NF-E2 related factor (Nrf2) controls a pleiotropic cellular defense, where multiple antioxidant/detoxification pathways are up-regulated in unison. Although small molecule inducers of Nrf2 activity have been reported to protect neurons in vitro, whether similar pathways can be accessed in vivo is not known. We have investigated whether in vivo toxicity of the mitochondrial complex II inhibitor 3-nitropropionic acid (3-NP) can be attenuated by constitutive and inducible Nrf2 activity. The absence of Nrf2 function in Nrf2−/− mice resulted in 3-NP hypersensitivity that became apparent with time and increasing dose, causing motor deficits and striatal lesions on a more rapid time scale than identically treated Nrf2+/+ and Nrf2+/- controls. Striatal succinate dehydrogenase activity, the target of 3-NP, was inhibited to the same extent in all genotypes by a single acute dose of 3-NP, suggesting that brain concentrations of 3-NP were similar. Dietary supplementation with the Nrf2 inducer tert-butylhydroquinone attenuated 3-NP toxicity in Nrf2−/− mice, but not Nrf2−/-, confirming the Nrf2-specific action of the inducer in vivo. Increased Nrf2 activity alone was sufficient to protect animals from 3-NP toxicity because intrastratial adenovirus-mediated Nrf2 overexpression significantly reduced lesion size compared with green fluorescent protein overexpressing controls. In cultured astrocytes, 3-NP was found to increase Nrf2 activity leading to antioxidant response element-dependent gene expression providing a potential mechanism for the increased sensitivity of Nrf2−/− animals to 3-NP toxicity in vivo. We conclude that Nrf2 may underlie a feedback system limiting oxidative load during chronic metabolic stress.

The Cap “n” Collar transcription factor NF-E2 related factor (Nrf2)1 controls the coordinated expression of important antioxidant and detoxification genes (Phase 2 genes) through a promotor sequence termed the antioxidant response element (ARE) (1–3). Phase 2 genes work in synergy to constitute a pleiotropic cellular defense that scavenes reactive oxygen/nitrogen species (ROS/RNS), detoxifies electrophiles and xenobiotics, and maintains intracellular reducing potential (4–8). Normally, Nrf2 is sequestered in the cytoplasm by the actin-bound regulatory protein, Keap1 (9, 10). Multiple cysteine residues allow Keap1 to act as a molecular “switch” by responding to electrophiles/ROS with a conformational change, which releases Nrf2 for nuclear translocation and activation of Phase 2 gene expression (11–13). Thus, Nrf2 provides an important mechanistic link between oxidative stress leading to cell death and antioxidant gene expression supporting cell survival.

Nrf2−/− mice are particularly maladapted when challenged with toxicity paradigms such as hypoxic lung injury, butylated hydroxytoluene-induced acute pulmonary injury, acetaminophen-induced liver toxicity, benzo[a]pyrene-induced tumorigenesis in the forestomach, or cigarette smoke-induced emphysema (14–19). Although the protective function of Nrf2 is apparent in peripheral tissues, the role of Nrf2 in the brain, where substantial ROS/RNS production can occur because of a high rate of metabolism, remains unclear. We hypothesize that Nrf2 function is critical for supporting neuronal survival during neurodegenerative disease when aberrant ROS/RNS production is known to exacerbate (20). To study the effect of Nrf2 activity on in vivo neurodegeneration, we systemically administered 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase (SDH), which causes striatal-specific cell loss leading to impairment of motor function (21). Metabolic inhibition by 3-NP produces oxidative stress in the brain through multiple mechanisms. 1) ATP depletion, membrane depolarization, increased neuronal/glial glutamate release, overactivation of N-methyl-D-aspartate ionotropic glutamate receptors, accumulation of intracellular Ca2+, and excessive mitochondrial ROS production (22, 23). 2) Secondary activation of Ca2+-dependent enzymes such as phospholipase A2 (production of inflammatory mediators) and nitric-oxide synthase (peroxynitrite formation) (24, 25). 3) Excessive dopamine release, which can generate H2O2 when metabolized (26).

