SCIREA Journal of Clinical Medicine

http://www.scirea.org/journal/CM

October 14, 2016

Volume 1, Issue1, October 2016

A dietary polyphenol resveratrol acts to provide neuroprotection in recurrent stroke models by reducing energy requirements during ischemia

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Abstract

Polyphenol resveratrol (RSV) has been associated with Silent Information Regulator T1 (SIRT1) and AMP-activated protein kinase (AMPK) metabolic stress sensors and probably responds to the intracellular energy status. Our purpose is to investigate the neuroprotective effects of RSV and the association with SIRT1 and AMPK signalling in recurrent ischemia models. In this study, elderly male Wistar rats received a combination of two mild transient Middle Cerebral Artery Occlusions (tMCAO) as *in vivo* recurrent ischemic model. Primary cultured cortical neuronal cells subjected to combined oxygen–glucose deprivation (OGD) were used as *in vitro* recurrent ischemic model. RSV administration significantly reduced infarct volumes, improved behavioral deficits and protected neuronal cells from cell death in recurrent ischemic stroke models *in vivo* and *in vitro*. RSV treatments significantly increased the intracellular NAD^+/NADH ratio, AMPK and SIRT1activities, decreased energy assumption and restored cell energy ATP level. SIRT1 and AMPK inhibitors and specific siRNA for SIRT1 and AMPK significantly abrogated the neuroprotection induced by RSV. AMPK-siRNA and inhibitor decreased SIRT1 activities; however, SIRT1-siRNA and inhibitor had no impact on p-AMPK levels. These results indicated that the neuroprotective effects of RSV increased the intracellular NAD^+/NADH ratio as well as AMPK and SIRT1 activities, thereby reducing energy ATP requirements during ischemia. SIRT1 is a downstream target of p-AMPK signaling induced by RSV in recurrent ischemic stroke model.

Keyword(s): RSV Neuroprotection;Recurrent Stroke Model;SIRT1 and AMPK signaling; Intracellular NAD^+/NADH ratio; Intracellular ATP level

Introduction

Of patients with an ischemic stroke, about 23% are reported to have a prior transient ischemic attack (TIA) or minor stroke (Rothwell et al, 2005, Correia et al. 2006). More than half of these secondary strokes occurred within 48 hours of the initial attack (Chandratheva et al 2009). The occurrence of a TIA or minor stroke provides a distinct opportunity for timely intervention to help prevent or reduce damage from a potential second subsequent stroke (Johnston and Hill 2004; MacDougall et al. 2009; Spence, 2010 Spence, 2010).

Improved diet offers promise as a safe and effective way to reduce both stroke risk and injury. Resveratrol (RSV) (3,4',5-trihydroxystilbene) is a promising candidate (Baur et al.,2006). RSV is a dietary polyphenol found in a wide variety of common foods such as berries, nuts, grape skins and red wine, and is speculated to be responsible for the 'French Paradox' (Kopp, 1998). The number of studies aimed at identifying potential therapeutic roles of RSV in human health is growing because of its various beneficial biological effects, e.g., antioxidant, anti-inflammation, anti-aging, release of neurotransmitter and neuromodulator, modulate mitochondrial dysfunctions and control of cell cycle and apoptosis (Dal et al.,2011; Das et al.,2010; Li et al.,2010; Raval et al.,2006; Sakata et al.,2010; Shin et al.,2010; Tsai et al.,2007; Yang et al.,2010; Yousuf et al.,2009; Zhang et al.,2010). RSV has also been shown to mimic a calorie-restriction diet that extends lifespan and stress resistance by linking to the longevity gene SIRT1. SIRT1 is one member of the mammalian family of Sirtuins, a highly conserved family of NAD^+-dependent deacetylases that regulate cellular energy and lifespan in both lower organisms and mammals (Wood. et al., 2004; Pallàs et al., 2009). Studies have shown heightened SIRT1 activity diminishes oxidative stress-related decline of cardiac function, promotes restoration of intracellular energy balance and the resistance of neurons to a number of neurodegenerative diseases (Ferrara N et al., 2008; Pallàs et al.,2008, Murayama. et al.,2008).

AMPK is a key metabolic and stress sensor/effector that is activated under conditions of nutrient deprivation, hypoxia, oxidative stress, as well as vigorous exercise and dietary hormones (Turnley. et al, 1999). AMPK acts as an intracellular energy sensor (AMP:ATP ratio) and plays a pivotal role in maintaining energy balance within the cell (Carling, D. 2004; Hardie, D.G. 2007). Emerging evidence indicates that phosphorylation of AMPK (p-AMPK) is critical for preserving cellular energy and for mitochondrial biogenesis in response to energy deprivation (Suwa, M et

al 2003). Recently, it has been reported that AMPK also enhances the activity of SIRT1 which links with cellular energy balance (Finkel, T., et al 2009, Cantó C., et al 2009).

RSV has been suggested as a potential agent in cardioprotection (Penumathsa et al.,2009), and has been shown to reduce ischemic brain damage in animal models(Li et al.,2010; Huang et al.,2001; Raval et al.,2006; Sakata et al.,2010; Tsai et al.,2007; Yousuf et al.,2009). It is currently not known whether RSV might also be protective in a model of elderly recurrent stroke. Thus the major goal of the present study is to determine whether RSV treatments are effective in reducing neuronal damage following an initial stroke and prior to a recurrent stroke in elderly. This study will also investigate the neuroprotection of RSV associated with the AMPK and SIRT1 signaling pathways and cellular energy status in recurrent stroke models.

Materials and Methods:

Ethics statement and RSV treatment

Experiments were performed on elderly male Wistar rats at age of 19 to 21 months (Charles River, Wilmington, Del). All animals were handled in accordance with the guidelines provided by the Chinese Council on Animal Care, and experiments were approved by the ShanDong University and TianJin University Animal Care and Ethics Review Committees (Approved protocol RO8068). The experiments followed the international guidelines which laid down by the NIH in the US regarding the care and use of animals for experimental procedures.

