

# Resveratrol Preconditioning Induces a Novel Extended Window of Ischemic Tolerance in the Mouse Brain

Kevin B. Koronowski, BS; Kunjan R. Dave, PhD; Isabel Saul, BS; Vladimir Camarena, PhD; John W. Thompson, PhD; Jake T. Neumann, PhD; Juan I. Young, PhD; Miguel A. Perez-Pinzon, PhD

**Background and Purpose**—Prophylactic treatments that afford neuroprotection against stroke may emerge from the field of preconditioning. Resveratrol mimics ischemic preconditioning, reducing ischemic brain injury when administered 2 days before global ischemia in rats. This protection is linked to silent information regulator 2 homologue 1 (Sirt1) and enhanced mitochondrial function possibly through its repression of uncoupling protein 2. Brain-derived neurotrophic factor (BDNF) is another neuroprotective protein associated with Sirt1. In this study, we sought to identify the conditions of resveratrol preconditioning (RPC) that most robustly induce neuroprotection against focal ischemia in mice.

**Methods**—We tested 4 different RPC paradigms against a middle cerebral artery occlusion model of stroke. Infarct volume and neurological score were calculated 24 hours after middle cerebral artery occlusion. Sirt1-chromatin binding was evaluated by ChIP-qPCR. Percoll gradients were used to isolate synaptic fractions, and changes in protein expression were determined via Western blot analysis. BDNF concentration was measured using a BDNF-specific ELISA assay.

**Results**—Although repetitive RPC induced neuroprotection from middle cerebral artery occlusion, strikingly one application of RPC 14 days before middle cerebral artery occlusion showed the most robust protection, reducing infarct volume by 33% and improving neurological score by 28%. Fourteen days after RPC, Sirt1 protein was increased 1.5-fold and differentially bound to the uncoupling protein 2 and BDNF promoter regions. Accordingly, synaptic uncoupling protein 2 level decreased by 23% and cortical BDNF concentration increased 26%.

**Conclusions**—RPC induces a novel extended window of ischemic tolerance in the brain that lasts for at least 14 days. Our data suggest that this tolerance may be mediated by Sirt1 through upregulation of BDNF and downregulation of uncoupling protein 2. (*Stroke*. 2015;46:2293-2298. DOI: 10.1161/STROKEAHA.115.009876.)

**Key Words:** BDNF ■ cerebral ischemia ■ preconditioning ■ resveratrol ■ UCP2

With few and limited clinically approved treatments, ischemic stroke remains a devastating pathology that plagues the world population.<sup>1</sup> Our ability to predict those who are predisposed to or at risk for stroke has significantly improved in recent years, making prophylactic treatment a viable option for neuroprotection. There is an identifiable population in the millions that are at an increased risk for stroke and are ideal candidates for prophylactic therapeutic intervention. Indeed, studies have demonstrated the immense potential of a prophylactic approach, using forthcoming pharmacological agents derived from the field of ischemic preconditioning (IPC).

IPC refers to the ability of a brief, nondamaging ischemic episode to induce tolerance against a subsequent, damaging ischemic insult.<sup>2</sup> Ischemic tolerance is characterized to have an early window of protection, which is activated immediately and lasts for several hours, as well as a late window of protection, which is active 2 days later.<sup>2</sup> Several pharmacological agents can mimic the effects of IPC, including resveratrol.<sup>3</sup>

Resveratrol is a polyphenol synthesized naturally in several species of plants.<sup>4</sup> The therapeutic potential of resveratrol has been widely investigated in many pathological conditions,<sup>5</sup> although the investigation of resveratrol as an agent in stroke treatment is still in its infancy.

Similar to IPC, resveratrol preconditioning (RPC) induces ischemic tolerance, at least in part, by activation of silent information regulator 2 homologue 1 (Sirt1).<sup>3,6</sup> Sirt1 is a versatile NAD<sup>+</sup>-dependent deacetylase that acts on both histone and nonhistone proteins to regulate many biological processes. Additionally, Sirt1 has a host of neuroprotective properties in pathological states, such as neurological disorders.<sup>7</sup> More recently, Sirt1 has been implicated in cerebral ischemia, fostering ischemic tolerance through common targets, such as HIF, PGC1 $\alpha$ , and p53.<sup>8</sup> Additional targets of Sirt1 include mitochondrial uncoupling protein 2 (UCP2), a proton channel found in the inner mitochondrial membrane that uncouples oxidative phosphorylation,<sup>6</sup> and brain-derived neurotrophic

Received April 24, 2015; final revision received May 21, 2015; accepted June 11, 2015.

From the Cerebral Vascular Disease Research Laboratories (K.B.K., K.R.D., I.S., J.W.T., J.T.N., M.A.P.-P.), Department of Neurology (K.B.K., K.R.D., I.S., J.W.T., J.T.N., M.A.P.-P.), and Neuroscience Program (K.B.K., K.R.D., J.I.Y., M.A.P.-P.), HUSSMAN Institute for Human Genetics (V.C., J.I.Y.), University of Miami Miller School of Medicine, Miami, FL.

The online-only Data Supplement is available with this article at <http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.115.009876/-/DC1>.

Correspondence to Miguel A. Perez-Pinzon, PhD, Department of Neurology, D4-5, University of Miami, Miller School of Medicine, PO Box 016960, Miami, FL 33101. E-mail [perezpinzon@miami.edu](mailto:perezpinzon@miami.edu)

© 2015 American Heart Association, Inc.

*Stroke* is available at <http://stroke.ahajournals.org>

DOI: 10.1161/STROKEAHA.115.009876

factor (BDNF),<sup>9</sup> a growth factor involved in many neuronal processes, including synaptic plasticity.

Our previous work demonstrates that RPC reduces ischemic brain injury when administered 2 days before cardiac arrest in rats<sup>6</sup> as well as middle cerebral artery occlusion (MCAo) in mice.<sup>9a</sup> In terms of neuronal function, ischemic tolerance was associated with an increase in Sirt1 activity, a decrease in UCP2, and enhanced mitochondrial function in the hippocampus. The goal of this study was to identify the conditions of RPC that most robustly induce neuroprotection against focal cerebral ischemia in mice and investigate further the contribution of the Sirt1 signaling axis.

