Resveratrol Preconditioning Protects Against Cerebral Ischemic Injury via Nuclear Erythroid 2–Related Factor 2

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Background and Purpose—Nuclear erythroid 2 related factor 2 (Nrf2) is an astrocyte-enriched transcription factor that has previously been shown to upregulate cellular antioxidant systems in response to ischemia. Although resveratrol preconditioning (RPC) has emerged as a potential neuroprotective therapy, the involvement of Nrf2 in RPC-induced neuroprotection and mitochondrial reactive oxygen species production after cerebral ischemia remains unclear. The goal of our study was to study the contribution of Nrf2 to RPC and its effects on mitochondrial function.

Methods—We used rodent astrocyte cultures and an in vivo stroke model with RPC. An Nrf2 DNA binding ELISA and protein analysis via Western blotting of downstream Nrf2 targets were performed to determine RPC-induced activation of Nrf2 in rat and mouse astrocytes. After RPC, mitochondrial function was determined by measuring reactive oxygen species production and mitochondrial respiration in both wild-type and Nrf2−/− mice. Infarct volume was measured to determine neuroprotection, whereas protein levels were measured by immunoblotting.

Results—We report that Nrf2 is activated by RPC in rodent astrocyte cultures, and that loss of Nrf2 reduced RPC-mediated neuroprotection in a mouse model of focal cerebral ischemia. In addition, we observed that wild-type and Nrf2−/− cortical mitochondria exhibited increased uncoupling and reactive oxygen species production after RPC treatments. Finally, Nrf2−/− astrocytes exhibited decreased mitochondrial antioxidant expression and were unable to upregulate cellular antioxidants after RPC treatment.

Conclusions—Nrf2 contributes to RPC-induced neuroprotection through maintaining mitochondrial coupling and antioxidant protein expression. (Stroke. 2015;46:1626-1632. DOI: 10.1161/STROKEAHA.115.008921.)

Key Words: ischemic preconditioning • mitochondria • reactive oxygen species • resveratrol

In the United States, 1 in 20 deaths can be attributed to stroke, whereas >80% of the 800,000 people who experience stroke each year survive and require long-term rehabilitation.1 Ischemic preconditioning has emerged as a potential therapy that could mitigate the morbidity of cerebral ischemic injury; previous studies from our group have shown that ischemic preconditioning treatment induced neuroprotection in rodent models of global cerebral ischemia.2 This protection has been recapitulated using the polyphenolic compound resveratrol as a pharmacological preconditioning agent.4 The use of resveratrol as a preconditioning agent has been substantiated by numerous studies in a diverse range of in vitro and in vivo models.5,6

However, previous preconditioning studies have focused mainly on neuronal physiology and amelioration of neuronal cell death after cerebral ischemia. As a result, the role of astrocytes in mediating ischemic preconditioning is often neglected, despite the well-known functions of astrocytes in mediating several neuroprotective mechanisms.7 Astrocytes have been suggested to have increased resistance to ischemic injury when compared with neurons8; however, astrocyte dysfunction has been shown to exacerbate various neurodegenerative conditions9,10 and increase susceptibility of neurons to ischemia.11 Rodents have both a lower astrocyte:neuron ratio and fewer astrocytic processes compared with humans.12 Indeed, the relative differences in cytoarchitecture between rodents and humans may have contributed to the relative plateau of clinically translatable neuroprotective agents.

One of the many neuroprotective functions of astrocytes includes supplying neurons with antioxidants, the production of which is primarily controlled by the transcription factor nuclear erythroid 2 related factor 2 (Nrf2). Nrf2 has been previously suggested to be highly expressed in astrocytes as opposed to neurons and has been shown to increase the antioxidant proteins thioredoxin and NAD(P)H-quinone oxidoreductase 1 (NQO-1).13 Because oxidative stress is a major consequence of cerebral ischemia, the function of Nrf2 to mitigate this stress makes Nrf2 and related downstream pathways attractive targets to combat cerebral ischemic injury.
In light of the aforementioned studies, the focus of our investigation was to determine whether resveratrol preconditioning (RPC) treatment could induce neuroprotection through Nrf2 activation. We found that the absence of functional Nrf2 protein reduced RPC-induced neuroprotection in a mouse model of focal cerebral ischemia. In addition, RPC treatment failed to increase mitochondrial and cellular antioxidants in cultured astrocytes when Nrf2 was absent. These studies highlight the contribution of astrocyte Nrf2 to RPC-induced protection and a novel role of Nrf2 in maintaining mitochondrial function.