A total of 58 elderly rats were used in this study. The mortality rate was 34.5%. Rats were placed in randomized groups treated with either a vehicle (a solution of 15-20 % Jello powder in water) or trans-RSV (Sigma-Aldrich, Oakville, Ont). A dose of 25mg/kg RSV was used in this study; based on earlier reports, RSV has demonstrated its neuroprotective property in MCAO models in doses ranging from 20 to 50 mg/kg (Sinha et al., 2002; D.K). 32 elderly rats were randomized to receive either a single mild (30 min tMCAO) or a recurrent mild (2 x 30 min tMCAO) stroke. Animals designated to receive a single mild stroke were pre-treated with RSV (25mg/kg p.o) or a vehicle daily for 3 days prior to the tMCAO injury. The behavioral test was performed on the second day of MCAO and continued daily for 3 days. Brain removal was performed at day 6 (Fig 1, A). Animals designated to receive recurrent stroke received first mild tMCAO at day 0, then

received RSV (25mg/kg p.o) or vehicle treatment daily for three days. A second stroke was performed at day 3 one hour after RSV administration, with the behavioral test and brain removal being performed on the same day with the single mild stroke animals (Fig 1, B). Among these animals, six rats were used to determine physiological variables.

Bioavailability of RSV in Blood, Brain and Liver

RSV levels in blood and tissue were assessed using high performance liquid chromatography (HPLC) in elderly rats treated with either RSV (25mg/kg n=3) or vehicle (n=3). Blood samples were obtained at various time points following treatment with either RSV or the vehicle as described above. After 2 hours of the fourth dose on day 3 of treatment, the brain and liver were dissected and the tissues of brain and liver were homogenized in RIPA buffer on ice. RSV concentration in supernatant was analyzed by Liquid Chromatography - Mass Spectrometry and Liquid Chromatography (LC/MS/MS) analysis. This was carried out with an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, USA) coupled with an API 4000 triple quadrupole Mass Spectrometer equipped with a Turbo-Ionspray Interface (AB Sciex, Concord, Ont., Canada).

Recurrent Stroke Model

Procedures for transient focal ischemia were achieved by placing a microaneurysm clip (Codman, size#1) on the middle cerebral artery through a small burr hole in the skull, as previously described (Qiao et al.,2009). The animal was anesthetized with isoflurane (4% induction, 2% maintenance). Rectal temperature was maintained using a servo-regulated overhead heating lamp at 37.0-37.5℃ during both surgery and recovery periods until the animal regained consciousness. The tail artery was cannulated with PE50 tubing to continuously monitor arterial blood pressure (MABP) and to obtain blood samples for determination of blood gas and glucose levels during the procedure. Both common carotid arteries were isolated and then an incision was made in the temporalis muscle. The skin and muscle were retracted to expose the skull. A small craniotomy was made at the point where the MCA (middle cerebral artery) crossed the rhinal fissure. Prior to removing the dura over the MCA, 2 additional burr holes were made to allow for Laser Doppler Flowmetry (LDF) determination of blood flow. One hole was made 3mm dorsal to the site of the MCA (referred to as proximal) and the other 3mm posterior to the first hole (referred to as distal). Once baseline blood flow measurements were obtained, the dura was removed to expose the MCA which was then subsequently occluded with the microaneurysm clip. Concurrently, both

common carotid arteries were occluded using vascular clamps. Blood flow reduction was confirmed with LDF. Animals were excluded if the criterion of 90% blood flow reduction in the core was not met. At the end of the 30 minute occlusion, the microclip was removed and the carotid artery clamps were removed.

For animals that were to undergo a second stroke, artificial dura (Gore Preclude MVP, Better Hospital Supplies Corp., Miami, FL) was placed over the exposed MCA to minimize adhesion to the vessel and fibrosis infiltration. All wounds were securely sutured and topical anesthetic was applied (Lidocaine, Sigma-Aldrich.com). Analgesia (0.03 mg/kg buprenorphine s.c, Sigma-Aldrich.com) was administered to minimize pain and animals were monitored closely during recovery. Three days later, the rats that were to receive a recurrent stroke underwent tMCAO in a similar manner as described above. The position of the second clipping of the MCA was immediately dorsal to the original clip position to minimize trauma to the vessel. Following surgery rats were given softened food in order to minimize weight loss.

Infarct Volume Measurements: Infarct volume was measured by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) Staining and magnetic resonance imaging (MRI) Scan.

TTC Staining: TTC staining was performed according to the procedure described previously (Lin. et al., 1993). Infarct volume was determined using an image analyzer (Axiovision LE 4.1, Carl Zeiss, Jena, Germany).

Magnetic Resonance Imaging (MRI): 3 days after MCAO, MRI scans were acquired using a 9.4-T/21-cm horizontal bore magnet (Magnex), an Avance console (Bruker, Germany) and a 3 cm quadrature coil according to the procedure previously mentioned (Qiao et al.,2009). In brief, T2 imaging was performed using a multi-echo spin-echo sequence with a TE = 10 ms between echoes for a total of 32 echoes (TR = 5000 ms). The field of view was 2.5 cm^2 and the data matrix was 256 × 128 for slices at a thickness of 1 mm through the cerebrum. Calculation of infarct volume was performed by the summation of the "infarct area of each of the slices in T2 images multiplied by Slice Thickness" using the software program Marevesi. A percentage of the infarct volume with respect to the contralateral normal hemisphere was taken for analysis.

Behavioral tests

Rat behavior was evaluated on a horizontal ladder task and cylinder task on the second day of single or double MCAO and continued daily for 3 days (Smith et al., 2007). Horizontal ladder task is designed to assess sensory–motor function. The ladder test consisted of a 115cm long horizontal ladder, with a distance of 3.5cm between successive rungs. The ladder was placed at a height of 3cm above the glass walkway. Sugar pellets were placed as a reward in a blacked out box at one end of the apparatus. Animals then ran across the apparatus three times per testing session with the number of paw-placement errors quantified each time from video tape. Error rate was determined as the percentage of slips made while traversing the variably spaced segment of the apparatus.