## Materials and Methods

Full descriptions of the methods sections can be found in the online-only Data Supplement.

### Animals and Treatments

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Miami and were in accordance with institutional regulations. Eight- to 12-week-old C57Bl/6J male mice obtained from The Jackson Laboratory (n=108) were randomly assigned to different treatment groups. Mice were injected IP with 10 mg/kg transresveratrol (3,4',5-trihydroxy-*trans*-stilbene; Sigma) in 1.5% dimethyl sulfoxide (DMSO; Sigma) and 0.9% saline (Hospira Inc; RPC) or the control 1.5% DMSO and 0.9% saline (Veh). All injections, experimental procedures, and analysis throughout this study were conducted in a blinded fashion.

### Focal Cerebral Ischemia by Reversible MCAo

Right-side MCAo was produced as previously described.<sup>10</sup> Cerebral blood flow was monitored using a Laser-Doppler (Perimed System, Stockholm, Sweden) probe. MCAo was induced by inserting a silicone-coated 8-0 monofilament nylon surgical suture into the internal carotid artery and circle of Willis via the proximal external carotid. After 60 minutes of MCAo, the suture was removed.

### Neurological Scoring and Infarct Volume

Twenty-four hours after MCAo, animals were scored based on a neurobehavioral battery,<sup>10</sup> and infarct volume was quantified by TTC staining<sup>11</sup> as previously described.

### Chromatin Immunoprecipitation and Subsequent Quantitative PCR

Mouse brain cortices were dissected, frozen, pulverized in liquid nitrogen, and resuspended in cross-linking solution (1% formaldehyde). Nuclei were lysed, chromatin was sonicated to 200 to 500 bp fragments, centrifuged, and diluted in ChIP dilution buffer. The sonicated, cleared chromatin supernatant was used for immunoprecipitation with 5 µg of anti-Sirt1 antibody or control IgG overnight at 4°C. Beads were washed sequentially with 1 mL each of low salt wash, high salt wash, LiCl wash, and TE wash. Beads were resuspended in elution buffer and cross-linking was reversed. DNA was purified by phenol–chloroform extraction and EtOH precipitation. Quantitative real-time PCR was performed using SYBR Green reagent (Roche) in the LightCycler 480 II (Roche Applied Science) and analyzed with the  $\Delta\Delta$ CT method. DNA-relative enrichment was determined by normalizing to input genomic DNA and preserum IgG as background.

### Isolation of Synaptosomes via Percoll Gradient Fractionation

Percoll gradients were used to isolate synaptosomes according to Dunkley and colleagues<sup>12</sup> with slight modifications. Mice were decapitated under isoflurane anesthesia and their cortices immersed in isolation

medium at 4°C. Tissue was chopped, homogenized, diluted to give 10% wt/vol and centrifuged at 500g for 5 minutes (Sorvall RC5 centrifuge, Newton CT). The supernatant was layered on a Percoll (Sigma) gradient and centrifuged at 32500g for 5 minutes. Synaptosomes were collected between 23% to 15% and 15% to 10% Percoll junctions, washed once with isolation medium by centrifugation at 15000g for 10 minutes and once with 0.25 mol/L sucrose. The final pellet was resuspended in radioimmunoprecipitation assay buffer and processed for protein analysis according to the Western blot protocol.

### Western Blotting

Mouse cortices were homogenized in radioimmunoprecipitation assay buffer. Protein concentration was measured by Bradford assay (BioRad). Equal amounts of protein were run on 12% SDS-polyacrylamide gels and transferred to a nitro-cellulose membrane (BioRad). The next steps in order were block 2 hours at room temperature; probe with primary antibody overnight at 4°C; incubate secondary antibody for 1 hour at RT; addition of substrate for 3 minutes; protein bands detected with x-ray film (Denville Scientific). Films were analyzed densitometrically using Image J. Two normalized values (for the same sample) from different blots were averaged to give the values presented.

### BDNF ELISA Assay

BDNF concentration was quantified using a ChemiKine™ BDNF Sandwich ELISA Kit (Millipore) according to the manufacturer's instructions.

### Statistical Analysis

All data are represented as mean±SEM. For statistical analysis, Student's *t*-tests were used to compare differences between 2 groups. Differences were considered significant at the 95% confidence interval (\**P*<0.05, \*\**P*<0.01).