Up-regulation of Nrf2 activity in the brain is an attractive strategy for mitigating ROS/RNS overproduction during neurodegenerative disease. To test the hypothesis that augmenta...
tion of Nrf2 could protect neurons in vivo, we increased Nrf2 activity in the brain using dietary administration of the small molecule inducer, tert-butyldihydroquinone (tBHQ) or adenosinergic-mediated overexpression of Nrf2 (6). In previous studies, these treatments protected cultured neurons from toxicity paradigms that mimic aspects of in vivo neurodegeneration, such as glutamate-induced glutathione depletion, dopamine toxicity, direct exposure to H$_2$O$_2$, metabolic inhibition with mitochondrial toxins (rotenone), platelet activating factor-induced inflammatory responses, and increased intracellular Ca$^{2+}$ (6, 27–30). Importantly, neuronal cultures derived from Nrf2$^{-/-}$ mice showed increased susceptibility to neurotoxicity paradigms and were not protected by Nrf2 inducers, but could be rescued by overexpression of Nrf2 (27, 28).

Our in vitro and in vivo data suggest that Nrf2 activation is a protective response to counter the toxic effects of metabolic inhibition and ROS/RNS production in the brain. Loss of Nrf2 function in Nrf2$^{-/-}$ mice exacerbated motor deficits and striatal lesions caused by 3-NP administration. Conversely, pre-activation of the Nrf2 response (using dietary administration of a Nrf2 inducer or viral gene transfer) attenuated 3-NP toxicity. Nrf2 activation may be a viable strategy to prime the antioxidan
capacity of the brain, thereby decreasing injury caused by progressive neurodegeneration.

**MATERIALS AND METHODS**

**Chemicals**—All chemicals were obtained from Sigma unless otherwise stated.

**In Vivo 3-NP Dosage Regimen**—All experiments were approved by the University of British Columbia Animal Care Committee and were conducted in strict accordance with guidelines set by the Canadian Council on Animal Care. Adult male Wistar rats (Charles River, Canada) (250–350 g) and Nrf2$^{-/-}$ mice (C57Bl/6SV129 background), originating from the laboratory of Dr. Yuet Wai Kan (32), were maintained in a 12-h light-dark cycle with food and water ad libitum. 3-NP was prepared in 0.1 M phosphate-buffered saline (PBS) and adjusted to pH 7.4 with NaOH and maintained at 4 °C for up to 1 week. For Nrf2 mice, a similar number of males and females (aged 10–16 weeks) were used. The 3-NP treatment regimen consisted of 9 total intraperitoneal injections with one injection given every 12 h at successively increasing doses: 20 mg/kg × 4, 40 mg/kg × 4, and 60 mg/kg × 1 (33). No significant difference was observed between males and females with respect to behavior, weight loss, or lesion size in this study. For rats, daily intraperitoneal injections of 3-NP (n = 10) or PBS control (n = 10) were administered by one of two dosage regimens: 40, 40, and 20 mg/kg or 40, 20, and 20 mg/kg, each regimen resulting in the animal receiving a total of 100 mg/kg of 3-NP over 3 or 4 days. Both dosage regimens were tested to overcome possible preconditioning effects because of adenovirus infection. However, the average lesion volumes produced by the two dosage regimens were not different after statistical analyses (two-tailed t test), and the groups were pooled. Because virus was injected unilaterally we conservatively only included in the study animals with measurable 3-NP lesions by histology. Animals were sacrificed 24 h after the last injection or upon displaying severe motor behavior deficits such as recumbence (complete loss of motor control).

**Semi-Quantitative Behavior Scoring of Mice**—We have developed a detailed description for the assessment of 3-NP induced motor deficits described previously (33). Briefly, the scoring system involved the evaluation of 5 major symp
toms each with a 3-tiered scoring scale (0, 1, or 2), with 0 = normal behavior and 2 = severe deficit. Symptoms included: (a) hind limb clasp
ing, (b) reduced general locomotor activity, (c) hind limb dystonia (in
creased muscle tone leading to abnormal jerky movements), (d) trunkal dystonia, and (e) postural instability. Animals received a cumulative score of 10 for each trial. Behavior was assessed by an experimenter blinded to the animal genotype and treatment before each scheduled injection.