The cylinder task measures spontaneous limb preference during exploration of a vertical Plexiglas cylinder under red light conditions. To determine forelimb-use asymmetry, rats were placed in a transparent cylinder (20cm diameter and 30cm height); independent use of the left or right forelimb versus the simultaneous use of both forepaws was assessed for: (i) the initial contact of the cylinder wall a full rear; (ii) all weight bearing lateral movements along the wall. Limb use was assessed over 10 independent rear views.

Primary Neuronal Cell Culture

Primary cortical neurons were isolated from embryonic day 18 Wistar rat fetuses obtained from pregnant females using a previously described method with some modifications (Domoki.et al., 2009). After isolation, cortical cells were placed on poly-D-lysine-coated plates (BD Bioscience) at a density of ~100 cells/cm^2 and were maintained in growth medium consisting of neurobasal medium supplemented with B27 (2%, Gibco), L-glutamine (0.5 mM, Invitrogen), Pen-Strep (100IU, Invitrogen) and KCl (25 mM). Cultures were treated with cytosine β-D-arabinofuranoside (Ara-C, 10μ; Sigma-Aldrich) on days 3-6 to prevent the growth of astrocytes. This method resulted in culture observed to be ~99% pure neurons at day 7. Experiments were carried out on 6–10 day old cultures.

RSV Administration and Recurrent Oxygen-Glucose Deprivation (OGD) Model

The neuroprotective effects of RSV on neuronal cells were investigated in double OGD model. An intermediated dose of 0.5uM RSV was selected based on the dosing curve of RSV and peak

concentration in plasma in our pre-experiments. The dose of 0.5μM RSV is about the same as the peak level of 0.45μM measured in the plasma. Cortical neuronal cells were pre-treated with 0.5μM RSV or vehicle for 3 hours, then were exposed to OGD environment (glucose-free DMEM, 5% CO_2, <1% O_2 and 95% N_2) for 30 min followed by **re-oxygenation** for 3 hours under normal culture environment in regular DMEM media. After 3 hours **re-oxygenation**, the cells received a secondary OGD treatment for 30 min. Cell viability was measured by 0.4% Trypan blue staining.

SIRT1 Deacetylase Activity Assay and Western-Blot

SIRT1 deacetylase activity was evaluated in crude nuclear extract from penumbra cortex tissue 3 days after MCAO and cortical neuronal cells 3 hours after OGD. Penumbra cortex was determined by using hematoxylin–eosin (H&E) staining on the alternating cryosections (20μm). The morphological differences between necrosis and apoptosis regions were observed, the necrosis ischemia core was removed and the apoptosis penumbra region was kept. SIRT1 activity was measured using a deacetylase fluorometric assay kit (#CY-1151, CycLex, MBL International Corporation,) per manufacturer's instructions described previously (Ferrara et al.,2008). A standard calibration curve (RFU) was prepared with a known amount of the deacetylated standard included in the kit. All determinations were performed in triplicate on 6 different samples.

SIRT1 expression was tested by Western-blot assay. The samples of nuclear extract from penumbra cortex tissue and neuronal cells were separated by SDS–PAGE, and blotted using mouse anti-purified recombinant fragment of human SIRT1 monoclonal first antibodies which react with rat, human and money (1:1000) (ab104833, tested applications include WB, IHC-P, Flow Cyt, ICC/IF, ELISA. Abcam, Cambridge, MA, USA). Blots were developed with goat anti-mouse HRP-linked secondary antibodies (1:2000) (#115-035-003, Jackson ImmunoResearch,) and enhanced by chemiluminescence (GE healthcare Amersham). The intensities (density area) of individual bands were measured by densitometry (Model GS-700, Imaging Densitometer, Bio-Rad).

AMP Kinase activity Assay

AMP kinase activity was evaluated in protein extract from penumbra cortex tissue 3 days after MCAO and cortical neurons 3 hours after OGD reperfusion with or without RSV treatment.

Western blots and phosphorylation assays were performed as described previously (Liu et al., 2010). Rabbit Anti-phospho-AMPK (Thr-172) of human AMPKalpha monoclonal first antibodies which react with rat, mouse and human (1:1000) (#2535, tested application include WB, IP, IHC-P. Cell Signaling, Danvers, MA.) were used for immunoblotting to detect AMPK activity. The Blots were developed with goat anti-rabbit HRP-linked secondary antibodies (1:2000) (#111-035-003, Jackson ImmunoResearch). The intensity was measured in the same way as SIRT1, mentioned above.

Measurement of NAD^+/NADH Ratio: NAD^+/NADH were measured using the NAD^+/NADH Assay Kit according to the manufacture's protocol (ab65348, Abcam, www.abcam.com. Canada).

Measurement of ATP Levels

Intracellular ATP levels were measured in penumbra brain tissue 3 days after tMCAO and cortical neurons 3 hours after OGD exposure. The ATP content in the supernatants was determined using an ATP Bioluminescence Assay kit CLSII (#1699695, Roche Diagnostics, Barcelona, Spain), following the procedure indicated by the manufacture (Troyano et al., 2001). Results were normalized according to the protein content of the extracts.

The Effects of SIRT1 and AMPK inhibitors and specific siRNA on RSV neuroprotection

In order to demonstrate a direct correlation of the neuroprotection of RSV treatment to regulation of SIRT1 and AMPK, cortical neurons were exposed to the SIRT1 inhibitor Sirtinol 100 μM (Calbiochem, La Jolla, CA,USA) or the AMPK inhibitor Dorsomorphin dihydrochloride 20 μM (Tocris Bioscience.USA) overnight before being treated with 0.5μM RSV for 3 hours, then undergoing single or recurrent OGD insults. Cell viability was measured as described above.

In order to further confirm the direct correlation of protection by RSV treatment to regulation of SIRT1 and AMPK, cortical neurons were transfected with SIRT1-siRNA and AMPK-siRNA respectively. SIRT1 siRNA was synthesized by Eurogentec (Guarani et al.,2011) (5′ GAAGTTGACCTCCTCATTG 3′), and AMPK siRNA was purchased from B-Bridge International (5' GAGGAGAGCTATTTGATTA 3') (Nakano.et al.,2010). A scrambled siRNA (5′-TTCTCCGAACGTGGCACGA-3′) was used as a negative control for SIRT1, and siControl (B-Bridge International) was used as a negative control for AMPK. Cells were transfected with the indicated siRNAs (50 nM) using the Lipofectamine 2000 (Invitrogen) according to the

manufacturer's protocol. After incubation with siRNAs for 24 h, the cells received RSV and OGD treatment and were then harvested and analyzed.