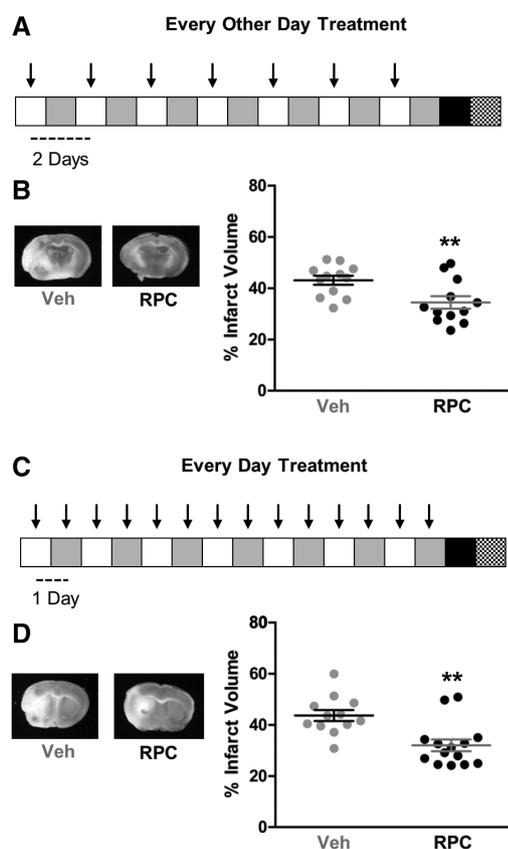
## Results

### Repetitive RPC Treatment Confers Ischemic Tolerance Against Focal Ischemia

Based on our previous findings that RPC induces ischemic tolerance when administered 2 days before injury,<sup>3,6</sup> we sought to evaluate RPC in the context of focal cerebral ischemia using the MCAo mouse model. Our first hypothesis was that repetitive RPC over an extended period of time induces ischemic neuroprotection. To this end, mice were given RPC or Veh every other day for 14 days before MCAo, mimicking the standard preconditioning paradigm repeated 7 times (Figure 1A). Animals that did not display ≥80% reduction in cerebral blood flow were excluded—full documentation of cerebral blood flow, exclusion, and mortality can be found in Table. Compared with Veh, RPC significantly reduced infarct volume by 20% (*P*<0.01, Veh n=12, RPC n=12; Figure 1B). Next, we hypothesized that increasing the frequency of RPC treatment could enhance this protection. Thus, mice were given RPC or Veh every day for 14 days before MCAo (Figure 1C). Similar to every other day treatment, everyday treatment significantly reduced infarct volume by 27% compared with Veh (*P*<0.01, Veh n=12, RPC n=14; Figure 1D). From this, we conclude that repetitive RPC induces ischemic tolerance against focal ischemia.

### RPC Induces a Novel Extended Window of Ischemic Tolerance Against Focal Ischemia

Because there was no significant difference between the protection afforded by every other day and every day treatment, we rationalized that perhaps less frequent RPC could also



**Figure 1.** Repetitive resveratrol preconditioning (RPC) induces ischemic tolerance against focal ischemia. **A**, Every other day treatment schematic. **B**, Every other day RPC reduces infarct volume. Veh n=12; RPC n=12; \*\* $P<0.01$  compared with Veh. **C**, Every day treatment schematic. **D**, Every day RPC reduces infarct volume similar to every other day RPC. Veh n=12; RPC n=14; \*\* $P<0.01$  compared with Veh. Individual arrows indicate an RPC or Veh treatment; middle cerebral artery occlusion (MCAo) is indicated by the black square; TTC is indicated by the checkered square. Representative TTC-stained brain sections are shown on the left.

provide ischemic tolerance. Our previous studies show that IPC induces ischemic tolerance that lasts for at least 4 days in organotypic slice cultures.<sup>3</sup> Therefore, our next hypothesis

was that intermittent RPC could induce an extended window of ischemic tolerance. To test this hypothesis, we used a geometric growth pattern of intermittent RPC consisting of 4 applications at intervals of 1, 2, 4, and 8 days, with 14 days between the last treatment and MCAo (Figure 2A). Strikingly, compared with Veh, intermittent RPC significantly reduced infarct volume by 24% ( $P<0.01$ , Veh n=15, RPC n=16; Figure 2B). Before any conclusions can be drawn from this intermittent RPC paradigm, an important distinction to make is whether it is the culmination of 4 applications of RPC that give rise to this long-lasting ischemic tolerance or if simply one application of RPC can induce ischemic tolerance for this extended period of time. To test this, mice were given RPC or Veh once, 14 days before MCAo (Figure 2C). Remarkably, one application of RPC 14 days before MCAo significantly reduced infarct volume by 33% compared with Veh ( $P<0.01$ , Veh n=9, RPC n=10; Figure 2D). To confirm that this reduction in infarct volume was accompanied by improved neurological performances, we calculated neurological score 24 hours after MCAo and found that compared with Veh, RPC significantly improved this functional outcome measure by 28% ( $P<0.05$ ; Figure 2D). From these results, we conclude that a single application of RPC induces a novel extended window of ischemic tolerance that lasts for at least 14 days.

### Sirt1 and Its Targets Are Augmented 14 Days Following RPC

Given that Sirt1 is a well-established target of resveratrol and that in our previous studies blockade of Sirt1 ablates ischemic tolerance,<sup>3,6</sup> we investigated whether changes in Sirt1 expression occurred in this extended window of ischemic tolerance. Fourteen days after a single application of RPC, cortical Sirt1 levels were significantly increased 1.5-fold of Veh ( $P<0.01$ , n=10; Figure 3A). To assess whether this increase had functional implications, we turned our attention to putative chromatin binding sites for Sirt1. Based on known interactions with Sirt1<sup>13,14</sup> and our previous studies implicating BDNF<sup>9</sup> and UCP2<sup>6</sup> in ischemic tolerance, we evaluated Sirt1 binding to promoter regions within the BDNF and UCP2 genes. Chromatin immunoprecipitation and subsequent qPCR 14 days after a

**Table. MCAo CBF,\* Exclusion,† and Mortality‡**

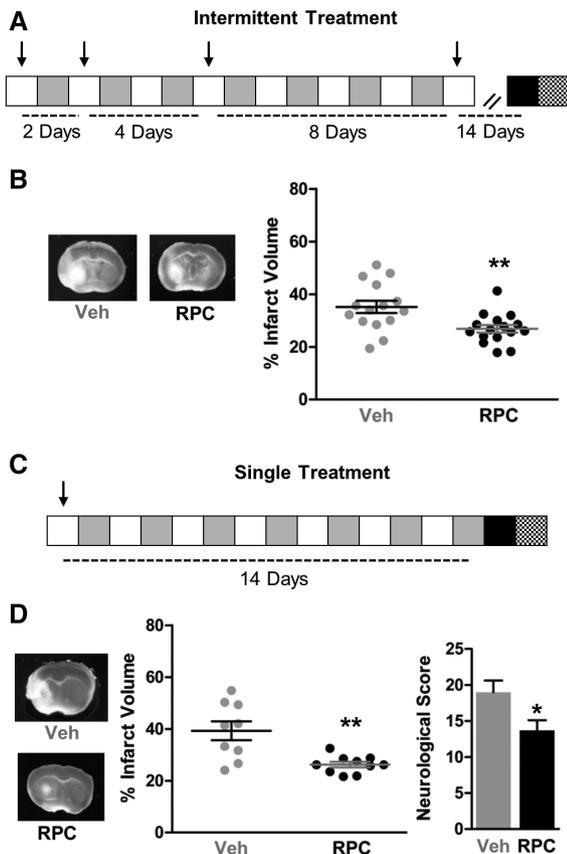
Group	Baseline CBF	Ischemia CBF	Reperfusion CBF	Excluded (n)	Mortality (n)
Every other day Veh	100	11.9±4.46	62.2±25.32	0	6
Every other day RPC	100	10.0±4.09	75.4±56.28	4	2
Every day Veh	100	11.9±4.89	72.2±23.93	3	2
Every day RPC	100	9.7±3.89	59.4±36.87	2	2
Intermittent Veh	100	11.3±3.17	67.4±28.34	2	6
Intermittent RPC	100	11.5±3.76	68.5±28.14	4	4
Single Veh	100	11.61±3.36	65.43±17.33	1	0
Single RPC	100	11.35±5.32	71.24±22.27	0	1
Total				16	23

