Published in final edited form as:

Neuroscience. 2009 March 31; 159(3): 993–1002. doi:10.1016/j.neuroscience.2009.01.017.

RESVERATROL PRETREATMENT PROTECTS RAT BRAIN FROM CEREBRAL ISCHEMIC DAMAGE VIA A SIRT1 – UCP2 PATHWAY

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Abstract

Resveratrol is a natural polyphenol found in grapes and wine and has been associated with protective effects against cardiovascular diseases. *In vitro*, both resveratrol preconditioning (RPC) and ischemic preconditioning (IPC) require activation of sirtuin 1 (SIRT1), an NAD⁺-dependent deacetylase, to induce neuroprotection against cerebral ischemia. In the present study, we tested two hypotheses: a) that neuroprotection against cerebral ischemia can be induced by RPC *in vivo*; and b) that RPC neuroprotection involves alterations in mitochondrial function via the SIRT1 target mitochondrial uncoupling protein 2 (UCP2). IPC was induced by two minutes of global ischemia (temporary bilateral carotid artery occlusion with hypotension), and RPC, by intraperitoneal injection of resveratrol at 10, 50 and 100 mg/Kg dosages. 48 hours later, we compared the neuroprotective efficacy of RPC and IPC in vulnerable CA1 hippocampal pyramidal neurons using a rat model of asphyxial cardiac arrest (ACA). SIRT1 activity was measured using a SIRT1-specific fluorescent enzyme activity assay. In hippocampal mitochondria isolated 48 hours after IPC or RPC, we measured UCP2 levels, membrane potential, respiration, and the mitochondrial ATP synthesis efficiency (ADP/O ratio). Both IPC and RPC induced tolerance against brain injury induced by cardiac arrest in this *in vivo* model. IPC increased SIRT1 activity at 48 hours, while RPC increased SIRT1 activity at 1 hour but not 48 hours after treatment in hippocampus. Resveratrol significantly decreased UCP2 levels by 35% compared to sham-treated rats. The SIRT1-specific inhibitor sirtinol abolished the neuroprotection afforded by RPC and the decrease in UCP2 levels. Finally, RPC significantly increased the ADP/O ratio in hippocampal mitochondria reflecting enhanced ATP synthesis efficiency. In conclusion, *in vivo* resveratrol pretreatment confers neuroprotection similar to IPC via the SIRT1-UCP2 pathway.

Keywords

Cardiac arrest; cerebral ischemia; mitochondria; neuroprotection; tolerance; bioenergetics

Stroke and cardiac arrest are the leading causes of serious long-term disability (Krause et al., 1986). Numerous studies have demonstrated that brief sub-lethal periods of ischemia are able

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Section Editor: Cellular Neuroscience - M. Segal

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to reduce the deleterious effects of a subsequent longer duration of an ischemic episode in heart, brain and in other organs (Dirnagl et al., 2003). This endogenous protection is known as ischemic preconditioning (IPC).

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol found in grapes and wine (Hao and He, 2004). Several studies have shown that regular consumption of red wine reduces the risk of atherosclerosis and cardiovascular disease (Hao and He, 2004) and resveratrol protects heart, brain, and kidney from ischemia-reperfusion injury (Raval et al., 2008). *In vitro*, resveratrol pre-treatment mimics the neuroprotection afforded by IPC (Raval et al., 2006). SIRT1, a member of the sirtuin family of NAD⁺-dependent deacetylases (Borra et al., 2005), was identified as a critical mediator of this neuroprotection (Raval et al., 2006). In other studies, resveratrol leads to activation of SIRT1 by as much as 8-fold (Borra et al., 2005).

SIRT1 is implicated in many processes, such as cell-cycle regulation, fatty acid metabolism, axonal degeneration, muscle cell differentiation, and life span extension (Michan and Sinclair, 2007). One mediator of SIRT1-induced protection is mitochondria. Bordone et al. demonstrated that SIRT1 represses mitochondrial uncoupling protein 2 (UCP2) transcription by binding directly to its promoter (Bordone et al., 2006). Moynihan et al. observed lower levels of UCP2 in islets isolated from transgenic mice overexpressing SIRT1 in pancreatic beta cells (Moynihan et al., 2005). The same study demonstrated that lower UCP2 levels induced by SIRT1 overexpression results in increased ATP production and enhanced insulin secretion in isolated islets. Other studies have demonstrated the role of UCP2 in modulating reactive oxygen species (ROS) production (Lee et al., 1999; Arsenijevic et al., 2000). UCP2 is also essential for mitochondrial Ca²⁺ uptake, a crucial factor in regulating the rate of oxidative phosphorylation (Trenker et al., 2007). The resveratrol-SIRT1 signalling pathway and its targets are summarized in Figure 1.