Analysis of SIRT1 and AMPK signaling pathway induced by RSV

In order to understand whether SIRT1 and AMPK act in the same pathway or if they represent separate parallel pathways, we measured the effects of AMPK siRNA and inhibitor Dorsomorphin dihydrocholoride on SIRT1 activity levels, and the effects of SIRT1 siRNA and inhibitor Sirtinol on p-AMPK levels as well. Cortical neurons were exposed to SIRT1 and AMPK siRNA for 24 hours, and then treated with RSV for 3 hours. SIRT1 activity and p-AMPK levels were tested as described above.

Statistical Analysis

Statistical analyses were performed with SigmaStat (SPSS, Chicago, IL, USA). Differences between groups were assessed with **t-test** or a one-way analysis of variance **(ANOVA)** and followed by a Tukey (Student-Neumann-Keuls) multiple comparisons of means tests. Data are expressed as means ± SD. A value of $P < 0.05$ was considered to be statistically significant.

Results

Detection of RSV's bioavailability in animals

RSV levels in plasma reached peak levels (450±89 nM) soon after feeding (10 min) and fell quickly, nearly to the baseline, over the first hour (Figure 1C). RSV was detected in significant amounts in the liver (100 ± 17.8 nM) and brain homogenates (55 ± 11.8 nM) when measured 2 hours after the fourth dose of 25mg/kg RSV (Figure 1D). Very small amounts of RSV were also observed in the tissue of control animals; however, this was considered to arise from RSV levels that might have been present in the routine rat lab diet or that could have been artifactual background in the assay.

RSV treatment significantly reduced infarct volumes and improved recovery of behavioural deficits in recurrent stroke models.

At the time of tMCAO, the cerebral blood flow measured within the core ipsilateral to the occlusion decreased to 7.6±2.8% and 7.9±2.6% in the vehicle and in RSV treated single stroke

groups respectively; 6.4±2.3% and 6.2±3.2% during the first occlusion period in the vehicle and RSV treated recurrent stroke groups respectively; and 7.6±2.8% and 8.0±3.5% during the second occlusion period in the recurrent stroke groups respectively. Because no significant differences were found in the flows detected either in the proximal or distal cortical regions between RSV and vehicle treated groups at the onset or the end of ischemia (Figure 2A), the recurrent ischemic model was considered to be successful.

As shown in Fig 2B and 2C, MRI and TTC assays have demonstrated that RSV treatments significantly decrease infarct volume, especially in the recurrent MCAO model. In single mild stroke rats, the infarct volumes of the control group were small (35±7.2mm^3) but RSV treatment reduced infarct volumes to an even smaller size (18±4.3 mm^3) (t-test: P<0.05; RSV treatment vs control). Recurrent ischemic treatment caused larger volumes (140±30.1mm^3, t-test: P<0.001. Recurrent ischemia vs single ischemia); RSV treatment significantly decreased infarct volume to 74±14.5 mm^3 (t-test: P<0.001; RSV treatment vs control)

As expected, both single and recurrent tMCAO lead to significant behavioral deficits as measured by either test (Tukey test: P<0.01; recurrent and single tMCAO vs control; recurrent vs single tMCAO). As shown in Fig. 2D and 2E, behavioural deficits were significantly more severe in recurrent tMCAO insulted rats (stepping error rate=45±7.8 and asymmetry score= 36±7.6) than in single tMACO insulted rats (stepping error rate=10.3±3.9 and asymmetry score=9.3±4.1) (one-way ANOVA: P=0.0002; recurrent tMCAO vs single tMACO). RSV treatment significantly decreased the stepping error rate to 29.6 ± 6 (one way ANOVA: P=0.0047; RSV treatment vs vehicle treatment) and the asymmetry score to 21.5±5.3 (one-way ANOVA: P=0.0056; RSV treatment vs vehicle treatment) in recurrent tMCAO rats. These results indicate that RSV treatment significantly improved sensory–motor function of rats subjected to tMCAO.

RSV protected neurons from cell death

As shown in Figure 3, OGD insults resulted in significant cell death, especially in the recurrent OGD model (Tukey test: P<0.01; recurrent and single tMCAO vs control; recurrent vs single tMCAO). Low concentrations of RSV (0.5μM) significantly protected neuronal cells from cell death both in single and recurrent OGD models, giving a 32±4.1% and a 51±6% increase in the neurons' viability in single and recurrent OGD models respectively (one-way ANOVA:

P=0.0003; RSV treatment vs vehicle treatment). These results demonstrate that RSV protects neuronal cells from cell death induced by OGD, especially in recurrent OGD model.