**Histology and Fluorojade Staining**—Mice were deeply anesthetized with euthanol (Bimed-MTC) and trans-cardially perfused with room temperature PBS followed by ice-cold 4% paraformaldehyde in PBS. The brains were post-fixed overnight in paraformaldehyde and then cryoprotected with 30% sucrose for 2 days before cryostat sectioning. Cresyl violet staining was performed with standard protocols on 40-μm sections mounted on SuperFrost Plus slides (Fisher). Lesion volume was calculated according to the principle of Cavalieri (volume = 4/3 × π × d$_3$ × d$_4$ + s$d_2$ + s$d_3$ + s$d_4$), where $s =$ lesion surface area and $d =$ distance between the 2 sections. Adjacent 20-μm slide-mounted sections were examined for Fluorojade (Histochrome Inc.) staining as previously de
described (34). Cresyl violet sections were scanned at 600 d.p.i. on an Epson Colorio scanner and lesion area was quantified by an experimenter blinded to the animal genotypes using ImageJ software (Na
tional Institutes of Health). Four anatomical levels of the striatum were examined (Bregma (in mm) +1.10, +0.40, –0.26, –0.92 according to the mouse brain atlas (35). Fluorojade-positive neurons were only as
sessed in the core of the striatal lesion. Fluorojade images were col
clected using an Axioskop microscope (Zeiss). Appropriate filters were used to enhance the contrast of the fluorochrome. Images were captured on a digital camera (QImaging). For rats, perfusion freezing and cresyl violet staining were performed as described for mice. Total lesion volumes were calculated using the following formula $V_o = V_t + V_1 + V_2 + \ldots + V_n$, where $V_o =$ m$m_0$, and $V_i =$ $m$m$_i$. where $h$ is the distance between two sections and $r$ is the radius of the lesion area using sections from Bregma (in mm) 1.70, 1.20, 0.70, 0.20, –0.28, and –0.80 according to the rat brain atlas (36).

**Succinate Dehydrogenase Assay**—Male or female mice from each genotype were acutely injected with 60 mg/kg of 3-NP and were always paired with PBS-injected littermates of the same sex and genotype. Mice were examined with halothane anesthesia before measurement on a microplate reader at 490 nm. Protein concentra
tion was determined using the bicinchoninic acid method (Pierce).

**Brain Homogenates** were serially diluted for the assay and data analy
ses were performed on values within the linear range of the SDH reaction. We also used 4 Nrf2$^{-/-}$ mice to examine the dose-response relationship between acute SDH inhibition and various injected 3-NP concentrations (20, 40, or 60 mg/kg). Both 20 and 40 mg/kg doses produced little or no change 2 h after injection, compared with PBS injection (data not shown), whereas 60 mg/kg produced an observable decrease in activity. The 60 mg/kg dose was used for further SDH assays.

**Preparation of tBHQ-supplemented Diets**—All rats and mice were normally fed Lab Diet 5001, which contains no tBHQ, but trace amounts of BHA for preserving animal fats (2 ppm). For tBHQ feeding, food pellets were powdered in a coffee grinder and dry mixed with tBHQ (1% w/w). Distilled water was added to the powder (equal v/w), and the mixture was resuspended into food pellets. The pellets were then baked at 60 °C for 3 h. Control food was processed in the same fashion without the addition of tBHQ. Initially, we determined 5% tBHQ in diet was not consumed by Nrf2$^{-/-}$ mice (n = 3), and 0.5% tBHQ, although consumed, only slightly preserved motor function during 3-NP treatment (n = 3). For these reasons, a concentration of 1% tBHQ was used in all further feeding experiments.