The main goals of this study were: 1) to determine the capacity of resveratrol to mimic IPC in protecting the CA1 region of rat hippocampus *in vivo*; and 2) to define the involvement of the SIRT1-UCP2 pathway in the induction of ischemic tolerance following resveratrol preconditioning.

EXPERIMENTAL PROCEDURES

Drugs

Resveratrol (Sigma Chemical, St Louis, USA) was dissolved in 7:3 saline (0.9 % NaCl): Solutol (BASF, Wyandotte, USA) and injected intraperitoneally (i.p.). Sirtinol: a specific inhibitor of SIRT1 activity (Grozinger et al., 2001), (Sigma) was dissolved in dimethyl sulfoxide (DMSO) at concentration of 1 mM. 10 µl (3.94 µg) was injected into the left intracerebral ventricle (ICV) (bregma: -0.8 mm, lateral: 1.5 mm, depth: 3.5 mm) at the rate of 60 µl/h (Zhang et al., 2006).

Experimental design

Descriptions for all groups are as follows (Figure 2):

Group 1: **Sham Asphyxial Cardiac Arrest (ACA)** (n=6) - Sham surgery was performed.

Group 2: **Test Ischemia/Vehicle control** (n=10) - 8 minutes of ACA was induced 48 hours after i.p. injection of saline (2 ml/Kg body weight).

Group 3: **IPC + ACA** (n=6) - 2 minutes of global cerebral ischemia was induced 48 hours before the induction of 8 minutes of ACA.

Group 4: **Resveratrol 10 mg/Kg + ACA** (n=8) - Resveratrol was injected i.p. at 10 mg/Kg body weight 48 hours before the induction of 8 minutes of ACA.

Group 5: **Resveratrol** 50 mg/Kg + **ACA** (n=8) - Resveratrol was injected i.p. at 50 mg/Kg body weight 48 hours before the induction of 8 minutes of ACA.

Group 6: **Resveratrol** 100 mg/Kg + **ACA** (n=8) - Resveratrol was injected i.p. at 100 mg/Kg body weight 48 hours before the induction of 8 minutes of ACA.

Group 7: **DMSO (ICV vehicle)** + **ACA** (n=5) - 10 µl of DMSO was infused ICV 48 hours before the induction of 8 minutes of ACA.

Group 8: **Resveratrol** + **DMSO** + **ACA** (n=5) - 10 µl of DMSO was infused ICV immediately after i.p injection of resveratrol (10 mg/Kg body weight). Eight minutes of ACA was induced 48 hours later.

Group 9: **Sirtinol ICV** (n=5) - 3.94 µg of sirtinol was infused ICV.

Group 10: **Sirtinol ICV** + **ACA** (n=5) - 3.94 µg of sirtinol was infused ICV 48 hours before the induction of 8 minutes of ACA.

Group 11: **Resveratrol** + **Sirtinol** + **ACA** (n=5) - 3.94 µg of sirtinol was infused ICV immediately after i.p injection of resveratrol (10 mg/Kg body weight). Eight minutes of ACA was induced 48 hours later.

Induction of Ischemic preconditioning (IPC)

All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Animal Care and Use Committee of the University of Miami. For all surgical procedures, Sprague Dawley (SD) rats weighing 250 to 350g were anesthetized with 4% isoflurane and a 30:70 mixture of oxygen and nitrous oxide followed by endotracheal intubation. IPC was induced using two minutes of global cerebral ischemia via two vessel occlusion (tightening carotid ligatures bilaterally and 45-50 mmHg hypotension) (Perez-Pinzon et al., 1997). Blood flow was restored by loosening the carotid ligatures and slowly re-infusing the shed blood (maintained at 36-37 °C) to restore normotension. Animals were maintained on a respirator until spontaneous respiration resumed.