Effects of RSV on SIRT1 deacetylase activity and SIRT1 protein Expression

As shown in the first panel of Figure 4A, dots indicate scattered cell death in the penumbra area; solid shadow indicates the pan necrosis in the infarct core. H&E stained sections (Figure 4A, the second panel) depict scattered cell death in the penumbra area and extensive cell necrosis in the infarct core. The extensive necrosis core was removed and the dot area (penumbra area) was kept for further study. Figure 4 B and C show that RSV significantly increased SIRT1 deacetylase activities in sham, single tMCAO and recurrent tMCAO rats after administration of RSV daily for 3 days (Figure 4B, t-test: *P*<0.001, *P*=0.0014, and *P*=0.002, respectively**;** RSV treatment vs vehicle treatment in sham, single and recurrent tMCAO respectively); RSV also significantly increased SIRT1 deacetylase activities in control, single OGD and recurrent OGD neuronal cells after exposure to RSV for 3 hours (Figure 4C, t-test: *P*=0.0003, *P*=0.0014 and *P*=0.002, respectively; RSV treatment vs vehicle treatment in control, single and recurrent OGD respectively). Single tMCAO or OGD insults alone also slightly increased SIRT1 activity (Figure 4B and 4C, t-test: *P*=0.04, single tMCAO and ODG vehicle groups vs sham and control vehicle groups), but this phenomenon was not seen in recurrent tMCAO/OGD insulted groups (t-test: *P*=0.065; recurrent tMCAO and ODG vehicle groups vs sham and control vehicle groups). Surprisingly, SIRT1 protein expression did not show differences (Figure 4D, Tukey test: *P*>0.05; RSV treatment vs vehicle treatment in sham, single and recurrent tMCAO groups respectively; RSV treatment in single and recurrent tMCAO vs sham vehicle) after administration of 25mg/kg RSV for 3 days in brain tissue, whereas SIRT1 protein expression did increase in cultured neurons after treatment of 0.5 μM RSV for 3 hours (Figure 4E, Tukey test: *P*<0.01; RSV treatment vs vehicle treatment in control, single and recurrent OGD respectively; RSV treatment in single and recurrent OGD vs control vehicle group). The mechanisms are presently unknown.

RSV increased AMP kinase activity

RSV significantly increased AMPK phosphorylation in sham, single tMCAO and recurrent tMCAO rats (Figure 5A, t-test: *P*=0.03, *P*=0.019 and P=0.016, respectively; RSV treatment vs vehicle treatment in sham, single and recurrent tMCAO, respectively) and also significantly

increased AMPK phosphorylation in control, single OGD and recurrent OGD insulted neuronal cells (Figure 5B, t-test: P=0.027, P=0.001 and P=0.001, respectively; RSV treatment vs vehicle treatment in control, single and recurrent OGD, respectively). Interestingly, single tMCAO or OGD insults alone also slightly increased AMPK phosphorylation (Figure 5A,B, t-test: #P=0.04; single tMCAO and OGD vehicle vs sham and control vehicle), however, this phenomena was not seen in recurrent tMCAO and OGD insulted groups (Figure 5A,B, t-test: P>0.05; recurrent tMCAO and OGD vehicle vs sham and control vehicle).

RSV increased NAD^+/NADH ratio and promoted restoration of intracellular ATP levels

Because SIRT1 deacetylase activity is driven by NAD+ levels (Imai, S. et al., 2000), we examined whether AMPK indirectly activates SIRT1 by altering the intracellular NAD^+/NADH ratio in recurrently ischemic stroke models. Our results show that tMCAO and OGD insults significantly decreased intracellular NAD^+/NADH ratio (Figure 6A&B; one-way ANOVA: P=0.012; P=0.001; single tMCAO and OGD vehicle vs sham and control vehicle), especially in recurrent tMCAO and OGD models, the ratio decreased to only 28±16% of sham and 54±21% of control levels (Figure 6A&B; one-way ANOVA: P<0.001; recurrent tMCAO and OGD vehicle vs sham and control vehicle). RSV treatments significantly increased the intracellular NAD^+/NADH ratio to 239±18% in single MCAO and 204±25% in recurrent MCAO groups (Figure 6A, one-way ANOVA: P<0.001; RSV treatment vs vehicle treatment in single and recurrent tMCAO); the same phenomenon was observed in cultured neuronal cells, where the NAD^+/NADH ratio was increased to 279±22% in single OGD and to 243±19% in recurrent OGD groups (Figure 6B, one-way ANOVA: P<0.001; RSV treatment vs vehicle treatment in single and recurrent OGD). These results further support the hypothesis that change in NAD^+ levels translate AMPK effects onto SIRT1 activity.

As shown in Figure 6C and 6D, tMCAO and OGD insults significantly decreased intracellular ATP levels (One-way ANOVA: P<0.01; single tMCAO and OGD vehicle vs sham and control vehicle), this is especially evident for the recurrent MCAO and OGD insults which caused an almost complete depletion of ATP (only19±8% of sham and 17±7% of control levels; one-way ANOVA: P<0.001; recurrent tMCAO and OGD vehicle vs sham and control vehicle). RSV treatment significantly restored ATP content and increased intracellular ATP levels to 127±21% in single MCAO and 97±24% in recurrent MCAO (Figure 6C, one-way ANOVA: P<0.001; RSV

treatment vs vehicle treatment in single and recurrent tMCAO); the same phenomenon was observed in cultured cortical neurons, where ATP levels were restored to 190±37% in single OGD and 170±45% in recurrent OGD groups (Figure 6D, one-way ANOVA: $P<0.001$; RSV treatment vs vehicle treatment in single and recurrent OGD).

SIRT1 and AMPK inhibitors and specific siRNA significantly abrogated RSV induced neuroprotection

Figure 7A and 7B show that the endogenous SIRT1 and AMPK expression was knocked down dramatically both in mRNA and protein level after the siRNA treatment. Cell viability assay shows that neither SIRT1 siRNA and inhibitor nor AMPK siRNA and inhibitor treatments caused neuronal cell death in 24 hours (Figure 7C,D and E,F; t-test: $P>0.05$, SIRT1- and AMPK-siRNA and inhibitor treatments vs vehicle treatment in control group); 0.5μM RSV treatment significantly protected neuronal cells from cell death induced by single and recurrent OGD treatments (Figure 7C,D and E,F; t-test: $**P<0.001$; RSV treatment vs vehicle treatment in single and recurrent OGD groups). SIRT1- and AMPK-specific siRNA and inhibitors significantly increased neuronal cell death (Figure 7C,D and E,F; t-test: $P<0.01$; SIRT1- and AMPK-siRNA and inhibitor treatments vs vehicle treatment in single and recurrent OGD groups) and also abolished RSV-induced neuroprotection in single and recurrent OGD models (Figure 7C.D and E,F; t-test: $\$\$P<0.001$; SIRT1- and AMPK-siRNA and inhibitor plus RSV treatments vs RSV treatment alone in single and recurrent OGD groups). These results confirmed that RSV-induced neuroprotection is directly correlated with the regulation of SIRT1 and AMPK activities.