CBF indicates cerebral blood flow; MCAo, middle cerebral artery occlusion; and RPC, resveratrol preconditioning.

\*Laser-Doppler flowmetry was used to measure CBF for inclusion/exclusion. Measurements are taken (1) baseline before occlusion, (2) ischemia immediately after occlusion is initiated, and (3) reperfusion immediately after occlusion is removed. Values are represented as percent of baseline±SD.

†Mice were excluded from the study if they did not reach ≤80% drop in blood flow from baseline.

‡Mortality figures illustrate mice who died <24 h after MCAo.



**Figure 2.** Resveratrol preconditioning (RPC) induces a novel extended window of ischemic tolerance against focal ischemia. **A**, Intermittent treatment schematic. **B**, Intermittent RPC reduces infarct volume. Veh  $n=15$ ; RPC  $n=16$ ;  $**P<0.01$  compared with Veh. **C**, Single treatment schematic. **D**, A single application of RPC reduces infarct volume and improves neurological score. Veh  $n=9$ ; RPC  $n=10$ ;  $*P<0.05$ ;  $**P<0.01$  compared with Veh. Individual arrows indicate an RPC or Veh treatment; middle cerebral artery occlusion (MCAO) is indicated by the black square; TTC and neurological scoring is indicated by the checkered square. Representative TTC-stained brain sections are shown on the left.

single application of RPC revealed that Sirt1 binding to both the BDNF and UCP2 promoters was significantly enhanced, 2.66-fold and 1.14-fold, respectively (BDNF  $P<0.01$ , UCP2  $P<0.05$ ,  $n=3$ ; Figure 3B). This enhancement was not seen in Veh-treated mice. Accordingly, we found that cortical BDNF levels were significantly increased by 27% ( $P<0.05$ ,  $n=7$ ) and synaptic UCP2 levels were significantly decreased by 23% ( $P<0.05$ ,  $n=6$ ) compared with Veh 14 days after a single application of RPC (Figure 3C and 3D). These data suggest that Sirt1 may be modulating BDNF and UCP2 expression at 14 days where ischemic tolerance is observed.

## Discussion

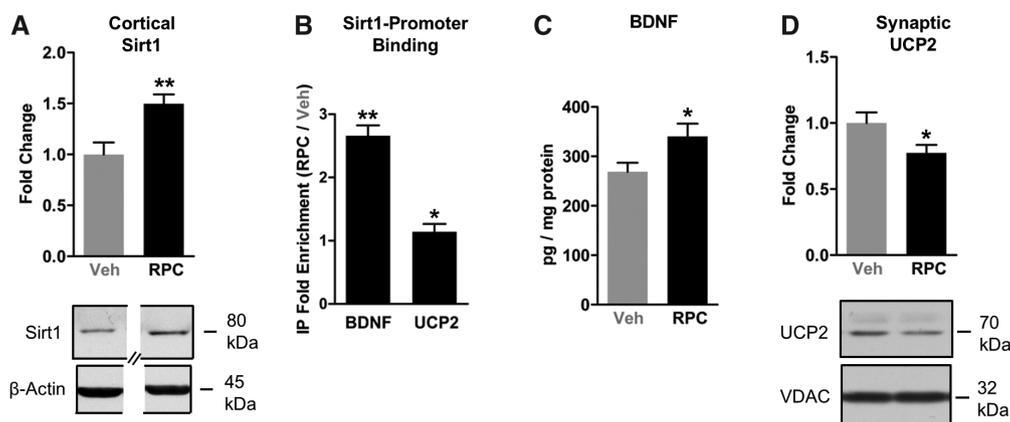
The major finding of this study—that a single application of RPC can protect against focal ischemia 14 days later—is both novel and intriguing. To our knowledge, this is the first evidence that a single pharmacological preconditioning treatment can induce a state of long-lasting ischemic tolerance. This is a new extended window of preconditioning beyond the 2 to 3 day time point or classical late window commonly studied.

In work before this study, evidence hinted that an extended window may exist. In organotypic slices, we showed that one application of IPC affords protection that lasts at least 4 days.<sup>3</sup> Additionally, a paradigm of repetitive hypoxia preconditioning (varying O<sub>2</sub> exposure over 2 weeks) conferred protection from focal ischemia for  $\leq 2$  months after treatment.<sup>15</sup> Elucidating the long-term effects of preconditioning will provide a framework for the development of novel clinical applications in the future. The major argument for resveratrol's immense potential is that it has already been deemed safe for clinical use in humans.<sup>16</sup>

Resveratrol either directly or indirectly activates or upregulates Sirt1.<sup>17,18</sup> Previously we have shown increased Sirt1 to be a common mechanism of preconditioning-mediated protection, where protection was lost in the presence of the Sirtuin inhibitor Sirtinol.<sup>3</sup> The proposed acute mechanisms for Sirt1-mediated ischemic tolerance include modulating the neuroinflammatory response via NF- $\kappa$ B,<sup>19</sup> reducing/inhibiting proapoptotic factors, such as p53,<sup>20</sup> as well as enhancing mitochondrial function via PGC1 $\alpha$ <sup>21</sup> and UCP2.<sup>6</sup> Here, we found Sirt1 to be upregulated at 14 days after a single RPC treatment. A chronic upregulation of Sirt1 could promote changes in gene expression that are maintained overtime that give rise to this extended window of ischemia tolerance. In line with this notion, 14 days after RPC, we find enhanced Sirt1 binding to UCP2 and BDNF promoter regions.