Asphyxial cardiac arrest (ACA)

As described previously (Dave et al., 2004), ACA was induced by disconnecting the ventilator from the endotracheal tube. Eight minutes after initiation of asphyxia, resuscitation was initiated by administering a bolus injection of epinephrine (0.005 mg/Kg, i.v.) and sodium bicarbonate (1 meq/Kg, i.v.) followed by mechanical ventilation with 100% oxygen and manual chest compressions at a rate of 200/minute until MAP reached 60 mmHg maintained by spontaneous heart beats for more than 10 seconds. Once the animal was hemodynamically stable and spontaneously breathing, usually by 10-15 minutes after restoration of spontaneous circulation (ROSC), the catheters were removed and the animal extubated, and 100% O₂ was delivered via face mask. Head and body temperatures were maintained at 37°C using heating lamps for 1 hour. Control animals (sham ACA) underwent surgical procedures similar to the ACA animals except induction of ACA. Post-surgical body temperature and body weight were monitored until 7 days and were not significantly different between all groups.

Histopathology

At the end of 7 days of reperfusion, rats were anesthetized with isoflurane and perfused with a mixture of 40% formaldehyde, glacial acetic acid, and methanol, 1:1:8 by volume (Perez-Pinzon et al., 1997). The brains were then removed from the skull, and coronal brain blocks were embedded in paraffin; coronal sections of 10 µm thickness were cut and stained with hematoxylin and eosin. The entire hippocampus (anterior to posterior) was examined. Normal

neuronal counts were made within the CA1 region of hippocampus at the level of 3.8 mm from posterior to bregma. Neurons exhibiting ischemic cell change were identified by: (1) eosinophilic cytoplasm, (2) dark-staining triangular shaped nuclei, and (3) eosinophilic-staining nucleolus. An investigator masked to the experimental conditions in all groups counted ischemic neurons, 18 fields per sections, along the medial to lateral extent of the CA1 region of the hippocampus.

Measurement of SIRT1 Activity

To measure SIRT1 activity, hippocampal tissue was collected at 1 or 48 hours after either IPC or RPC, and the nuclear extract was fractionated using techniques previously described in detail (Raval et al., 2006). The enzyme activity of SIRT1 was measured using a SIRT1 fluorescent activity assay kit (BIOMOL International, USA) based on Fluor de Lys-SIRT1 substrate peptide. Suramine-sensitive activity was considered as the SIRT1 enzyme activity.

Cell Fractionation and Western Blot Analysis

For the Western blot analysis, mitochondrial fractions were prepared according to previously described procedures (Lee CP, 1993). The mitochondrial fraction was analyzed for protein contents using the Bio-Rad protein assay kit, based on the method of Bradford et al. (Bradford, 1976). Equal amounts of protein from each group were run on the same gel and analyzed at the same time. Proteins were transferred to Immobilon-P (Millipore, USA) membrane and incubated with the primary antibody anti-UCP2 (1:1000) (Calbiochem, USA) and anti-COX IV (1:1000) (Molecular Probes, USA). Immunoreactivity was detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech, UK). The digitized immunoblots were subjected to densitometric analysis.

RNA isolation and TaqMan gene expression assay

Total RNA was isolated from rat hippocampus using QIAGEN (Valencia, CA) miRNeasy mini kit. 1 μ g of total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription kits according to the manufacturer's instructions. Real-time TaqMan PCR was performed to evaluate the transcript level of UCP2 in sham-treated and resveratrol-treated experimental groups. β -actin was used as the internal control for the calculation of ΔC_T . The means of the ΔC_T for the sham-treated samples at each time point were used as the "calibrator" for the calculation of $\Delta\Delta C_T$. The mean $\Delta\Delta C_T$ for each group was used to calculate the relative expression of UCP2 ($2^{-\Delta\Delta C_T}$). Thus, the relative expression levels reported here are calibrated by the sham-treated group and normalized by the internal control. For further details on these calculations please see reference (Pfaffl et al., 2002). The following gene-specific probe/primer pair mixtures were used: β -actin (Rm00667869_m1) and UCP2 (Rn01754856_m1). PCR were carried using 15 ng cDNA template on a 7300 PCR Sequence Detection System. PCR thermal cycle was performed according to the manufacturer's instructions. All reagents and instruments were obtained from Applied Biosystems, USA. Measurements were performed in triplicate.