Methods
Additional detailed methods are described in the online-only Data Supplement. All animal protocols were approved by the Animal Care and Use Committee of the University of Miami. Minimum Essential Medium, Hanks’ Balanced Salt Solution, and fetal bovine serum were purchased from Life Technologies (Grand Island, NY). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Preparation of Primary Cultures and In Vitro Preconditioning Studies
Astrocyte cultures were prepared as previously described.15 Cortical tissue was harvested from postnatal day 2–4 Sprague Dawley rats, C57Bl/6J wild-type (WT) mice, or Nrf2−− mice. After treatment with 0.25% trypsin and 0.1% DNase, single cell suspensions were plated onto cell-culture dishes and maintained for 10 to 14 days before experimental use. After reaching ≈80% confluence, cultures were trypsinized and passed. Passages 1 to 3 were used for experiments. For RPC treatment, astrocyte cultures were exposed to 2 hours of resveratrol (25 μmol/L) or dimethyl sulfoxide (vehicle) 48 hours before RPC treatment, astrocyte cultures were exposed to 2 hours of resveratrol (25 μmol/L) or dimethyl sulfoxide (vehicle) 48 hours before harvesting cell lysates or nuclear fractions for downstream analysis.

ELISA and Western Blotting
Nuclear and cytoplasmic fractionations were prepared according to the manufacturer’s protocol using a nuclear extract kit (Active Motif Cat. No.: 40010). Nuclear and cytoplasmic extracts were probed with both Lamin B and GAPDH to establish purity of the nuclear and cytoplasmic fractions, respectively. A total of 10 μg of nuclear samples were used for the TransAM Nrf2 ELISA kit (Active Motif) to measure DNA binding of activated Nrf2 nuclear protein, as determined by absorbance measurements at 450 nm. For immunoblotting, cells were lysed in radioimmunoprecipitation assay buffer and immunoblotted for Nrf2, uncoupler protein 2 (UCP2), GAPDH, β-actin, manganese superoxide dismutase (MnSOD), or Lamin B. Proteins were detected using enhanced chemiluminescence system (Pierce, ThermoScientific) and densitometry was performed using ImageJ (National Institute of Health).16

Animal Model of Focal Cerebral Ischemia
For our focal cerebral ischemia model, the left middle cerebral artery (MCA) was occluded for 1 hour using an intraluminal filament model as previously described.17 C57Bl/6J WT or Nrf2−− male mice between 7 and 11 weeks were randomly assigned to 2 treatment groups: resveratrol (10 mg/kg IP) or dimethyl sulfoxide (vehicle). The investigator was blinded to the administration of these agents 48 hours before MCA occlusion (MCAO). Twenty-four hours after reperfusion, infarct volume was assessed with 2,3,5-triphenyltetrazolium chloride (TTC) and quantified using ImageJ software. Exclusion criteria for these studies included (1) >30% of baseline MCA blood flow persisting through the occlusion phase of the MCAO injury as measured by laser Doppler flowmetry and (2) lack of detectable infarct after TTC staining.

Polarography
Mitochondrial respiration studies were conducted as previously described.18 In brief, nonsynaptic mitochondria were isolated from WT or Nrf2−− mice 48 hours after resveratrol or vehicle treatment. The ratio of state III/state IV respiration was measured using a Clark-type oxygen electrode. This ratio represented the respiratory control index (RCI), an established measure of mitochondrial coupling.19

Measurement of Mitochondria ROS Production
Mitochondrial ROS production was determined using a spectrophotometer following a previously established protocol.20 Isolated nonsynaptic mitochondria from Nrf2−− or WT animals were added to a microplate containing horseradish peroxidase, Amplex Red Ultra, and superoxide dismutase. H2O2 emission was measured spectrophotometrically at 555 nm excitation/590 nm emission wavelengths. After establishing baseline measurements, respiratory substrates were added in a similar manner to the polarographic studies. Rates of H2O2 emission were recorded for each complex-specific substrate/inhibitor pair and normalized to baseline H2O2 production for each sample.

Statistical Analysis
All data are expressed as mean±SD. Statistical analysis between 2 groups was performed using the unpaired Student t test. Statistical analysis between >2 groups was performed using a 1-way ANOVA with Bonferroni multiple comparison post hoc test unless otherwise specified. P≤0.05 was considered statistically significant (GraphPad Prism v5.00 for Windows, GraphPad Software, San Diego, CA).