AMPK and SIRT1 signaling pathway induced by RSV in recurrent ischemic stroke

Results show that RSV significantly increased SIRT1 activities p-AMPK levels in control, single OGD and double OGD insulted cortical neurons (Figure 8A,B, Tukey test: $**P<0.001$; RSV treatment vs vehicle treatment in control, single and double OGD groups respectively; RSV treatment in single and double OGD groups vs control vehicle group), both AMPK siRNA and inhibitor significantly abolished SIRT1 activity levels induced by RSV (Figure 8A, t-test: ## and $\$\$ P<0.001$; AMPK-siRNA and inhibitor plus RSV treatments vs RSV treatment alone in each group); however, the impact of SIRT1-siRNA and inhibitor on p-AMPK levels were not observed (Figure 8B, t-test: $P>0.05$; SIRT1-siRNA and inhibitor plus RSV treatments vs RSV treatment alone in each group). These results indicate that SIRT1 is one of the downstream

targets of RSV/AMPK signaling; they also indicate that AMPK is one of the upstream molecules in RSV/SIRT1 signaling.

Discussion

Because more than half of secondary strokes associated with TIA or minor strokes occur within 24 hours to 1 week of the initial attack (Roquer et al., 2010), greater clinical effectiveness of neuroprotective drugs would be expected if one could administer treatment prior to the recurrent stroke, particularly if the treatment had few or no side effects. In order to seek potential clinically effective therapies for recurrent stroke, a novel translational relevant model of elderly recurrent stroke consisting of an initial mild stroke followed by a second stroke was developed successfully in this study. This model is a very useful tool in investigating the molecular mechanisms of recurrent stroke and will assist in the translation to clinical testing of pre-clinical stroke prevention strategies. Epidemiological evidence shows that the elderly, in comparison to the general population, are at higher risk and disproportionately suffer more disability and mortality following stroke (Paolucci S et al 2003). In this study, we used elderly rats (19-21 months) to construct the recurrent stroke model, making the aspects of the model more translationally relevant. Since clinical statistical data shows that more than half of recurrent strokes associated with TIA or minor strokes occur within 24 hours to 1 week of the initial presentation, we selected a 72-hour delay to induce the secondary stroke in our elderly animal model to reflect this life phenomenon. The delayed phase of ischemia and reperfusion brain damage is present from day 3 onwards (Shichita T et al 2011). We then evaluated and analyzed the ischemic lesion and behavioral deficits post 2nd stroke beyond the 72 hours acute period which significantly increased the relevance of the work presented.

Numerous studies indicate that RSV's effects appear organ-specific and vary depending on dosage. In these studies, intermediate doses tend to be the most cardioprotective or neuroprotective (la-Morte et al., 2009). RSV, the unique phytoalexin present in red wine, delivers either a survival signal or a death signal to the ischemic myocardium depending on the dose (Dudley J et al, 2009, 2012). The 25mg/kg RSV dose selected for our elderly recurrent stroke model falls within the 20 to 50 mg/kg doses, demonstrated previously to have neuroprotective effects in MCAO models (Sinha et al., 2002). The dose of 0.5μM RSV which is

about the same as the peak level of 0.45μM measured in the plasma was used in primary cultured cortical neuronal cells. Results show that 25mg/kg of RSV significantly reduced infarct volume and improved behavioral deficits in recurrent ischemic stroke and that 0.5μM of RSV protected neuronal cells from cell death induced by OGD. RSV was detected in significant but different amounts in the liver (100 ± 17.8 nM) and brain homogenates (55 ± 11.8nM). Drug metabolism and disposition studies show that RSV demonstrates high oral absorption but rapid and extensive metabolism. Extremely rapid sulfate conjugation by the intestine/liver appears to be the rate-limiting step in RSV's bioavailability and organ specificity (Walle T et al., 2004; 2009; la-Morte et al., 2009). This may explain our results since the RSV concentration obtained was much higher in the liver than in the brain when measured 2 hours after oral administration of the fourth dose. The length of time and the RSV dosage required to obtain optimal concentration in the brain tissue along with the optimal route of administration both need further investigation.

Our results show that SIRT1 expression was increased in cultured neuronal cells treated with 0.5μM RSV for 3 hours. Surprisingly, SIRT1 expression was not affected in the brain tissue of rats treated with 25mg/kg RSV daily for 3 days. We suspect that SIRT1 expression and SIRT1 activities are probably related to *in vivo* and *in vitro* micro-environments, time-course and post-translational alterations as well. Clearly, brain tissues are more complex than the *in vitro* cortical neuronal culture. Non-neuronal cell types such as astrocytes, microglia or oligodentrocytes in brain tissue were activated and they released different inflammatory cytokines following an ischemic stroke (Benjelloun N et al., 1999). RSV treatment reduced neurodegeneration therefore resulting in decreased inflammation (Baur, J.A,et al., 2006). However, RSV would not exert the same effect on the pure culture cortical neurons because the other cell types were not present. The SIRT1 expression and SIRT1 activities are probably related to time-course as well. Previous studies have shown that RSV treatment increased SIRT1 activity in organotypic hippocampal slices at 30 minutes after treatment and SIRT1 activation returned to baseline levels by 48 hours (Raval et al, 2006). SIRT1 activity was significantly increased at 48 h after ischemic preconditioning, but did not change at 30 minutes (Raval et al., 2006). We suspect that the SIRT1 expression had increased at an earlier time and had returned to the baseline after 72 hours in this study. Moreover, the incongruity between SIRT1 expression and activity (*in vivo*) may relate to the post-translational alterations of SIRT1 that enhance activity without increasing

expression. In conclusion, the mechanisms of SIRT1 expression and activities need further investigation.