Isolation of Hippocampal mitochondria and Polarographic studies

Hippocampal mitochondria were isolated according to previously described procedures (Lee CP, 1993). Substrate oxidation rates and phosphorylation capacities of isolated mitochondria were determined polarographically and were carried out as described earlier (Hofhaus et al., 1996). In brief, oxygen consumption was measured polarographically in a medium containing 150 mM sucrose, 25 mM Tris-HCl (pH 7.4) and 10 mM potassium phosphate buffer (pH 7.4). The respiratory control index (RCI) and ADP/O ratios were measured in presence of 5 mM pyruvate and 2.5 mM malate. The RCI is defined as the ratio of the respiratory rate in presence and absence of ADP (i.e., state 3/state 4) (Chance and Williams, 1956). ADP/O ratio is defined as moles of ADP phosphorylated per moles of oxygen consumed.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was determined using 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC1) as described earlier (Dave et al., 2008). At the end of the experiments, FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, 1 μ M) was added as a negative control. Mitochondrial membrane potential is expressed as ratio of fluorescence at 590 nm divided by fluorescence at 525 nm.

Statistics

All data are expressed as mean $\pm$ SEM. Statistical evaluation of the data was performed using ANOVA test, followed by Bonferroni's post hoc test. $p < 0.05$ was considered significant.

RESULTS

In vivo resveratrol pretreatment mimics ischemic preconditioning

We tested the hypothesis that neuroprotection against cerebral ischemia could be induced *in vivo* by pretreatment with i.p. injection of resveratrol. Before and after the induction of ACA or sham ACA, physiological parameters including $p\text{CO}_2$, $p\text{O}_2$, HCO_3^- and plasma glucose concentration were similar among all experimental groups (Table 1). During the induction of ACA, all cardiac-arrest groups showed immediate bradycardia when apnea was induced followed by hypotension to 50 mmHg (Supplemental Figure 1). The ECG pattern returned to normal within 5 minutes after restoration of spontaneous circulation (ROSC, data not shown). No significant differences in physiological parameters were found between groups.

We performed a histological assessment of neuronal death in the CA1 region of the hippocampus 7 days after induction of ACA and resuscitation (Figure 3A and 5A). The number of normal neurons in the hippocampus of sham-operated rats was 1226 ± 72 ($n=6$). After cardiac arrest, the number of normal neurons decreased to 199 ± 11 ($n=10$) in the vehicle treated group. RPC (10 or 50 mg/kg, $n=8$) or IPC ($n=6$) significantly increased the number of normal neurons by 221, 175 and 261%, respectively (Figure 3A and B). In agreement with our previous *in vitro* study (Raval et al., 2006), the high dose of resveratrol (100 mg/Kg) ($n=8$) was not neuroprotective (Figure 3A and B). These data demonstrate that RPC and IPC share similar signaling pathways.

SIRT1 activity increases in the rat hippocampus after *in vivo* IPC and RPC

Our previous *in vitro* study demonstrated increased SIRT1 activity 48 hours after IPC and 30 min after RPC (Raval et al., 2006). Based on these previous results, we measured SIRT1 activity in rat hippocampus at 1 and 48 hours after RPC or IPC. In the case of RPC, we chose the lowest protective dose (10 mg/Kg). IPC significantly increased SIRT1 activity in the hippocampus by 29% ($p < 0.05$, $n=4$) at 48 hours, but not at 1 hour after treatment. In contrast, RPC significantly increased SIRT1 activity in the hippocampus by 36% ($p < 0.05$, $n=4$) at 1 hour, but not at 48 hours after treatment (Figure 4A). Intracerebral-ventricular (ICV) injection of the SIRT1 inhibitor sirtinol (3.94 μ g) blocked the resveratrol-induced increase in SIRT1 activity (Figure 4B). ICV injection of sirtinol without preconditioning reduced SIRT1 activity by almost 70% in the hippocampus of naïve rats (Figure 4B).