Results
Loss of Nrf2 Decreases RPC-Induced Neuroprotection After Focal Cerebral Ischemia
Previous studies from our laboratory have shown that RPC can induce neuroprotection against cerebral ischemia in vivo.4 Therefore, we wanted to determine whether the loss of functional Nrf2 decreased RPC-induced neuroprotection in a mouse model of focal cerebral ischemia. Nrf2−− mice were verified by standard genotyping and immunoblotting for NQO-1 (Figure I in the online-only Data Supplement). WT and Nrf2−− mice were subjected to MCAO injury 48 hours after either RPC or vehicle treatment. Quantification of TTC-stained brain slices from each treatment group indicated that RPC treatment of WT mice significantly reduced infarct volume compared with vehicle treatment (44.43±13.90%, n=9 versus 28.31±9.93%, n=6, respectively; P<0.05; Figure 1A and 1B). However, no significant difference was observed in Nrf2−− mice between RPC and vehicle-treated groups (40.86±17.10%, n=10 versus 34.47±14.25%, n=9; P=0.39). In addition, there was no significant difference between WT and Nrf2−− vehicle-treated groups. Therefore, the results from Figure 1 indicate that RPC-induced neuroprotection was decreased in the absence of functional Nrf2.

Loss of RPC-Induced Neuroprotection in Nrf2−− Mice Is Not Due to Altered Cerebral Blood Flow
A previous group has suggested that the cerebral blow flow is altered in Nrf2−− mice, which led to their use of a modified in vivo model to achieve adequate MCA occlusion.21 To determine whether the observed infarct volumes from Figure 1 were attributable to strain differences in cerebral blood flow, we analyzed blood flow throughout the MCAO injury via laser Doppler flowmetry for each treatment group of mice. The average blood flow for each group during the phases of the MCAO injury (baseline, occlusion, and reperfusion)
were compared and expressed as a percentage of baseline laser Doppler Flowmetry measurements (Figure 2A); our results show that there was no statistically significant difference between blood flow of any of the treatment groups for each phase of the MCAO injury. In Figure 2B, the average age and weight for each treatment group were analyzed and once again there was no statistically significant differences observed. Therefore, the loss of RPC-induced neuroprotection in Nrf2−/− mice is not attributable to altered cerebral blood flow.

RPC Treatments Activate Nrf2 in Rat Astrocytes

Given that the reduction in RPC-induced neuroprotection in Nrf2−/− mice was not due to altered cerebral blood flow (Figure 2), the results from Figure 1 suggest that RPC-induced neuroprotection partially requires Nrf2. As there is greater Nrf2 protein abundance in astrocytes, we next sought to determine whether RPC can activate Nrf2 in vitro using astrocyte cultures. Using the TransAM Nrf2 ELISA kit, RPC treatment increased the amount of activated Nrf2 in nuclear astrocyte fractions at 48 hours compared with vehicle-treated cultures as determined by absorbance measured at 450 nm (0.983±0.458 versus 0.625±0.352; P<0.05; n=3; Figure 3A), which were not observed at earlier time points (1 or 24 hours) after RPC treatment. To determine whether downstream pathways of Nrf2 were increased after RPC, whole-cell astrocyte lysates were probed for NQO-1, an Nrf2-dependent gene target. 48 hours after RPC treatments, NQO-1 protein levels were significantly increased in astrocyte cultures by ≈2.1 fold compared with vehicle treatments (Figure 3B). The results indicate that RPC activates astrocyte Nrf2 by increasing Nrf2 DNA binding and Nrf2-dependent gene transcription.

RPC Induces Uncoupling in WT and Nrf2−/− Mitochondria

Resveratrol has previously been implicated in modifying cerebral mitochondrial function. Therefore, we investigated mitochondrial coupling (represented as RCI) in isolated nonsynaptic mitochondria from WT and Nrf2−/− mouse cortex after RPC treatment in vivo. We used nonsynaptic mitochondria because this fraction contains more astrocyte-derived mitochondria than the synaptic fraction and therefore would better represent functional changes because of the loss of Nrf2. RPC-induced treatment induced a mild uncoupling in both WT and Nrf2−/− mitochondria compared with vehicle treatments of each respective mouse strain (WT: vehicle 5.84±0.55 versus RPC 3.93±0.42; P<0.05 and Nrf2−/−: vehicle 3.27±0.67 versus RPC 2.27±0.15; P<0.05; Figure 4A). In addition, we observed a significant difference between Nrf2−/− and WT mice RCI values between vehicle groups, suggesting that Nrf2−/− mitochondria have an innate respiratory dysfunction. Therefore, RPC treatment had similar effects on the RCI in both mouse strains, with RPC treatment further reducing the already decreased RCI in Nrf2−/− mice.
RPC Induces Increased UCP2 Expression in Nrf2−/− Astrocytes

Previous studies from our laboratory have suggested an interaction between resveratrol and UCP2, an uncoupler protein which if altered by RPC could explain RCI values and provide a molecular understanding for the results observed in Figure 4A. As isolation of astrocyte mitochondria from the brain was not feasible, we took advantage of highly enriched astrocyte cultures to look at the effects of Nrf2 on UCP2 expression in vitro. Therefore, 48 hours after RPC treatment, whole cell mouse WT and Nrf2−/− astrocyte lysates were prepared and probed for UCP2. Compared with vehicle treatments, UCP2 was significantly increased 48 hours after RPC treatment in WT and Nrf2−/− astrocyte cultures (4.05±1.01-fold and 1.89±0.26-fold, respectively; P<0.05; Figure 4B and 4C). Therefore, RPC treatment increased UCP2 protein expression in WT and Nrf2−/− mouse astrocyte cultures.