As mentioned earlier, Sirt1 is known to negatively regulate UCP2 by binding directly to its promoter.<sup>14</sup> This is supported in our current study as we observed a decrease in synaptic UCP2 protein levels. In our previous studies, we observed a reduction in UCP2 2 days after RPC in rats where protection from cardiac arrest was seen. Neuroprotection and decreased UCP2 were also linked to enhanced mitochondrial efficiency.<sup>6</sup> In another line of evidence, UCP2 knockout mice were found to be resistant to permanent focal ischemia.<sup>22</sup> In these mice, protection was attributed to increased antioxidant defenses and a reduction in oxidative damage. It should be noted that UCP2's effects in regards to cerebral ischemia are still somewhat controversial. Other groups have shown that overexpression of UCP2 is also protective against cerebral ischemia<sup>23</sup> and that the same UCP2 knockout mice mentioned above were actually more susceptible to a model of transient focal ischemia.<sup>24</sup> Discrepancies could be as a result of variation across species, genetic compensation in knockout mouse models, the type of ischemia model used, or choice of preconditioning agent.

Sirt1 is also known to upregulate BDNF expression; the proposed mechanisms are indirect and include Sirt1 deacetylation of MeCP2 (which regulates expression of BDNF by binding at its promoter)<sup>25</sup> and Sirt1 inhibition of miR-134 expression (a micro-RNA that downregulates expression of BDNF).<sup>13</sup> In addition, binding of Sirt1 to the BDNF promoter has been identified in the mouse cerebellum, suggesting that a direct interaction might also occur.<sup>26</sup> Moreover, BDNF was decreased in the brains of Sirt1 null mice.<sup>27</sup> BDNF is an important growth factor that promotes survival and growth of existing neurons, as well as the differentiation of new ones. It also impacts synaptic plasticity and learning and memory.<sup>28</sup> Our previous work shows enhanced BDNF as a mechanism



**Figure 3.** Silent information regulator 2 homologue 1 (Sirt1) and its targets are differentially regulated 14 days after a single application of resveratrol preconditioning (RPC). **A**, Cortical Sirt1 protein was increased 14 days after a single application of RPC. Representative bands for Sirt1 are shown below the quantification graph. #Nonadjacent bands from the same blot.  $n=10$ ;  $**P<0.01$  compared with Veh. **B**, ChIP-qPCR analysis revealed Sirt1 binding to brain-derived neurotrophic factor (BDNF), and uncoupling protein 2 (UCP2) promoter regions was enhanced 14 days after a single application of RPC.  $n=3$ ;  $*P<0.05$ ;  $**P<0.01$ . **C**, Cortical BDNF levels were increased 14 days after a single application of RPC. Values are expressed as pg/mg of protein.  $n=7$ ;  $*P<0.05$ . **D**, Synaptic UCP2 protein levels were decreased 14 days after a single application of RPC. Values were normalized to the mitochondrial marker voltage dependent anion channel (VDAC). The functional UCP2 dimer (70 kDa) was used for quantification; no 35 kDa monomer band was detected. Representative band are shown below the quantification.  $n=6$ ;  $*P<0.05$ .

for ischemia tolerance after IPC or protein kinase C epsilon-induced preconditioning,<sup>9</sup> in which increased BDNF was associated with a delay in anoxic depolarization. Another group shows that intravenous BDNF administration after ischemia improves recovery possibly through a mechanism centered on neurogenesis.<sup>29</sup> Given that all of these changes seem to occur homogeneously throughout the cortex, we expect them to impact both the core and penumbral regions of the infarct.

The fact that one dose of resveratrol has protective effects that are apparent 2 weeks later suggests a promising prophylactic avenue. The validity of a preconditioning approach in the clinic was nicely described by Dirnagl and colleagues<sup>30</sup> who defined clinical scenarios where a preconditioning approach could have a direct impact. Resveratrol also protects against stroke when administered post-injury. Although not tested as a postconditioning paradigm, future studies are needed to test whether resveratrol could be used in a postconditioning setting. Also future studies can test whether pre- and post-resveratrol treatment in tandem can enhance neuroprotection.

Resveratrol has been used in several clinical trials and deemed safe for use in humans at doses up to a few grams per day. Our finding that tachyphylaxis to RPC did not occur when administered on an everyday basis suggests that multiple administrations are feasible, if required. Additionally, oral administration of resveratrol as a neuroprotective intervention in rodents has been validated by several groups and seems similar to that of intraperitoneal or intravenous administration.<sup>31</sup> Taken together, our current findings build on the body of preclinical evidence for resveratrol as a potential treatment in the stroke clinic.

### Sources of Funding

National Institute of Health Grants NS45676, NS054147, NS34773 (M.A. Perez-Pinzon), 1F31NS089356-01 (K.B. Koronowski), and 5R01NS073779-04 (K.R. Dave) and American Heart Association Grant 13POST16720001 (J.T. Neumann).

### Disclosures

None.