ICV injection of sirtinol blocks resveratrol-induced neuroprotection

To determine whether an increase in SIRT1 activity is necessary for RPC-induced ischemic tolerance, we asked if sirtinol blocks RPC-induced neuroprotection. ICV injection of sirtinol, 48 hours before ACA, was able to block resveratrol-induced neuroprotection (number of normal neurons 366 ± 52 , $p < 0.05$) compared to IPC and RPC (resveratrol 10 and 50 mg/Kg). ICV injection of DMSO (the vehicle for sirtinol) did not affect cell death (243 ± 80) while RPC

(lowest protective dose) plus ICV injection of DMSO was also able to induce neuroprotection (647 ± 58 , $p < 0.05$) (Figure 5A and B). Sirtinol alone, used as control, did not change the number of normal neurons (1115 ± 84 , $n=5$); nor did sirtinol exacerbate cell death after ACA (212 ± 49 , $n=5$) (Figure 5A and B). Thus, our results with the SIRT1 antagonist, sirtinol, show that SIRT1 activity is critical for RPC. Because SIRT1 modulates UCP2 expression in mitochondria, we next asked if RPC targets mitochondrial function.

RPC decreases UCP2 levels in mitochondria isolated from the rat hippocampus

Because SIRT1 activity has been previously demonstrated to modulate UCP2 levels (Moynihan et al., 2005; Bordone et al., 2006), we next tested whether or not RPC can reduce UCP2 levels in hippocampal mitochondria. RPC (10 mg/Kg, the most protective dose) decreased levels of UCP2 to $65.1 \pm 2.1\%$ ($p < 0.05$, $n=4$) at 48 hours after the resveratrol injection when compared to the vehicle treated group ($n=4$) (Figure 6A). The SIRT1 inhibitor sirtinol, prevented the RPC-induced decrease in UCP2 level ($118 \pm 14\%$, $n=4$) (Figure 6B). These data implicate the SIRT1-UCP2 pathway in resveratrol preconditioning.

A potential mechanism by which SIRT1 may reduce UCP2 levels is by depressing UCP2 mRNA levels. We tested this hypothesis by using quantitative real-time PCR. We measured the levels of UCP2 mRNA in hippocampus of rats treated with resveratrol relative to the levels of β -actin and calibrated by the levels of UCP2 mRNA in the vehicle-treated group. No significant difference in the relative expression of UCP2 was detected at 6 hours (0.87 ± 0.34 , $n=6$), 12 hours (0.93 ± 0.17 , $n=6$), or 24 hours (1.23 ± 0.39 , $n=3$).

Effect of RPC on mitochondrial physiology

Changes in mitochondrial UCP2 levels may lead to altered $\Delta\Psi_m$. We measured $\Delta\Psi_m$ in hippocampal mitochondria at 48 hours after RPC or vehicle treatments (Mills et al., 2002). The $\Delta\Psi_m$ in the resveratrol group (31.4 ± 3.6 , $n=4$) (ratio of fluorescence at 590 nm divided by 525 nm) was not significantly different compared to the vehicle group (32.3 ± 3.4 , $n=4$) (Figure 7A). This suggests that decreased UCP2 levels do not significantly affect $\Delta\Psi_m$.

Another possible target of RPC is ATP production (Moynihan et al., 2005; Bodyak et al., 2007). Thus, we next tested if RPC had any effect on mitochondrial ATP synthesizing capacity by measuring mitochondrial respiration. The mean respiration rates of state 3 (respiratory rate in presence of ADP) and state 4 (respiratory rate in absence of ADP) did not significantly differ between the RPC group ($n=4$), vehicle ($n=4$), and resveratrol plus sirtinol ($n=4$) groups (Figure 7B). Next, we compared the respiratory control index (RCI) and ADP/O ratio (as defined in the methods section) between the three groups as an index of mitochondrial electron transport chain efficiency. No significant differences were observed in RCI values between the three groups (Figure 7C). The RCI was 3.75 ± 0.52 ($n=4$) in the resveratrol group compared to 3.98 ± 0.35 in the vehicle group ($n=4$), and to 3.88 ± 0.67 ($n=4$) in the resveratrol plus sirtinol group. Typical oxygen consumption traces for all three treated groups are shown in Figure 7D. However, we found that the ratio of the rate of ATP synthesis and the rate of oxygen consumption (ADP/O ratio) significantly increased by 23% in hippocampal mitochondria isolated from RPC animals compared to vehicle treated animals (3.03 ± 0.10 vs. 3.73 ± 0.10 , vehicle vs. RPC, $n=4$; $p < 0.02$) (Figure 7E). Sirtinol was able to block the resveratrol-induced increase in the ADP/O ratio (3.11 ± 0.25 , $n=4$, $p < 0.05$) (Figure 7E). These data suggest that lower UCP2 levels after resveratrol treatment may be responsible for increased mitochondrial efficiency as observed in terms of ADP/O ratio.