RPC Treatment Increases ROS Production in WT and Nrf2−/− Mitochondria

As Nrf2−/− mice are expected to have decreased antioxidant capacity, we also measured mitochondrial H2O2 production as a measure of ROS generation in nonsynaptic mitochondria isolated from WT and Nrf2−/− mouse cortex after RPC treatment in vivo. RPC treatment significantly increased the fold of H2O2 production compared with baseline production following rotenone-induced complex I inhibition (WT: vehicle 3.66±1.19 versus RPC 8.29±1.56-fold of baseline P<0.05 and Nrf2−/−: vehicle 3.90±2.82 versus RPC 8.35±2.65-fold; P<0.05) and antimycin-induced complex III inhibition (WT: vehicle 7.54±2.43 versus RPC 31.06±10.17; P<0.005 and Nrf2−/−: vehicle 9.59±6.32 versus RPC 23.25±10.05; P<0.05) for both strains of mice (Figure 5A). However, there were no significant differences between WT and Nrf2−/− mice for the same treatment group. These findings suggest a role of RPC in increasing ROS production at complex I and III in WT and Nrf2−/− cortical mitochondria.
RPC-Induced Antioxidant Enzyme Expression in WT and Nrf2−/− Astrocytes

Although RPC-induced increase in mitochondrial ROS production occurred in both WT and Nrf2−/− mice, we hypothesized that this phenomenon was detrimental in Nrf2−/− mice and could explain loss of RPC-induced neuroprotection in this population. Therefore, we immunoblotted WT and Nrf2−/− astrocyte culture lysates for the antioxidants MnSOD and NQO-1 48 hours after RPC treatment. (Figure 5B). RPC treatment significantly increased NQO-1 protein expression (normalized to actin) in WT astrocytes compared with vehicle treatment (1.91±0.35-fold increase; n=6; *P<0.05 (WT vehicle vs WT RPC); #P<0.05 (Nrf2 vehicle vs Nrf2−/− RPC). B. Western blots of astrocyte cultures for manganese superoxide dismutase (MnSOD), NAD(P)H-quinone oxidoreductase 1 (NQO-1), and actin (loading control) 48 hours after RPC or vehicle treatment. C. Quantification of WT astrocyte NQO-1 levels and (D) WT and Nrf2−/− astrocyte MnSOD protein levels, normalized to actin protein levels. n=3 to 6. *P<0.05 and **P<0.005.

Discussion

In the present study, we investigated the contribution of Nrf2 to RPC-induced neuroprotection and the effect of Nrf2 on
cortical mitochondrial function. We investigated the role of Nrf2 in RPC-induced neuroprotection in a rodent model of focal cerebral ischemia. Our MCAO studies suggest that in the absence of Nrf2 protein, RPC-induced neuroprotection is not effective in significantly ameliorating cerebral infarct in mice (Figure 1). Given that RPC treatment increased ROS production in both WT and Nrf2−/+ cortical mitochondria (Figure 4B), and that RPC was unable to induce antioxidant expression in Nrf2−/+ mice (Figure 5), we believe this evidence suggests that RPC-induced neuroprotection can be attributed to ROS-mediated signaling pathways, which ultimately activates astrocytic Nrf2 and confers cerebral ischemic tolerance.

The role of astrocyte pathways has not been fully elucidated in preconditioning research, but their importance to neuronal disease has been extensively studied. Previous studies by Bell et al.24 highlighted that astrocytic Nrf2 was necessary for ischemic tolerance in murine neuronal cultures. In direct contrast, Haskew-Layton et al.25 determined that physiological levels of H2O2 could induce ischemic tolerance in neurons independent of Nrf2. Although our study is a different preconditioning paradigm (ie, RPC), our current studies indicate that RPC is indeed able to increase Nrf2 DNA binding and downstream expression of NQO-1, an Nrf2-dependent gene.

Similar to a previous study,21 we did not observe any difference in infarct volume between vehicle-treated WT and Nrf2−/− mice at 24 hours. In contrast, we observed significant neuroprotection with RPC treatment in WT mice after MCAO, but there was no significant reduction of infarct volume in RPC-treated Nrf2−/− mice. Although resveratrol has been shown to activate a multitude of pathways, resveratrol was still unable to significantly ameliorate infarct injury in Nrf2−/− mice after MCAO. This suggests that Nrf2 activation is a key pathway for RPC-induced neuroprotection.