**Quantitative Real-time PCR Assays for Brain Tissue**—Brain and liver tissue were dissected in ice-cold PBS and homogenized with 10 strokes of a Dounce homogenizer in tissue buffer containing 25 mm Tris (pH 7.4) and 250 mm sucrose. Crude homogenates were centrifuged at 15,000 × g for 10 min at 4 °C. Tissue homogenate supernatants were collected and promptly assayed for enzyme activity. NQO1 enzyme activity was de
termined by calculating the dicumarol-sensitive fraction of 2,5-dichro
ylene-indo-phenol reduction (38). Reactions contained 40 μL of 5 mm Tris-HCl buffer (pH 7.4) with 0.7 mg/ml bovine serum albumin, 5 μM FAD, 200 μM NADH, 30 μg/ml protein, with and without 20 μM dicum
arol were preincubated for 10 min at 25 °C (final concentrations in 200 μl of reaction volume). 2,6-dichlorophenol-indo-phenol was then added to a final concentration of 36 μM (20 μl volume), and the reaction wa
t was started by the addition of the enzyme. The reaction rates were deter
ded by calculating the initial reaction velocity, which for 2,5-dichlorophenol-indo-phenol was 2.1 × 10$^{-5}$ m$^{-1}$ s$^{-1}$. The GST assay consisted of 1 mL 1-chloro-2,4-dinitrobenzene, 1 mL glutathione, and 100 μg/ml protein at 37 °C in 100 mM potassium phosphate buffer (pH 6.5) (final concentra
tion in 150 μl of reaction volume) (39). The GST reaction was mon

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itor at 340 nm and the spontaneous non-enzymatic sub-tractor was from the total observed slope. The extinction coefficient for 1-chloro-2,4-dinitrobenzene was 9600 M⁻¹ cm⁻¹. The lactate dehydrogenase (LDH) activity assay consisted of 3.3 mM pyruvate, 0.34 mM NADH, and 100 µM protein in PBS at 37 °C (final concentrations in 150 µl of reaction volume) (40). The LDH reaction was monitored at 340 nm and the spontaneous non-enzymatic slope was subtracted from the total observed slope. Purified LDH enzyme standards were used to ensure that values were obtained within the linear range of the assay. The extinction coefficient for NADH was 6219 M⁻¹ cm⁻¹. Protein concentra-
tion was determined as described previously (41). The lactate dehydrogenase activity assay was performed in triplicate.

**Stereotaxic Injections—**Rats were anesthetized by intraperitoneal injection of Somnitol (MTC Pharmaceuticals, Cambridge, ON) and placed in a stereotaxic frame (David Kopf Instruments). A burr hole was drilled and injection was performed into the right striatum at coordinates: 1.0-mm anterior-posterior from Bregma, 2.6-mm medial-lateral, and at both ~4.50- and ~5.50-mm dorsal-ventral, using a 10-µl Hamilton gas-tight syringe with a 26-gauge needle (type 2 tip). The virus was delivered by manually pressing the syringe plunger 3 times at 4-min intervals. Ad-GFP or Ad-Nrf2 was delivered at a concentration of 5 × 10⁶ total plaque forming units in 3 µl of PBS. Wounds were irrigated with 0.9% sterile saline solution and sutured. Animals were allowed to recover for 3 days before 3-NP treatment was initiated.

**Immunohistochemistry—**For rats, frozen 10-µm brain sections were thawed in PBS for 3 min at room temperature. Mouse monoclonal anti-O4 (Chemicon) or rabbit polyclonal anti-glial fibrillary acid protein antibodies were diluted 1:400 and 1:200, respectively, in Ab buffer (3% bovine serum albumin and 0.3% Triton X-100 in PBS) and incubated overnight at 4°C. The next day, the sections were rinsed with PBS for 5 min in PBS and incubated with goat anti-mouse IgM Alexa 546-conjugated or goat anti-rabbit Alexa 546-conjugated secondary antibodies (both Molecular Probes, 1:500) in a humid chamber for 1 h at room temperature. Sections were rinsed three times for 10 min in PBS prior to mounting with Fluoromount-G (Southern Biotechnology Asso-
ciates, Inc.). Images were captured using a Zeiss LSM Meta 510 confocal microscope and analyzed using NIH Image (for Mac) or ImageJ (for PC).

**Preparation of Primary Glial-enriched and COS-1 Cells—**Enriched glial cultures were prepared from 0–2 day post-natal Wistar rat pups as described previously (6). The conditions used largely results in a population of Type I and II astrocytes as assessed by anti-glia fibrillary acid protein staining. COS-1 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 54 mM pyruvate, 2 mM glucose, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