DISCUSSION

Several studies in animals and humans have clearly demonstrated the capacity of IPC to protect organs against ischemic injury (see reviews (Dirnagl et al., 2003; Yenari et al., 2008)). Various pathways have been implicated including activation of protein kinase C (PKC), ATP-sensitive K⁺ channels, PI3-Kinase, adenosine receptors, and glutamate receptors (see reviews (Dirnagl et al., 2003; Gidday, 2006)). One of the ultimate goals of understanding the pathways involved in IPC is to design novel therapies to mimic IPC pharmacologically. A number of different chemicals mimic IPC, such as sulphonylurea, volatile anaesthetics, levosimendan, erythropoietin, opioids, and estrogen, among others (Dirnagl et al., 2003; Gidday, 2006; Yenari et al., 2008).

Biologically active trans-resveratrol protects a variety of tissues such as heart, brain, and kidney from ischemia-reperfusion injury (Raval et al., 2008). Here we have demonstrated that *in vivo* resveratrol pre-treatment induces tolerance against an otherwise lethal episode of ischemia and that this neuroprotection depends on SIRT1 activation. We showed that pre-treating rats with low doses of resveratrol 48 hours before induction of lethal ischemia can induce neuroprotection by activating pathways similar to IPC. The resveratrol-induced neuroprotection was blocked by sirtinol, a SIRT1 specific inhibitor, demonstrating the pivotal role of SIRT1 activation (Figure 5). Earlier we demonstrated that sirtinol also blocks IPC induced neuroprotection in vitro (Raval et al., 2006).

Previous studies in pancreatic β -cells have established that SIRT1 activity modulates levels of the mitochondrial uncoupling protein, UCP2 (Bordone et al., 2006). UCP2 is a member of a family of inner mitochondrial membrane proteins capable of driving the ATP synthase pathway via regulation of the proton electrochemical gradient (Esteves and Brand, 2005). However, the exact role of UCP2 in mitochondrial function is not fully understood. UCP2 has been implicated in thermogenesis, diabetes, aging, and diseases of the immunological, circulatory and neurological systems (Brand and Esteves, 2005).

Resveratrol pretreatment induced a decrease in UCP2 levels in hippocampal mitochondria when measured at 48 hours. The decrease in UCP2 was blocked by sirtinol (Figure 6B). Thus, *in vivo* resveratrol pretreatment regulation of UCP2 levels requires SIRT1 activation. Despite lower UCP2 levels in hippocampal mitochondria in RPC-treated rats, we did not detect any change in $\Delta\Psi_m$. This could be due to different compensatory mechanisms present in the mitochondrial membrane (Scheffler, 1999). In contrast to the lack of change in $\Delta\Psi_m$ we observed a significant increase in ADP/O ratio (Figure 7E), which is an indicator of increased mitochondrial ATP synthesis efficiency. Brain ATP levels may be maintained for a longer duration when subjected to ischemia due to increased mitochondrial ATP synthesizing capacity. Our data are in agreement with previous studies in cardiomyocytes and in the liver in which increased ATP levels were reported to occur when UCP2 levels were decreased (Bodyak et al., 2007; Evans et al., 2007). These data suggest that resveratrol-induced downregulation of UCP2 protects against lethal ischemia, at least in part, by increasing mitochondrial ATP production efficiency (Figure 1).