Our current investigation has also shown that resveratrol induced an increase in ROS production in WT and Nrf2−/− mitochondria, which can be detected 48 hours after treatment. This increase in ROS after resveratrol treatment has been seen previously in yeast cells,26 human adipocytes,24 and cancer cell lines.25 In addition, the increase in ROS after preconditioning treatments is a well-observed phenomenon, and inhibition of ROS production has been shown to ameliorate preconditioning-induced neuroprotective effects.26

We also present findings that RPC treatment increased UCP2 expression in astrocyte cultures, and that this increase was several fold more in WT versus Nrf2−/− astrocytes (Figure 4). Although our previous findings described an RPC-induced decrease in UCP2 protein expression in adult rat hippocampal mitochondria, our current studies investigated UCP2 in cortical postnatal mouse astrocyte cultures. This discrepancy could be because of the use of different models and cell types, and future studies may serve to elucidate the dependence of cell type and brain region on the regulation of UCP2 by RPC treatment. Taken together, we propose that ROS production from RPC treatment induces UCP2 expression, leading to mild uncoupling and subsequent protection against oxidative stress. Furthermore, RPC-induced increase in UCP2 (Figure 4), basal MnSOD, and NQO-1 (Figure 5) protein levels were all decreased several fold in Nrf2−/− mice compared with WT mice; we believe that this implicates Nrf2 as a critical pathway in which RPC-mediated mitochondrial ROS production activates Nrf2, thus promoting the induction of antioxidant pathways and subsequent neuroprotection against focal cerebral ischemia.

After RPC treatment, WT and Nrf2−/+ exhibited reductions in their RCI, suggestive of a resveratrol-induced uncoupling effect. By further decreasing the coupling of Nrf2−/− mitochondria, resveratrol may exacerbate already dysfunctional cortical mitochondria in Nrf2−/− mice. Thus, decreased mitochondrial coupling, increased ROS production, and decreased antioxidant defenses could be plausible explanations as to explain why RPC was less effective in Nrf2−/− mice when compared to WT mice. Our findings that Nrf2−/+ nonsynaptic mitochondria exhibited decreased coupling were in line with previous studies, which suggested that Nrf2−/+ brain, heart, and liver mitochondria exhibit decreased RCI.27 Interestingly, studies by Fiskum et al.28 did not describe any changes to brain nonsynaptic mitochondrial respiration after activation of Nrf2 with sulforaphane. Future studies may serve to understand the relationship of Nrf2 to cortical nonsynaptic and synaptic mitochondria.

In conclusion, our investigation provides new insight into the mechanism of RPC-induced protection and implicates Nrf2 as an important pathway to induce RPC’s neuroprotective effects in the context of stroke.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL

Materials

Minimum Essential Medium (MEM), Hanks Balanced Salt Solution (HBSS) and Fetal Bovine Serum (FBS) were purchased from Gibco/Life Technologies (Grand Island, NY). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Animal Use

All animal protocols were approved by the Animal Care and Use Committee of the University of Miami. Experiments were conducted in accordance to ARRIVE guidelines. 16-17 day-pregnant Sprague-Dawley rats were purchased from Charles Rivers Laboratories and housed in a temperature controlled environment with 12 hr light -12 hr dark cycle and ad libitum food and water. 5 week old male and female homozygous knockout mice (Nrf2-/-, Jackson Laboratories) were bred to establish homozygous Nrf2-/- colonies. The targeting vector replaces exon 5 and part of exon 4 of Nrf2 with a lacZ reporter followed by a neomycin resistant cassette with a polyadenylation sequence\(^1\). This targeted sequence essentially negates the ability of Nrf2 to bind to its response element, rendering Nrf2 ineffective in upregulating antioxidant gene transcription. This construct was electroporated into 129X1/SvJ-derived JM-1 embryonic stem (ES) cells. Chimeric mice with the correct ES-targeted cells were bred with C57BL/6J mice to generate Nrf2-/- mice. This subsequent strain was backcrossed with C57BL/6J mice for at least 10 generations. DNA was harvested from ear punches of each mouse and analyzed by polymerase chain reaction (PCR) to confirm its genotype and confirmed by a third-party genotyping service (Transnetx).