**Plasmids and Adenoviruses—**PEF mammalian expression plasmids carrying cDNA encoding mouse Nrf2 and Nrf2DN were a generous gift from Dr. Jawed Alam (Alton Ochsner Medical Foundation, New Orle-
ans, LA) (1). Rat ARE sequences were obtained from the nqopl promoter. The human placentatalkine phosphatase (hPAP) reporter plasmid (45) was described as described previously (44). To make adenoviral vectors were generated using the Cre-lox system (Canadian Stroke Network core facility, University of Ottawa), as described previously (6, 42). The N terminus-tagged Nrf2-GFP vector was made by PCR amplifying the Nrf2 coding sequence from the PEF vector with specific primer sequences: forward, 5’-ACTGAGATCTCGAAGGATTGTTGATCCTT-3’ and reverse, 5’-TCGCTGGAAAAAGAA-3’. The resulting PCR product was restriction digested with XhoI and KpnI for ligation into the pEGFP-N1 vector (Invitrogen). The Keap1 vector was a generous gift from Dr. Masayuki Yamamoto (University of Tsukuba), later modified to Keap1-FLAG by Laurie

**Transfection, Infection, and Treatments for Astrocytes and COS-1 Cells—**Astrocytes and COS-1 cells seeded (1 × 10⁵ cells/ml) in 24-well plates were transfected using Lipofectamine 2000 (Invitrogen), accord-
ing to the manufacturer’s protocol with some modifications. In parti-
cular, for each well of a 24-well plate, 1 µl of DNA and 1 µl of Lipo-
fectamine were used. The transfection efficiency was typically ~20% for astrocytes and ~50% for COS-1 cells as assessed by β-galactosidase staining. For some experiments, adenosine infection of astrocytes as described previously (6), was initiated immediately after Lipofectamine transfection. All infected cultures were examined by fluorescence microscopy to ensure that data represented PCR products within the linear range of the reaction (supplemental materials Fig. 2).

**Glutathione—**Glutathione was quantified in glial cell culture media using the glutathione assay kit (Biovision, 1:500). One µg of RNA was reverse transcribed using random hexamer primers and Thermoscript reverse transcriptase enzyme (Invitrogen). The resulting cDNA was then amplified by PCR using various primer sets (27): mouse Nrf2 exon 5, 5’-TCTTCA-TGGCTGAAAAAAA-3’ and 5’-AATTGGTCTGCTGCTGCTGATT-3’; nqo1, 5’-CATTCTGAAGGCTGTTTGA-3’ and 5’-CATCCTTGAT- TGGTGTACG-3’; Xot, 5’-TTGCAAGCTCAAGAAACCT-3’ and 5’-GTCGAGTAGTACGAAAAA-3’; gc1, 5’-AACAGAGCCCGCCTTCG- GGT-3’ and 5’-CTCCAGGCTCTCTACTCTCC-3’; β-actin, 5’-ACGAAAG- CCTGCTGCTGCTCATT-3’ and 5’-GTAGATGAAGATTTGAGATTTT-3’. PCR conditions were as follows: initial denaturing step using 1 cycle at 95 °C for 3 min, followed by 26 cycles at 95 °C for 30 s, the appri-
ate primer annealing temperature (~55 °C) for 40 s, 72 °C for 1 min, and a final cycle at 72 °C for 5 min. For Xot and gc1, 36 and 31 cycles were used, respectively. The PCR products were separated on a 1.4% agarose gel containing ethidium bromide. cDNA was then visualized using an ultraviolet light source. cDNA templates for each sample were serially diluted and band densities were plotted. These plots were used to ensure that data represented PCR products within the linear range of the reaction (supplemental materials Fig. 2).

**Glutathione Assay—**Glutathione was quantified in glial cell culture media and tissue homogenates (100 mg/ml) by the method of Tietze (45), as described previously (6).

**Western Blot Analysis and Immunocytochemistry—**Western blot and immunocytochemistry was performed as described previously (6). Anti-
tibodies used include: rabbit anti-heme oxygenase 1 (Stressgen Inc., 1:500), goat anti-actin (Santa Cruz, 1:1000), sheep anti-rabbit horseradish peroxidase (Amersham Biosciences, 1:5000), donkey anti-goat horseradish peroxidase (Santa Cruz, 1:5000), mouse anti-FLAG M5 (Sigma, 1:1000), and goat anti-rabbit Texas Red (Molecular Probes, 1:2000).