### References

- Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2013 update: a report from the American Heart Association. *Circulation*. 2013;127:e6–e245. doi: 10.1161/CIR.0b013e31828124ad.
- Gidday JM. Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci*. 2006;7:437–448. doi: 10.1038/nrn1927.
- Raval AP, Dave KR, Pérez-Pinzón MA. Resveratrol mimics ischemic preconditioning in the brain. *J Cereb Blood Flow Metab*. 2006;26:1141–1147. doi: 10.1038/sj.jcbfm.9600262.
- Carrizzo A, Forte M, Damato A, Trimarco V, Salzano F, Bartolo M, et al. Antioxidant effects of resveratrol in cardiovascular, cerebral and metabolic diseases. *Food Chem Toxicol*. 2013;61:215–226. doi: 10.1016/j.fct.2013.07.021.
- Smoliga JM, Baur JA, Hausenblas HA. Resveratrol and health—a comprehensive review of human clinical trials. *Mol Nutr Food Res*. 2011;55:1129–1141. doi: 10.1002/mnfr.201100143.
- Della-Morte D, Dave KR, DeFazio RA, Bao YC, Raval AP, Perez-Pinzon MA. Resveratrol pretreatment protects rat brain from cerebral ischemic damage via a sirtuin 1-uncoupling protein 2 pathway. *Neuroscience*. 2009;159:993–1002. doi: 10.1016/j.neuroscience.2009.01.017.
- Zhang F, Wang S, Gan L, Vosler PS, Gao Y, Zigmund MJ, et al. Protective effects and mechanisms of sirtuins in the nervous system. *Prog Neurobiol*. 2011;95:373–395. doi: 10.1016/j.pneurobio.2011.09.001.
- Morris KC, Lin HW, Thompson JW, Perez-Pinzon MA. Pathways for ischemic cytoprotection: role of sirtuins in caloric restriction, resveratrol, and ischemic preconditioning. *J Cereb Blood Flow Metab*. 2011;31:1003–1019. doi: 10.1038/jcbfm.2010.229.
- Neumann JT, Thompson JW, Raval AP, Cohan CH, Koronowski KB, Perez-Pinzon MA. Increased BDNF protein expression after ischemic or PKC epsilon preconditioning promotes electrophysiologic changes that lead to neuroprotection. *J Cereb Blood Flow Metab*. 2015;35:121–130. doi: 10.1038/jcbfm.2014.185.
- Narayanan SV, Dave KR, Saul I, Perez-Pinzon MA. Resveratrol preconditioning protects against cerebral ischemic injury via nuclear erythroid 2-related factor 2. *Stroke*. 2015;46:1626–1632.
- Belayev L, Busto R, Zhao W, Fernandez G, Ginsberg MD. Middle cerebral artery occlusion in the mouse by intraluminal suture coated with poly-L-lysine: neurological and histological validation. *Brain Res*. 1999;833:181–190.

11. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, et al. Toll-like receptor 9: a new target of ischemic preconditioning in the brain. *J Cereb Blood Flow Metab.* 2008;28:1040–1047. doi: 10.1038/sj.jcbfm.9600606.
12. Dunkley PR, Jarvie PE, Robinson PJ. A rapid Percoll gradient procedure for preparation of synaptosomes. *Nat Protoc.* 2008;3:1718–1728. doi: 10.1038/nprot.2008.171.
13. Gao J, Wang WY, Mao YW, Gräff J, Guan JS, Pan L, et al. A novel pathway regulates memory and plasticity via SIRT1 and miR-134. *Nature.* 2010;466:1105–1109. doi: 10.1038/nature09271.
14. Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, et al. Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. *PLoS Biol.* 2006;4:e31. doi: 10.1371/journal.pbio.0040031.
15. Stowe AM, Altay T, Freie AB, Gidday JM. Repetitive hypoxia extends endogenous neurovascular protection for stroke. *Ann Neurol.* 2011;69:975–985. doi: 10.1002/ana.22367.
16. Patel KR, Scott E, Brown VA, Gescher AJ, Steward WP, Brown K. Clinical trials of resveratrol. *Ann N Y Acad Sci.* 2011;1215:161–169. doi: 10.1111/j.1749-6632.2010.05853.x.
17. Dai H, Kustigian L, Carney D, Case A, Considine T, Hubbard BP, et al. SIRT1 activation by small molecules: kinetic and biophysical evidence for direct interaction of enzyme and activator. *J Biol Chem.* 2010;285:32695–32703. doi: 10.1074/jbc.M110.133892.
18. Ghosh S, Liu B, Zhou Z. Resveratrol activates SIRT1 in a Lamin A-dependent manner. *Cell Cycle.* 2013;12:872–876. doi: 10.4161/cc.24061.
19. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.* 2004;23:2369–2380. doi: 10.1038/sj.emboj.7600244.
20. Vahtola E, Louhelainen M, Forstén H, Merasto S, Raivio J, Kaheinen P, et al. Sirtuin1-p53, forkhead box O3a, p38 and post-infarct cardiac remodeling in the spontaneously diabetic Goto-Kakizaki rat. *Cardiovasc Diabetol.* 2010;9:5. doi: 10.1186/1475-2840-9-5.
21. Zhu HR, Wang ZY, Zhu XL, Wu XX, Li EG, Xu Y. Icaritin protects against brain injury by enhancing SIRT1-dependent PGC-1alpha expression in experimental stroke. *Neuropharmacology.* 2010;59:70–76. doi: 10.1016/j.neuropharm.2010.03.017.
22. de Bilbao F, Arsenijevic D, Vallet P, Hjelle OP, Ottersen OP, Bouras C, et al. Resistance to cerebral ischemic injury in UCP2 knockout mice: evidence for a role of UCP2 as a regulator of mitochondrial glutathione levels. *J Neurochem.* 2004;89:1283–1292. doi: 10.1111/j.1471-4159.2004.02432.x.
23. Mattiasson G, Shamloo M, Gido G, Mathi K, Tomasevic G, Yi S, et al. Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. *Nat Med.* 2003;9:1062–1068. doi: 10.1038/nm903.
24. Haines BA, Mehta SL, Pratt SM, Warden CH, Li PA. Deletion of mitochondrial uncoupling protein-2 increases ischemic brain damage after transient focal ischemia by altering gene expression patterns and enhancing inflammatory cytokines. *J Cereb Blood Flow Metab.* 2010;30:1825–1833. doi: 10.1038/jcbfm.2010.52.
25. Zocchi L, Sassone-Corsi P. SIRT1-mediated deacetylation of MeCP2 contributes to BDNF expression. *Epigenetics.* 2012;7:695–700. doi: 10.4161/epi.20733.
26. MacIsaac KD, Lo KA, Gordon W, Motola S, Mazor T, Fraenkel E. A quantitative model of transcriptional regulation reveals the influence of binding location on expression. *PLoS Comput Biol.* 2010;6:e1000773. doi: 10.1371/journal.pcbi.1000773.
27. Libert S, Pointer K, Bell EL, Das A, Cohen DE, Asara JM, et al. SIRT1 activates MAO-A in the brain to mediate anxiety and exploratory drive. *Cell.* 2011;147:1459–1472. doi: 10.1016/j.cell.2011.10.054.
28. Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Prog Neurobiol.* 2005;76:99–125. doi: 10.1016/j.pneurobio.2005.06.003.
29. Schäbitz WR, Steigleder T, Cooper-Kuhn CM, Schwab S, Sommer C, Schneider A, et al. Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. *Stroke.* 2007;38:2165–2172. doi: 10.1161/STROKEAHA.106.477331.
30. Dirnagl U, Becker K, Meisel A. Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. *Lancet Neurol.* 2009;8:398–412. doi: 10.1016/S1474-4422(09)70054-7.
31. Singh N, Agrawal M, Doré S. Neuroprotective properties and mechanisms of resveratrol in in vitro and in vivo experimental cerebral stroke models. *ACS Chem Neurosci.* 2013;4:1151–1162. doi: 10.1021/cn400094w.