Polymerase Chain Reaction

Standard PCR was used to differentiate Nrf2-/- mice from WT mice according to Jackson Laboratory protocol. DNA from 2-3 mm ear punch samples were isolated by digestion in 75 µL lysis buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl pH 9.0, 0.1% Triton X-100, 0.4 mg/ml Proteinase K). Samples were incubated in lysis buffer for 1 hr at 95°C. After replacing samples on ice (4°C), 75 µL of neutralization reagent (25 mmol/L 10 N NaOH, 0.2 mmol/L EDTA) was added and samples were centrifuged for 30 s on a table-top centrifuge at 12000 RPM. For PCR reactions, 20 µM of each of the two primers (WT reverse: 5’-GGA ATG GAA AAT AGC TCC TGC C-3’; Nrf2-/- reverse: 5’-GAC AGT ATC GGC CTC AGG AA- 3’) were added to approximately 10 µg of DNA isolated from WT or Nrf2-/- mouse ear punch samples, along with 25 µL of 2x MangoMix (Bioline Inc., USA) and 3.5 mmol/L MgCl2. Samples were thermocycled according to Jackson Laboratory protocol for Nrf2-/- mouse strain (Stock#017009) and according to Chan et. al\(^1\).

Preparation of embryonic neuronal and post-natal astrocyte cultures

Astrocyte and neuronal cultures were prepared as previously described\(^2\) with slight modifications. For astrocyte cultures, Cortices from postnatal Sprague Dawley rat pups (P2-P4) were harvested, followed by digestion with 0.25% trypsin and DNase. Following tituration and filtration through a 70 µm filter, the resulting cell suspension was centrifuged at 200\(^1\) g for 5 minutes and plated in Minimal Essentials Media (MEM) supplemented with 20 mmol/L glucose, 1% GlutaMAX, 1% Penicillin/Streptomycin, and 10% FBS before plating. Complete media
changes were performed every 2-3 days until cultures reached 70% confluency 7 days following the initial plating. Astrocyte cultures were then passaged and plated in appropriate culture vessels at a density of 50,000 cells/cm², and allowed to reach full confluency and maintained for an additional 6-7 days before experimental use.

Neuronal cultures were prepared from embryonic (E15-16) rat pups. Embryonic cortices were harvested similarly to the post-natal rat pup astrocyte preparation, except titrated cells were initially plated onto poly-d-lysine-coated 10 cm dishes (2-3 hemispheres/dish) in MEM supplemented with 20 mmol/L glucose, 1% GlutaMAX, and 5% FBS. 3 days after the initial plating, cultures were treated with 5 µmol/L cytosine arabinoside for 48 hrs to terminate proliferation of contaminating cell populations and subsequent half media changes were performed every 3-4 days using neuronal maintenance media (MEM, 20 mmol/L glucose, 1% GlutaMAX). Cultures were used at 10-14 days in vitro (DIV) for immunoblotting experiments.

Subcellular fractionation
Subcellular fractionation was performed as previously described³ with minor modifications. For mitochondrial isolation, cells were suspended in an isotonic buffer consisting of 250 mM sucrose, 1 mM EDTA, 0.25 mM DTT, and 1 mg/mL Bovine Serum Albumin (BSA, fraction V). BSA was added to bind free fatty acids, and to improve the degree of coupling of isolated mitochondria.⁴ Cells were then homogenized using a teflon-glass homogenizer. The resulting homogenate was centrifuged at 1,000 x g for 5 min at 4°C. The resulting supernatant was further centrifuged at 13,000 x g for 10 min at 4°C to pellet mitochondria. The supernatant was collected, and this represented the cytoplasmic fraction of the cells. Mitochondrial pellets were washed twice with isolation buffer and resuspended in RIPA lysis buffer for Western blot analysis. Alternatively, mitochondria were resuspended in isolation buffer for mitochondrial subfractionation, and finally resuspended in 0.25 M sucrose for respiration studies. The nuclear fraction was isolated using a previously described protocol. In brief, cells plated on 100 mm cell-culture treated dishes were washed with 1x PBS and harvested in 1x PBS with protease and phosphatase inhibitor cocktail (Sigma). The cells were then centrifuged at 1000 g x 5 min at 4 deg C. The supernatant was discarded and the remaining cells were resuspended in hypotonic buffer consisting of 10 mM HEPES, 1.5 mmol/L MgCl210 mM KCl, and 0.5 mmol/L DTT, pH 7.9. The cells were allowed to swell for 15 minutes, after which NP-40 detergent was added to a final concentration of 0.1 %v/v and the cell suspension was vortexed vigorously for 10 seconds. The cell suspension was then centrifuged for 14000 g x 1 minute at 4 deg C, and the supernatant was collected and also corresponded to the cytoplasmic fraction. The nuclear pellet was washed once with hypotonic buffer and lysed in lysis buffer (Active Motif cat.# 16965838).