**Nrf2-GFP Localization in COS-1 Cells—**After 2 h of 3-NP treatment or 4 h of tBHQ treatment, COS-1 cells were fixed and stained. Cell count (and nucleus/cytoplasm delineation) was performed by an experi-
menter blinded to the treatment groups and directed to assess whether the Nrf2-GFP fluorescence was excluded from or enriched in the nucleus. We confirmed that fluorescence using a nuclear counterstain (Hoechst) and found in all other experiments that it was relatively straightforward to assess Nrf2 localization even without the counterstain because COS-1
cells have a well defined nuclear-cytoplasmic border.

Data Analysis—All data are shown as mean ± S.E. Statistical analyses were performed using Student’s t test and one-way or two-way ANOVA in GraphPad Prism (version 2.01).

RESULTS

Nrf2−/− Mice Are Hypersensitive to Systemic 3-NP Treatment—Nrf2−/− mice were challenged with 3-NP to examine how loss of Nrf2 function affects both motor system impairment and toxicity attributed to metabolic inhibition in vivo. Our hypothesis was that Nrf2−/− mice would be more sensitive to the effects of 3-NP-induced metabolic inhibition. Thus, in initial experiments we determined in vivo 3-NP dosing regimes that would provide significant impairment in Nrf2−/− mice and then assessed what impact these had on wild-type animals. The extent of 3-NP toxicity was monitored through both manifestation of overt motor symptoms (i.e., reduced general locomotor activity, hind limb and truncal dystonia, and postural instability) and development of bilateral lesions restricted to the dorso-lateral striata (33). An advantage of using behavioral assessment as an adjunct to histology is that it can be performed repeatedly on the same animals providing a measure of 3-NP toxicity progression.

FIG. 1. Behavioral scoring, weight loss, and lesions of mice with varied Nrf2 genotype during 3-NP challenge. Behavior (A) and weight (B) were evaluated before each scheduled injection. The 3-NP treatment regimen consisted of 9 total intraperitoneal injections with one injection given every 12 h at successively increasing doses: 20 mg/kg × 4, 40 mg/kg × 4, and 60 mg/kg × 1 (doses are depicted with black bars below the x axis). At 108 h, behavior was measured but no injections were made. All animals were sacrificed 1.5 days after the last dose of 3-NP (arrow). C, representative cresyl violet-stained slices from all genotypes after 3-NP treatment. Pallor in staining because of cell death and cell shrinkage is outlined by dotted lines. D, representative images from cresyl violet-stained slices (left column, scale bar = 1 mm), magnified image from cresyl violet striatal inset (middle column, scale bar = 100 μm), and magnified image of Fluorojade staining (marker of neuronal degeneration) from striatal inset (right column, scale bar = 100 μm). Arrow shows typical hemorrhaging seen in some cases with the Nrf2−/− group. E, lesion area from 4 different levels relative to Bregma within the striatum were measured and expressed as a percentage of total ipsilateral striatum. Left and right bars for each genotype correspond to left and right hemispheres. No significant differences were seen between hemispheres within the animals. F, Fluorojade-positive cells were counted in a region of interest within the core of the lesion. Counts from both hemispheres were averaged for each slice. Data represent the mean ± S.E. from n = 5 (Nrf2+/+), n = 7 (Nrf2−/−), n = 5 (Nrf2−/−). *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared with wild-type control group, two-tailed t test.

FIG. 2. Inhibition of brain SDH is not different among Nrf2 genotypes 2 h after acute 3-NP injection. Four mice from each genotype were injected with a single dose of 60 mg/kg 3-NP or PBS. 3-NP-injected mice were compared with PBS-injected littermates of the same genotype for each experiment. Animals were sacrificed 2 h after 3-NP injection when the peak of SDH inhibition has been shown to occur (47). Mitochondria samples were prepared from striatum and cortex and used in the SDH assay. A590 was normalized to mitochondrial protein content. Data represent the mean ± S.E. from n = 4 mice for each genotype and treatment group. *, p < 0.05, compared with PBS injected controls from same genotype, one-way ANOVA with Bonferroni post-hoc test.
Pre-activation of Nrf2 by tert-Butylhydroquinone Attenuates motor deficits and lesions in this genotype (data not shown).

