metabolism was correlated with attenuated neurodegeneration in Rsv-treated diabetic rats.

The STZ-induced diabetic animal model is characterized by hyperglycemia and insulin deficit. Hyperglycemia-induced ROS generation in brain tissue has been implicated in the development of diabetic encephalopathy. Rsv has been shown to ameliorate diabetic encephalopathy, attributed to its antioxidation efficacy [70]. Moreover, insulin has been shown to exert a neurotrophic factor-like effect to improve neuron survival. Insulin deficit thus could be viewed as a risk factor to neurodegeneration. In this regard, Rsv has been shown to enhance sensitivity to insulin *in vivo* and *in vitro* [71]. More recently, Rsv has been shown to prevent memory deficit through increasing acetylcholinesterase activity in the brain of STZ-induced diabetic rats [72]. In the present study, we also evaluated the effects of Rsv on normal rats; our results indicate that Rsv administration exerted no detected effect on the normal rat brain, but led to a small decrease in plasma glucose (data not shown), which was consistent with our previous study [36]. The exact mechanisms of the Rsv effects on diabetic encephalopathy remain to be further unraveled, although our study results are mostly descriptive, we believe that based on our work, future experiments may be designed for mechanistic exploration.

In conclusion, the present study showed that treatment of diabetic rats with Rsv significantly attenuated astrocyte activation and neurodegeneration in the hippocampus. The inhibition of MAPK signaling by Rsv, the TNF- α and IL-6 transcripts and the nuclear translocation of NF- κ B were repressed, suggesting an anti-inflammatory effect of Rsv in the brain under DM. In addition, Rsv

treatment reduced the VEGF expression in the hippocampus of diabetic rats and attenuated the structural alterations to the BBB. Moreover, Rsv treatment improved mitochondrial biogenesis, which was associated with an increased level of P-AMPK in the hippocampus of diabetic rats. Taken together, our results suggested that RSV could potentially be a new therapeutic agent for diabetic encephalopathy and neurodegeneration.

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