Oxygen Glucose Deprivation
To mimic IPC in vitro, astrocyte cultures were exposed to oxygen and glucose deprivation (OGD) as previously described⁵ for 1 hr. Through empirical testing, 1 hr was determined to be a sublethal duration of OGD that induced the highest degree of protection to astrocytes following a lethal OGD insult (6 hrs). The 6 hr time point was chosen because greater than 50% cell death occurred along with minimal cell detachment from the tissue culture dishes, allowing for more accurate lactate dehydrogenase release assays. To induce OGD, cells were washed two times with glucose-free HBSS (in mmol/L: CaCl₂ 1.26, KCl 5.37, KH₂PO₄ 0.44, MgCl₂ 0.49, MgSO₄
0.41, NaCl 136., NaHCO₃ 4.17, Na₂HPO₄ 0.34, sucrose 20, pH 7.4) and exposed to an oxygen-
free environment (90% nitrogen, 5% hydrogen, and 5% CO₂, 37°C) using a COY anaerobic
chamber (COY Laboratory Products Inc, Lake Charter Township, MI). OGD was terminated by
placing the cells back into glucose-containing maintenance media and returning cultures to a 5%
CO₂, 37°C incubator. Sham IPC was performed using similar number of washes and glucose-
Free HBSS, except glucose (20 mmol/L) was substituted for sucrose and cells were placed back
into normoxic incubator conditions.

**Western Blot**
Cells were lysed in RIPA Buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L
EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L
Na₃VO₄ and 1 mmol/L PMSF). Protein concentration was determined by BCA protein assay and
30 µg of protein was loaded onto a 12% SDS-polyacrylamide gel and electroblotted to
nitrocellulose. Membranes were blocked in 5% dry milk/TBST and hybridized with primary
antibodies overnight at 4°C. Blots were probed with rabbit anti-Nrf2 (1:500, Santa Cruz
Biotechnology, Dallas, TX), rabbit anti-UCP2 (1:500, Calbiochem Inc.), rabbit anti-GAPDH
(1:10000, Cell Signaling Technology, Danvers, MA), mouse anti-β-actin (1:10000, Sigma),
rabbit anti-MnSOD (1:2000, Cell Signaling Technology) or goat anti-Lamin-B (1:1000, Cell
Signaling Technology). Membranes were washed with TBST followed by incubation with anti-
mouse, anti-goat, or anti-rabbit HRP-conjugated secondary antibodies (1:5000, Pierce, Thermo
Scientific; Rockford, IL) for 1 hr at room temperature. Proteins were detected using enhanced
chemiluminescence (ECL) system (Pierce, Thermo Scientific). Western blot densitometry was

**ELISA**
Nuclear and cytoplasmic fractionation was performed according to manufacturer’s protocol by
using a nuclear extract kit (Active motif). Nuclear and cytoplasmic extracts were probed with
both Lamin B and GAPDH to establish purity of the nuclear and cytoplasmic fractions
respectively. 10 µg of nuclear samples were used for the TransAM Nrf2 ELISA kit (Active
Motif, catalogue# 50296) to measure DNA binding of activated Nrf2 nuclear protein, as
determined by absorbance measurements at 450 nm.

**Animal model of Focal Cerebral Ischemia**
For our focal cerebral ischemia model, the left middle cerebral artery (MCA) was occluded for 1
hr using a poly-d-lysine-coated 3-0 nylon monofilament as previously described in C57black/6
mice. To measure blood flow during the MCA occlusion (MCAO), a laser Doppler flowmetry
probe inserted in the temporal lobe to measure perfusion of the MCA territory. Regional cerebral
blood flow was measured over the middle cerebral artery territory via Laser Doppler flowmetry
(Perimed, Stockholm, Sweden). For lasser Doppler, a flexible 0.5-mm fiberoptic probe was
affixed to the exposed skull over the ischemic cortex at 2 mm posterior and 3 mm lateral to
bregma in mice. The suture was advanced into the external carotid artery and advanced through
the internal carotid artery until an approximately 70% decrease in blood flow to the middle
cerebral artery occurred as measured by LDF. 48 hrs prior to ischemia, animals of each strain
were randomized into treatment groups and a blinded investigator administered either agent
intraperitoneally (i.p.). C57Bl/6J WT or Nrf2⁻/⁻ male mice between 7-11 weeks were used for
these experiments, and each group of mice were further separated into 2 different treatment
groups: resveratrol (10 mg/kg i.p., Sigma) or DMSO (Vehicle injection, i.p.). Following 1 hr of
occlusion, the filament was removed and the animal was returned to their cages. 24 hr following
reperfusion, mice were anesthetized and perfused with 0.9 % saline, followed by decapitation
and rapid brain removal. Brains were sliced into eight 1 mm thick coronal sections using a
mouse brain matrix (RBM-200C, Activational Systems, Ann Arbor, MI, U.S.A.). These sections
were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in isotonic
saline and incubated at 37º C in the dark for 7 minutes. The sections were transferred to buffered
10% formalin for fixation and scanned into an image analysis system (M4, St. Catherine,
Ontario, Canada). Infarct areas were traced at each level and volumes were computed using a
direct and indirect method that corrects for edema using ImageJ software as described
previously. Blood flow during the ischemic phase of the MCAO injury was monitored by laser
Doppler flowmetry. Exclusion criteria for these studies included (1) greater than 30% of baseline
MCA cerebral blood flow persisting through the occlusion phase of the MCAO injury (as
measured by laser Doppler); and (2) lack of detectable infarct following TTC staining.