## Resveratrol Preconditioning Induces a Novel Extended Window of Ischemic Tolerance in the Mouse Brain

Kevin B. Koronowski, Kunjan R. Dave, Isabel Saul, Vladimir Camarena, John W. Thompson, Jake T. Neumann, Juan I. Young and Miguel A. Perez-Pinzon

*Stroke*. 2015;46:2293-2298; originally published online July 9, 2015;  
doi: 10.1161/STROKEAHA.115.009876

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231  
Copyright © 2015 American Heart Association, Inc. All rights reserved.  
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://stroke.ahajournals.org/content/46/8/2293>

Data Supplement (unedited) at:

<http://stroke.ahajournals.org/content/suppl/2015/07/09/STROKEAHA.115.009876.DC1.html>

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

**Reprints:** Information about reprints can be found online at:  
<http://www.lww.com/reprints>

**Subscriptions:** Information about subscribing to *Stroke* is online at:  
<http://stroke.ahajournals.org/subscriptions/>

## SUPPLEMENTAL MATERIAL

### Supplemental Methods:

#### Focal cerebral ischemia by reversible middle cerebral artery occlusion (MCAo)

Right-side MCAo was produced similarly as previously described<sup>1</sup>. Mice were anesthetized with 3% isoflurane in 100% O<sub>2</sub>, and maintained at 1 – 1.5% throughout the surgery. Rectal and temporalis muscle temperatures were constantly monitored and maintained at 37.0 °C using heating pads (Harvard Apparatus, Holliston, MA, USA). Cerebral blood flow was monitored before, during, and after MCAO using a Laser-Doppler (Perimed System, Stockholm, Sweden) probe over the skull. The right common carotid artery was exposed through a midline neck incision and dissected free from surrounding nerves and fascia. Next, the occipital artery branches of the external carotid artery were dissected and coagulated. MCAO was induced by inserting a silicone-coated 8-0 monofilament nylon surgical suture into the internal carotid artery and circle of Willis via the proximal ECA. After 60 min of MCAO the intraluminal suture was carefully removed, the neck incision was closed, and the animal was kept in a humidified neonatal incubator set at 32 °C for one hour before being returned to its home cage. The surgeon was blinded to each treatment. Animals that did not display a  $\geq 80\%$  reduction in cerebral blood flow were excluded. Full documentation of inclusion, exclusion, mortality (Table 1) and cerebral blood flow (Table 2) can be found in the supplemental information.

#### Neurological scoring

24 hours after MCAO, animals were scored based on a neurobehavioral battery (focal neurological score: movements such as body symmetry, gait, climbing, circling behavior, forelimb symmetry, compulsory circling, and whisker response) as previously described<sup>1</sup>. Focal score ranged from 0 – 28, where 0 is considered normal and 28 indicates severe neurological deficits. The investigator scoring neurological deficits was blinded to the experimental conditions.

#### Quantification of infarct volume

Mice were anesthetized with isoflurane and transcardially perfused with heparinized ice-cold saline. Animals were then decapitated and the brain was rapidly removed, sliced and stained with triphenyl tetrazolium chloride (TTC, Sigma)<sup>2</sup>. Brain slices were fixed in 10% formaldehyde (Corning) and imaged using a flat-bed image scanner. Images were corrected for auto color intensity and contrast. Infarct volume was measured using NIH image (Image J) and correct for edema as previously described<sup>3</sup>, where infarct volume = (contralateral hemisphere volume – volume of noninfarcted tissue of the ipsilateral hemisphere) / (volume of contralateral hemisphere)  $\times 100$ . Infarct volume was quantified by a blinded researcher.