RSV administration has been effective in reducing damage in a number of cell cultures and animal models of CNS ischemia (Dong et al. 2008; la-Morte et al. 2009; Raval et al. 2006; Yousuf et al. 2009). **However, none of these studies investigated the neuroprotection of RSV administration in a recurrent stroke model that could provide the basis for clinical investigation.** In this study, we demonstrate for the first time that RSV treatment remarkably reduces infarct volumes, dramatically improves recovery on behavioural deficits in elderly recurrent ischemic animal model and protects neuronal cells from cell death induced by recurrent OGD insults. With respect to understanding how RSV reduces damage with cerebral ischemia, there is much recent information regarding the nitric oxide and adenosine, NF-κB-COX2, SIRT1-UCP2, and/or AMPK signaling pathways may be affected by RSV (Bradamante S, 2003; Dasgupta et al.,2007; Della-Mortem D. et al., 2009; Yi CO.et al., 2011). RSV treatment resulted in a robust increase in AMPK Thr^{172} phosphorylation within 2 hours that persisted for up to 72 hours in neuro2A neuroblastoma cells (Dasgupta et al.,2007). OGD insults induced AMPK activated in 30 minutes, reach peak levels 2 hours after OGD and declined to control levels at 6 hours in endothelia cells (Liu et al.,2010). Our results show for the first time that RSV stimulates AMPK activity in cortical neurons in elderly recurrent stroke models (*in vivo)* up to 72 hours and lasts at least 3 hours in primary cultured neurons (*in vitro).* We demonstrate that RSV treatment increases the intracellular NAD^+/NADH ratio, increases SIRT1 activities, up-regulated AMPK phosphorylation, and promotes restoration of intracellular ATP levels in elderly recurrent stroke models. These results are consistent with a previous study showing that RSV activated SIRT1 by altering the interacelluar NAD^+/NADH ratio, leading to deacetylation, thus limiting rRNA transcription, reducing energy consumption and further protecting cells from energy deprivation-dependent apoptosis (Murayama et al.,2008). We also demonstrate that both SIRT1 and AMPK specific inhibitors and siRNA significantly abolish the RSV-mediated neuroprotection in recurrent OGD insulted models. These results confirm that RSV is an activator of SIRT1 and AMPK in neurons (Borra,MT, et al.,2005; Dasgupta, B. et al., 2007; Yi, et al., 2011) and establish that SIRT1 and AMPK are key mediators of neuroprotection after MCAO/OGD insults. RSV-mediated neuroprotection thus directly correlates with the regulation of SIRT1 and AMPK activities. However, it is not clear whether SIRT1 and AMPK act in the same pathway or

whether they represent separate parallel pathways since deletion of either prevents protection. We further measured the cross-effects of the AMPK siRNA and inhibitor on SIRT1 activity levels and the SIRT1 siRNA and inhibitor on p-AMPK levels. Our results show that AMPK siRNA and inhibitor decrease SIRT1 activity levels induced by RSV; however, the SIRT1 siRNA and inhibitor have no impact on p-AMPK levels. These results indicate that SIRT1 is a downstream target of p-AMPK activated by RSV; therefore RSV acts to provide neuroprotection in elderly recurrent stroke models through the RSV/p-AMPK/SIRT1 signaling pathway. Our results are consistent with the report that AMPK enhances the activity of SIRT1 by altering the intracellular NAD^+/NADH ratio (Canto´C et al 2009) and that the p-AMPK/SIRT1/PGC-1α signaling pathway plays a critical role in mitochondrial function facilitating adaptive energy metabolism in cells, including the neurons (Spiegelman B.M. 2007).

To summarize, the current study demonstrates for the first time that a translationally-relevant elderly recurrent ischemic stroke model has been developed successfully. A dietary polyphenol RSV achieves neuronal protective effects in elderly recurrent ischemic stroke models *in vivo* and *in vitro* by increasing p-AMPK levels, increasing the interacelluar NAD^+/NADH ratio and SIRT1activities thereby reducing energy ATP requirements during ischemia; RSV acts to provide neuroprotection through the RSV/p-AMPK/SIRT1 signaling pathway. Our results improve the understanding of the molecular mechanisms of RSV neuroprotedction and provide a foundation for a new pharmacological approach to neuroprotection in patients with high risk for a recurrent stroke. Both AMPK and SIRT1 proteins are involved in the cellular response to metabolic stress, nutrient deprivation and hypoxia; it will be interesting to investigate whether there are additional interactions between these two protein families in neurons and other cells.

Acknowledgments

We thank Dr. Darren. L. Clark for technical assistant in recurrent stroke model and cortical neuronal cell culture. We thank Dr. Robert Edwards for proof-reading the manuscript.

Abbreviations

AMPK: AMP-activated protein kinase

MRI: Magnetic Resonance Imaging

NAD/NADH: Nicotinamide Adenine Dinucleotide/Reduced form of NAD

OGD: Oxygen glucose deprivation

p-AMPK: phospho-AMPK

RSV: resveratrol

SIRT1: Silent Information Regulator T1

tMCAO: transient Middle Cerebral Artery Occlusions

TIA: transient ischemic attack

TTC: triphenyltetrazolium chloride

Sources of Funding

This work was supported by the National Natural Science Foundation of China (31130026), the State Program of National Natural Science Foundation of China for Innovative Research Group (No. 81021001), the Independent Innovation Foundation of Shandong University (IIFSDU) and the Natural Science Foundation of JiangXi Province (GJJ12591). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript."

Disclosures

None.

Figure Legends

Figure 1. Schematic representation of the experimental protocol and the bioavailability of RSV following RSV administration. (A&B) Schematic representation of the experimental protocol for developing the elderly single and recurrent stroke model. (C) RSV levels in the plasma reached peak levels (450 ± 89.1nM) after 10 min feeding and fell nearly to the baseline after the first hour after administration. (D) The concentration of RSV was 100 ± 17.8 nM in the liver and 55 ± 11.8 nM in the brain tissues 2 hours after the fourth dose on day 3.