**Polarography**
Mitochondrial respiration studies were conducted as previously described. In brief, non-
synaptic mitochondria were isolated from WT or Nrf2−/− mice treated with resveratrol (10 mg/kg
intraperitoneal injection, i.p.) or dimethyl sulfoxide (DMSO) vehicle. Mouse cortex were
homogenized in isolation medium (250 mM sucrose, 1 mg/ml bovine serum albumin (fraction V
essentially fatty acid free, BSA), 1.0 mM ethylenediaminetetra-acetic acid (EDTA), and 0.25
mM dithiothreitol, pH 7.4) Tissue was minced with a pair of scissors and rinsed thoroughly with
the isolation medium. The minced tissue was homogenized in a hand-operated Teflon glass
homogenizer by 7-8 strokes. The homogenate was diluted to yield 10% (w/v) homogenate and
centrifuged at 720 g for 5 min using a Sorvall (Newton, CT) RC5 centrifuge. The supernatant
was collected in another tube and centrifuged again at the same speed to reduce nuclear
contamination of the eventual mitochondria sample. To isolate glial and neuronal cell body
mitochondria, non-synaptic mitochondria was collected by layering the supernatant obtained
from the final slow-speed centrifuge on a 24% (v/v) percoll gradient (percoll diluted in isolation
media with BSA). The gradients were centrifuged at 32,500 g for 5 min. The resulting pellet was
washed once with isolation media and centrifuged at 15,000 g for 10 min. The pellet was again
washed with 0.25 M sucrose by centrifugation at 15,000 g for 10 min. The resulting pellet was
resuspended in 0.25 M sucrose, and protein content was determined by bicinchoninic acid (BCA)
assay. All mitochondrial isolation procedures were performed at 4°C. The rate of state III
mitochondrial oxygen consumption was determined using a Clark-type oxygen electrode in the
presence of 100 µg non-synaptic mitochondria, 5 mmol/L pyruvate, 2.5 mmol/L malate, and 5
mmol/L ADP (excess). To induce state IV respiration, 5 µmol/L oligomycin was added to the
polarographic chamber to inhibit ATP synthase and coupled respiration. Ratio of State III/State
IV respiration yielded the respiratory control index, or RCI, an established measure of
mitochondrial coupling.

**Measurement of mitochondria ROS production**
Mitochondrial ROS production was determined using a spectrophotometer following a
previously modified protocol. Isolated non-synaptic mitochondria from Nrf2−/− or C57Bl/6J
animals were added to a microplate. H2O2 emission was measured fluorescently at 555 nm
(excitation)/590 nm emission wavelengths. After establishing baseline measurements,
respiratory substrates were added in a similar manner to the polarographic studies. The
production of H$_2$O$_2$ was determined spectrophotometrically, and the electron transport chain site of this production could be determined based on the combination of substrates added. Rates of H$_2$O$_2$ emission were recorded for each complex-specific substrate/inhibitor pair, and normalized to baseline H$_2$O$_2$ production for each sampled well.

**Statistical Analysis**
All data are expressed as mean ± STDEV. Statistical analysis between two groups was performed using the unpaired Student’s *t*-test. Statistical analysis between more than two groups was performed using a one-way ANOVA with Bonferroni’s multiple comparison post hoc test unless otherwise specified. $p \leq 0.05$ was considered statistically significant.
Supplementary Figure I: Nrf2-/- mice present with reduced levels of the Nrf2-regulated antioxidant protein NQO-1. A) Standard PCR genotyping of Nrf2-/- mice and astrocyte cultures. Ear punch samples from Nrf2-/- mice were subjected to genotyping analysis. Using genomic DNA, the 400-bp PCR product was detected only in Nrf2-/- compared to the 262-bp product detected in WT mice. B) Western blot of whole brain cortical lysate from WT and Nrf2-/- mice. NQO-1 proteins levels were measured using Western blotting, along with Actin protein (loading control). n = 4-6.
References Cited:

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