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/cm^2, 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 titurated 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 described[3] 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 described[5] 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: $CaCl_2$ 1.26, KCl 5.37, KH_2PO_4 0.44, $MgCl_2$ 0.49, $MgSO_4$

0.41, NaCl 136., $NaHCO_3$ 4.17, Na_2HPO_4 0.34, sucrose 20, pH 7.4) and exposed to an oxygen-free environment (90% nitrogen, 5% hydrogen, and 5% CO_2, 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% CO_2, 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 Na_3VO_4 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 analyzed using ImageJ (NIH)[6].

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[7] 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[8,9]. 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[10]. 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[11].

Measurement of mitochondria ROS production

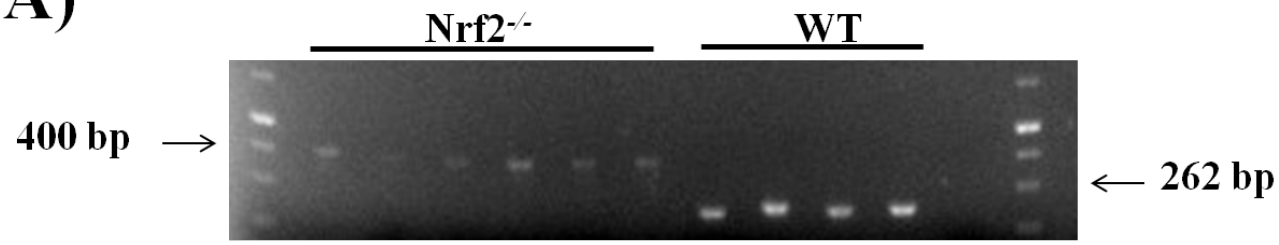
Mitochondrial ROS production was determined using a spectrophotometer following a previously modified protocol[12]. Isolated non-synaptic mitochondria from Nrf2$^{-/-}$ or C57Bl/6J animals were added to a microplate. H_2O_2 emission was measured fluorescently at 555 nm (excitation)/590 nm emission wavelengths. After establishing baseline measurements, respiratory substrates were added in a similar manner to the polarographic studies. The

production of H_2O_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_2O_2 emission were recorded for each complex-specific substrate/inhibitor pair, and normalized to baseline H_2O_2 production for each sampled well.

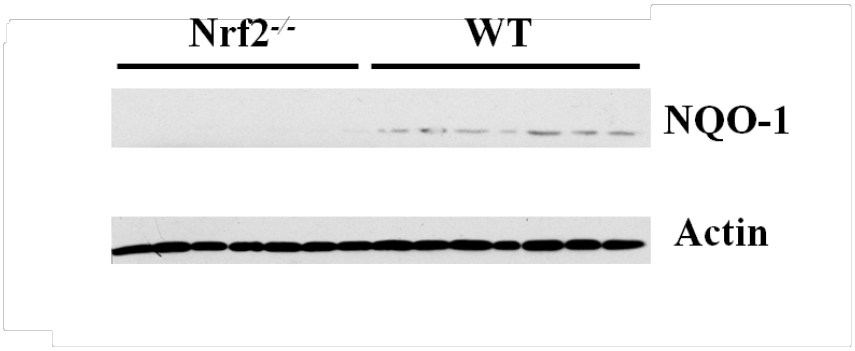
Statistical Analysis

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.

A)



B)



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:

1. Chan K, Lu R, Chang JC, Kan YW. Nrf2, a member of the nfe2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93:13943-13948
2. Kaech S, Banker G. Culturing hippocampal neurons. *Nature protocols*. 2006;1:2406-2415
3. Horn D, Al-Ali H, Barrientos A. Cmc1p is a conserved mitochondrial twin cx9c protein involved in cytochrome c oxidase biogenesis. *Molecular and cellular biology*. 2008;28:4354-4364
4. Tretter L, Mayer-Takacs D, Adam-Vizi V. The effect of bovine serum albumin on the membrane potential and reactive oxygen species generation in succinate-supported isolated brain mitochondria. *Neurochemistry international*. 2007;50:139-147
5. Kim EJ, Raval AP, Perez-Pinzon MA. Preconditioning mediated by sublethal oxygen-glucose deprivation-induced cyclooxygenase-2 expression via the signal transducers and activators of transcription 3 phosphorylation. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2008;28:1329-1340
6. Degasperi A, Birtwistle MR, Volinsky N, Rauch J, Kolch W, Kholodenko BN. Evaluating strategies to normalise biological replicates of western blot data. *PloS one*. 2014;9:e87293
7. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke; a journal of cerebral circulation*. 1989;20:84-91
8. 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
9. 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
10. Dave KR, DeFazio RA, Raval AP, Torraco A, Saul I, Barrientos A, et al. Ischemic preconditioning targets the respiration of synaptic mitochondria via protein kinase c epsilon. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008;28:4172-4182
11. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. *The Biochemical journal*. 2011;435:297-312

12. Starkov AA. Measurement of mitochondrial ros production. *Methods in molecular biology*. 2010;648:245-255