Figure 2. Blood flow measurement and neuroprotective effects of RSV on infarct volumes and behavioural recovery. (A) At the time of tMCAO, the cerebral blood decreased to 7.6±2.8% and 7.9±2.6% in the vehicle and RSV treated single stroke groups respectively; 6.4±2.3% and 6.2±3.2% during the first occlusion period in the vehicle and RSV treated recurrent stroke groups respectively; and 7.6±2.8% and 8.0±3.5% during the second occlusion period in the vehicle and RSV treated recurrent stroke groups respectively. No significant differences were found in the proximal or distal cortical regions between RSV and vehicle treated groups at either onset or end of ischemia. (B & C) The infarct volumes of control group were 35 ±7.2mm^3, RSV treatment reduced infarct volumes to smaller size 18±4.3mm^3 (P<0.01). Recurrent ischemic treatment caused larger infarct volumes (140±30.1mm^3), RSV treatment significantly decreased infarct volume to 74±14.5 mm^3 (P<0.001). (D&E) Behavioural deficits were significantly more severe in recurrent tMCAO insulted rats (stepping error rate=45±7.8 and asymmetry score= 36±7.6) than in single tMACO insulted rats (stepping error rate=10.3±3.9 and asymmetry score=9.3±4.1, P=0.0002). RSV treatment significantly decreased the stepping error rate (29.6 ± 6, P=0.0047) and the asymmetry score (21.5±5.3, P=0.0056) in recurrent MCAO rats. Values shown are the mean ± SD from three independent experiments; single asterisk (*) indicate P <0.05, double asterisks (**) indicate P < 0.001 as compared to vehicle (t-test or one-way ANOVA with the Neuman-Keuls post-test).

Figure 3. RSV protected neurons from cell death induced by Oxygen-Glucose-Deprivation. OGD insults resulted in significant cell death both in single and recurrent OGD models (P=0.007), and especially in the recurrent OGD model (P=0.0002). 0.5μM RSV treatment gave a 32 ±4.1 % and a 51±6% increase in cell viability in single and recurrent OGD models respectively (P=0.0003). Values shown are the mean ± SD from three independent experiments, single (*and #) indicate P <0.05, double asterisks (**) indicate P < 0.001 as compared with vehicle (t-test or one-way ANOVA with the Neuman-Keuls post-test).

Figure 4. Penumbra Determination and the Effects of RSV on SIRT1 expression and activities. (A) Penumbra area determination: Figure 4A panel 1, dots indicate scattered cell death in the penumbra area, solid shadow indicates the pan necrosis in the infarct core. Figure 4A panel 2, H&E stained sections depict scattered cell death in the penumbra area and extensive cell necrosis in the infarct core. (B) RSV significantly increases SIRT1 activity in tMCAO animal model (*in vivo)* and (C) OGD neuronal cells model (*in vitro)* (**P<0.001; P=0.0014; P=0.002 vs vehicle); (D) RSV treatment does not change SIRT1 expression *in vivo* (P>0.05, RSV vs sham vehicle). (E) RSV treatment increases SIRT1 expression *in vitro* (* P<0.01 RSV vs control vehicle). Values shown are the mean ± SD. from six independent experiments; single (# and *) indicate P <0.05, double asterisks (**) indicate P < 0.001 as compared to vehicle (t-test or one-way ANOVA with the Neuman-Keuls post-test).

Figure 5. **The Effects of RSV on AMPK activities.** (A) RSV significantly increases AMPK activity in tMCAO animal model (*in vivo)* and (B) OGD neuronal cells model (*in vitro)* (** P=0.027; P=0.001, P=0.001 vs vehicle); Single stroke alone also slightly increases AMPK activity (A,B. #P<0.05 vs sham vehicle).

Values shown are the mean ± SD. from three independent experiments; single (#) indicate P <0.05, double asterisks (**) indicate P < 0.001 as compared to vehicle (t-test or one-way ANOVA with the Neuman-Keuls post-test).

Figure 6. **RSV treatment increased NAD^+/NADH ratio and promoted restoration of intracellular ATP levels. (**A&B) Recurrent tMCAO and OGD insults caused severe decrease of intracellular NAD^+/NADH ratio (only 28±16% levels of sham rats and 54±21% of control neurons). RSV treatment significantly increased the intracellular NAD^+/NADH ratio to 239±18% and 204±25% in single and recurrent tMCAO groups respectively (Figure 6A, P<0.001 vs sham vehicle group); and 279±22% and 243±19% in single and recurrrent OGD neurons respectively (Figure 6B, P<0.001 vs control vehicle). **(C&D)** Recurrent tMCAO and OGD insults caused an almost complete depletion of ATP (only19±8% levels of sham rats and 17±7% of control neurons). RSV treatment significantly increased intracellular ATP levels to 127±21% and 97±24% in single and recurrent tMCAO rats respectively (**P<0.001 vs sham vehicle); and 190±37% and 170±45% in single and recurrent OGD neurons respectively (**P<0.001). Values shown are the mean ± SD from three independent experiments; single (#) indicate P <0.05, double asterisks (**) indicate P < 0.001 as compared to vehicle (t-test or one-way ANOVA with the Neuman-Keuls post-test).

Figure 7. SIRT1 and AMPK inhibitors and specific siRNA significantly abrogated RSV induced neuroprotection. (A&B) Endogenous SIRT1 and AMPK expression was knocked down dramatically both in mRNA and protein level after the siRNA treatments. (C,D & E,F) 0.5 μM RSV significantly protected neuronal cells from cell death induced by single and recurrent OGD treatments (**P<0.001); SIRT1 and AMPK specific siRNA and inhibitors treatments significantly increased neuronal cell death induced by single and recurrent OGD insults (##P<0.01 vs vehicle) and significantly abolished RSV induced neuroprotection in single and recurrent OGD models ($$ P<0.001 vs RSV treatment). Values shown are the mean ± SD from three independent experiments; single (#) indicate P <0.05, double (** and ## and $$) indicate P < 0.001 (t-test or one-way ANOVA with the Neuman-Keuls post-test).

Figure 8. AMPK and SIRT1 signaling pathway induced by RSV in recurrent ischemic stroke. (A) AMPK siRNA and inhibitor significantly abolished the increase of SIRT1 activity levels induced by RSV in control, single and recurrent OGD cortical neurons (P<0.001 vs RSV). (B) SIRT1 siRNA and inhibitor had no impact on p-AMPK levels after RSV treatments (P=0.07). Values shown are the mean ± SD from three independent experiments; double (**,## and $$) indicate P < 0.001 (t-test or one-way ANOVA with the Neuman-Keuls post-test).

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