#### Chromatin immunoprecipitation and subsequent quantitative PCR

Mouse brain cortices were dissected, frozen, pulverized in liquid nitrogen and resuspended in cross-linking solution containing 1% formaldehyde in PBS for 10 min at RT. The reaction was stopped by adding glycine to a final concentration of 125 mM and placing the samples on ice for 5 min. After two washes in cold PBS, the pellet was incubated in Cell lysis buffer (20 mM Tris-HCl, pH 8.0, 85 mM KCl, 0.5% NP-40) supplemented with protease inhibitors (1 mM PMSF and Mini Complete Protease Inhibitor Cocktail Tablet (Roche)) on ice for 10 min. After centrifugation, the supernatant was removed and the nuclei were resuspended in Nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS supplemented with protease inhibitors) and incubated for 10 min on ice. Next, chromatin was sonicated to 200-500 bp fragment size, centrifuged at maximum speed for 20 min and diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl,

supplemented with protease inhibitors). Input aliquots were saved at this point. The sonicated chromatin was pre-cleared by incubation with Protein G Magnetic Beads-salmon sperm DNA for 1h rotating at 4°C. The cleared supernatant was set up for chromatin immunoprecipitations with 5 ug of anti-Sirt1 antibody (Millipore) or control IgG overnight at 4°C rotating. The next day, salmon sperm DNA-blocked beads were added to the immunoprecipitation reactions and samples were incubated rotating for an additional hour. Beads were washed sequentially for 5 min at 4C on the rotator with 1 ml each of: Low salt wash (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 150 mM NaCl supplemented with protease inhibitors), High salt wash (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl wash (0.25 M LiCl, 1% NP-40, 1% Na deoxycholate, 1 mM EDTA, 20 mM Tris-HCl, pH 8.), and TE wash (10 mM Tris- HCl, pH 8.0, 1 mM EDTA). Beads were resuspended in 250 µl of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>) and incubated at RT for 15 min with frequent agitation. Eluted samples were re-eluted with another 250 µl of elution buffer. Cross-linking was reversed by adding NaCl to a final concentration of 0.2M and incubating for 4h at 65°C. Samples were treated with proteinase K for 1 h at 45°C. The DNA was purified by phenol-chloroform extraction and EtOH precipitation.

Quantitative real-time PCR was performed with the following primers:

BDNF Forward 5' – TGACAGGCTTGGCTTCTGT – 3'

BDNF Reverse 5' – GGCAGCGAGAGCAGTCCT – 3'

UCP2 Forward 5' – CGGGGCTAAGGAGGATAAAA – 3'

UCP2 Reverse 5' – CGAGGACAAACTCAGCAGGT – 3'

qPCR was carried out in triplicate using SYBR Green reagent (Roche) in the LightCycler® 480 II (Roche Applied Science) machine and analyzed with the  $\Delta\Delta CT$  method. DNA-relative enrichment was determined by normalizing to an input genomic DNA and pre-serum IgG as background control.

#### Isolation of synaptosomes via Percoll gradient fractionation

A Percoll gradient fractionation protocol was used to isolate synaptosomes according to Dunkley and colleagues<sup>4</sup> with slight modifications. Mice were decapitated under isoflurane anesthesia and their cortices immediately immersed in isolation medium at 4°C (250 mM sucrose, 1.0 mM ethylenediaminetetra-acetic acid, and 0.25 mM dithiothreitol at pH 7.4). Tissue was chopped with scissors, rinsed thoroughly in isolation medium and homogenized in a glass Teflon homogenizer with 7 up-and-down strokes. The homogenate was diluted to give 10% w/v and centrifuged at 500 × g for 5 min (Sorvall RC5 centrifuge, Newton CT). The supernatant was layered on a Percoll (Sigma) gradient (prepared in 12.0 ml polycarbonate tubes consisting of 2.0 ml each of 23, 15, 10 and 3% v/v Percoll). The sample layered gradient was centrifuged at 32,500 × g for 5 min. Synaptosomes were then collected between the 23-15% and 15-10% Percoll junctions, washed once with isolation medium by centrifugation at 15,000 × g for 10 min and once with 0.25 M sucrose at the same speed and duration. The final pellet here was resuspended in RIPA buffer (pH 8.0 consisting of 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, supplemented with 1% protease inhibitor cocktail (Sigma), 1% phosphatase inhibitor (PhosphoStop, Roche)) and processed for protein analysis according to the Western blot protocol below.

#### Western blotting

Mice were decapitated under isoflurane anesthesia, brains were removed and cortices dissected in a blinded fashion. This tissue was homogenized in RIPA buffer (see above) and incubated on ice for 30 minutes. Debris was then pelleted by a 12,000 × g spin for 20 minutes at 4°C. Protein

concentration was measured by Bradford assay (BioRad) and equal amounts of protein were run on 12% SDS-polyacrylamide gels. The separated proteins were then transferred to a nitrocellulose membrane (BioRad) and the blot was blocked with 5% blotting grade blocker (BioRad) in TBST (0.1% Tween-20) for 2 hours at room temperature. Probing with primary antibodies was achieved by overnight incubation at 4°C: Sirt1 (1:1000, Santa Cruz); UCP2 (1:500, Calbiochem); VDAC (1:2000, Cell Signaling);  $\beta$ -Actin (1:50,000, Cell Signaling). Next, blots were incubated with species specific HRP-conjugated secondary antibodies (1:5000, GE healthcare) for 1 hr at room temperature. Protein bands were developed by addition of ECL reagents (Thermo) and detected using x-ray film (Denville Scientific). Exposed films were digitalized and blots were analyzed densitometrically using Image J software. Two normalized values (for the same sample) from different blots were averaged to give the values presented.

#### BDNF ELISA assay

BDNF concentration was quantified using a ChemiKine™ BDNF Sandwich ELISA Kit (Millipore) according to the manufacturer's instructions. Samples were processed via the Western blot protocol above. Each sample was first normalized to 5  $\mu\text{g}/\mu\text{l}$  protein then diluted with sample dilution buffer (kit) to give 2.5  $\mu\text{g}/\mu\text{l}$  and 1.25  $\mu\text{g}/\mu\text{l}$ . These two concentrations were used in duplicate to calculate BDNF concentration in pg/ml.

1. Belayev L, Busto R, Zhao W, Fernandez G, Ginsberg MD. Middle cerebral artery occlusion in the mouse by intraluminal suture coated with poly-L-lysine: Neurological and histological validation. *Brain Res.* 1999;833:181-190
2. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, et al. Toll-like receptor 9: A new target of ischemic preconditioning in the brain. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.* 2008;28:1040-1047
3. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.* 1990;10:290-293
4. Dunkley PR, Jarvie PE, Robinson PJ. A rapid percoll gradient procedure for preparation of synaptosomes. *Nature protocols.* 2008;3:1718-1728