The neuroprotective role of UCP2 against cerebral ischemia is not clearly defined. On one hand, higher levels of UCP2 following IPC were shown to play a role in neuroprotection following cardiac and cerebral ischemia (Mattiasson et al., 2003; McLeod et al., 2005). In contrast, de Bilbao et al. observed that ischemic brain damage was lower in UCP2 knockout mice after focal cerebral ischemia (de Bilbao et al., 2004). In addition, a recent study showed that overexpression of UCP2 protected thalamic neurons against global cerebral ischemia but not other brain regions such as hippocampus (Deierborg et al., 2008). Moreover, another study demonstrated that UCP2 overexpression results in increased cell death after

hypoxiareoxygenation in adult rat cardiomyocytes (Bodyak et al., 2007). UCP2 overexpression led to decreased ROS generation and promoted a shift in hydrogen peroxide release from an intramitochondrial to an extramitochondrial site (de Bilbao et al., 2004). Thus, UCP2 overexpression altered cellular redox signalling (Arsenijevic et al., 2000; Mattiasson et al., 2003). In contrast, the UCP2-depleted condition led to increased ROS production and an increase in cellular ROS buffering capacity by means of increased reduced glutathione (GSH) and mitochondrial manganese superoxide dismutase levels (de Bilbao et al., 2004). The common denominator in both UCP2-overexpressed and -depleted conditions is decreased ROS. Our results suggest that besides increased ROS buffering capacity, UCP2-depleted conditions also increase mitochondrial ATP production capacity.

The importance of ROS in the induction of IPC and damage following cerebral ischemia has been extensively studied (Perez-Pinzon et al., 2005). The increased cellular ROS buffering capacity is likely to result in lower ROS induced damage to protein, DNA and lipids during the ischemia/reperfusion. Moreover, our ADP/O ratio results indicate more efficient mitochondrial ATP production. From these results we propose that lower levels of UCP2 following RPC leads to increased mitochondrial ATP production efficiency, which in turn protects cells from ischemia.

As we described in our previous study (Raval et al., 2006), although both RPC and IPC were neuroprotective against ischemia, the time course for the activation of SIRT1 was different between the two paradigms. As expected, resveratrol rapidly and transiently increased SIRT1 activity, whereas IPC-induced increase of SIRT1 activity was only observed before induction of ACA. However, at this stage we do not know the duration of the resveratrol-induced SIRT1 activity increase. Our data only indicate that this increase in activity returns to baseline by 48 hours. Early activation of SIRT1 following RPC resulted in a decrease in UCP2 protein levels at 48 hours, with not significant change in mRNA levels. Previous studies showed that the induction of UCP2 expression *in vivo* is regulated at translational level and not accompanied by a change in UCP2 mRNA (Pecqueur et al., 2001). The half-life of UCP2 is not clearly defined and varies greatly in different tissues (Pecqueur et al., 2001; Rousset et al., 2007). We conjecture that the observed delay in UCP2 down-regulation reflects a longer half-life of UCP2 in brain. Overall, the resveratrol results suggest that the transient increase in SIRT1 activity initiates a neuroprotective state lasting at least 48 hours. Loss of neuroprotection due to inhibition of SIRT1 activation during 48 hours of reperfusion after RPC confirms the fact that SIRT1 activation is critical for the induction of a neuroprotective state. Further studies are needed to further define the mechanisms by which SIRT1 activation promotes UCP2 protein levels to decrease. In addition, additional studies are required to define the time range whereby resveratrol promotes neuroprotection after a single administration.

To summarize, *in vivo* pre-treatment with intraperitoneal injection of 10 or 50 mg/Kg resveratrol mimicked ischemic preconditioning: resveratrol protected the CA1 region of the hippocampus against global cerebral ischemia. This neuroprotection depended on the activation of SIRT1, an NAD⁺ deacetylase linked with life-span extension by caloric restriction. We also demonstrated that resveratrol preconditioning afforded neuroprotection by decreasing UCP2 levels and increasing mitochondrial ATP synthesizing efficiency. Studies under way are aimed at defining other mechanisms involved in resveratrol-induced neuroprotection against ischemic injury. A better understanding of common mechanisms of neuroprotection shared by IPC and resveratrol could help us develop new strategies against cerebral ischemia. Our results provide a foundation for a new pharmacological approach to neuroprotection in patients with a high probability for stroke via improved mitochondrial metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Brant Watson for critical reading of this manuscript and Dr. Jessica Ranieri for technical support.

Sources and Funding This study was supported by PHS grants NS34773, NS05820, NS045676, NS054147 and, AHA Grant 0725314B.

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Abbreviations

ACA	asphyxial cardiac arrest
ADP	adenosine diphosphate
ADP/O	moles of ADP phosphorylated per moles of oxygen consumed
ATP	adenosine triphosphate
BPM	beats per minute
CA1	cornu ammonis 1
cDNA	complementary deoxyribonucleic acid
COX IV	cytochrome c oxidase subunit IV
DMSO	dimethyl sulfoxide
$\Delta\Psi_m$	mitochondrial membrane potential
ECG	electrocardiogram
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
ICV	intracerebral ventricle
i.p.	intraperitoneally
IPC	ischemic preconditioning
i.v.	intra venous
JC1	5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolyl-carbocyanine iodide
MAP	mean artery pressure
NAD⁺	nicotinamide adenine dinucleotide

PCR	polymerase chain reaction
PKC	protein kinase C
RCI	respiratory control index
ROS	reactive oxygen species
ROSC	restoration of spontaneous circulation
RNA	Ribonucleic acid
RPC	resveratrol preconditioning
SD	sprague dawley
SEM	standard error of the mean
Sir2	silent information regulator 2
SIRT1	sirtuin 1
UCP2	uncoupling Protein 2

Figure 1. Schematic diagram of pathways activated by resveratrol induced SIRT1 activation
 Resveratrol leads to SIRT1 activation which in turn decreases UCP2 levels. Subsequently the decrease in UCP2 level in mitochondria is coupled with increased mitochondrial ATP producing capacity.

Figure 2. Experimental design

A **B**

Figure 3. *In vivo* pretreatment with resveratrol mimics ischemic preconditioning in CA1 region of hippocampus

(A) Representative histological images and (B) number of normal neurons in the CA1 region of hippocampus are presented. Scale bar indicates 30 μ m. * P <0.05 as compared to vehicle, and resveratrol 100 mg/Kg of body weight. # P <0.05 as compared to IPC; 10, and 50 mg/Kg of resveratrol.

Figure 4. SIRT1 activity increases in rat hippocampus after IPC and after resveratrol pretreatment. Intracerebral Ventricular (ICV) injection of sirtinol blocks SIRT1 activation following resveratrol pre-treatment

(A) SIRT1 enzyme activity was measured in the rat hippocampus at 1 and 48 hours after IPC or treatment with 10 mg/Kg of body weight of resveratrol. * $P < 0.02$ as compared to sham group. (B) SIRT1 enzyme activity was measured in the rat hippocampus at 1 hour after DMSO ICV, treatment with 10 mg/Kg of body weight of resveratrol + sirtinol ICV, and sirtinol ICV alone. * $P < 0.02$ as compared to control group.

Figure 5. Sirtinol blocks resveratrol induced neuroprotection

(A) Representative histological images and (B) number of normal neurons in the CA1 region of hippocampus are presented. Scale bar indicates 30 μ m. * P <0.05 as compared to sirtinol + ACA, DMSO +ACA; and resveratrol 10 mg/Kg + sirtinol groups. # P <0.05 as compared to sirtinol ICV; and resveratrol 10 mg/Kg + DMSO groups.

Figure 6. Resveratrol pretreatment decreases UCP2 levels after 48 hours in mitochondria isolated from rat hippocampus

(A) UCP2 levels were measured in mitochondria isolated from rat brain hippocampus at 48 hours after treatment with vehicle and resveratrol (10 mg/Kg). (B) Sirtinol blocks the resveratrol-induced decrease in UCP2. The following treatments were used: resveratrol (10 mg/Kg) + intracerebral ventricular injection (ICV) of DMSO (sirtinol vehicle), and resveratrol (10 mg/Kg) + 3.94 μ g sirtinol ICV. * P <0.02 as compared with sham.

Figure 7. Effect of resveratrol on mitochondrial physiology

(A) $\Delta\Psi_m$, (B) state 3 and 4 respiration rates, (C) respiratory coefficient index (RCI), (D) typical polarographic traces. Arrows A and B indicate addition of pyruvate + malate, and adenosine diphosphate, respectively. (E) ADP/O ratio (ratio of rates of ATP synthesis and oxygen consumption) were measured in hippocampal mitochondria isolated at 48 hours after RPC (10 mg/Kg), vehicle treatment, and RPC + sirtinol ICV. * $P<0.02$ as compared with vehicle; and * $P<0.05$ as compared with RPC + sirtinol ICV.