**Striatal SDH Inhibition by 3-NP Is Not Different Among Genotypes**—Because we used systemic administration of 3-NP, it was possible that increased toxicity in Nrf2<sup>−/−</sup> mice was because of compromised peripheral detoxification systems (i.e. liver, kidney, and intestines), leading to a higher circulating concentration of 3-NP in the brain. To address this possibility, we directly quantified SDH activity in the brain after an acute systemic injection of 3-NP (60 mg/kg). The cortex and striatum were collected after 2 h when peak brain SDH inhibition was shown to occur (47). SDH activity was reduced by 40–50% in total tissue from cortex and striatum of all genotypes, consistent with previous studies (Fig. 2) (47). Importantly, we observed no difference in SDH activity between genotypes, suggesting that increased damage caused by 3-NP in Nrf2<sup>−/−</sup> mice is most likely because of an intrinsic sensitivity in their brains.

**Pre-activation of Nrf2 by tert-Butylhydroquinone Attenuates 3-NP Toxicity in Nrf2<sup>−/−</sup>, but Not Nrf2<sup>+/−</sup> Mice**—Nrf2 induction by small electrophilic molecules represents a practical method to increase the phase 2 response from broccoli sprouts have been shown to induce phase 2 genes in peripheral tissues and reduce toxicity associated with a number of pathologies, including stomach tumor formation and hyperpnea (18, 51). To determine whether in vivo induction of Nrf2 could provide resistance to 3-NP toxicity, mouse diets were supplemented with the potent Nrf2 inducer, tBHQ (1, 52).

The in vivo phase 2 response was assessed by measuring the activities of two prototypic phase 2 enzymes, glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1), as well as total glutathione (GSH) content. Nrf2<sup>+/−</sup> mice exhibited lower basal striatal and cortical activity for GST and NQO1, when compared with Nrf2<sup>−/−</sup> littermate controls (Table I, basal phase 2 enzyme levels). Interestingly, basal brain GSH levels were not altered, suggesting that constitutive synthesis of brain GSH does not rely on Nrf2 function, at least in the young adult mice examined in here. As observed in previous studies, GST, NQO1 activities, and total GSH levels were markedly reduced in the livers of Nrf2<sup>−/−</sup> mice (53–56). The housekeeping gene, *ldh*, is not regulated by Nrf2 and accordingly, no significant differences in activity were observed between genotypes.
Primarily Nrf2+/− and Nrf2−/− mice were used in tBHQ feeding studies because we were interested in observing reduced motor deficit and lesion size during 3-NP treatment, and Nrf2−/− mice did not develop these phenotypes. Mice received a tBHQ-supplemented diet for 1 week prior to and during 3-NP administration. Only Nrf2+/− and Nrf2−/− mice were studied because we were interested in observing reduced motor deficit and lesion size with tBHQ treatment, and Nrf2−/− mice did not develop these phenotypes. A, no significant differences were observed in body weight of Nrf2−/− mice between tBHQ and control diet. B, Nrf2−/− mice never fully adjusted to the tBHQ-supplemented diet where body weight of tBHQ-fed Nrf2−/− mice dropped by 10–20% during 1 week of feeding. C, control diet and tBHQ-fed Nrf2−/− mice consumed similar amounts of food throughout the experiment. D, Nrf2−/− mice consumed less food than Nrf2−/− mice with control diet. E, tBHQ-fed Nrf2+/− mice exhibited reduced motor deficit at the end of the 3-NP treatment regimen. F, an overall exacerbation of 3-NP-induced motor deficits was associated with tBHQ feeding of Nrf2−/− mice. G and H, tBHQ feeding prevented lesion development in Nrf2−/− mice, but had no effect on Nrf2−/− mice. Lesions are outlined by dotted lines. For the Nrf2−/− group, n = 8 and 6 animals were used for control and tBHQ-supplemented diets, respectively. For the Nrf2−/− group, n = 6 and 5 animals were used for control and tBHQ-supplemented diets, respectively. Data represents mean ± S.E., *, p < 0.05; **, p < 0.01, compared with Nrf2−/− mice receiving control diet, one-way ANOVA with Bonferroni post-hoc test.
7.6 ± 0.7 mg of tBHq/g of body weight over 1 week, respectively. The 1% tBHq diet increased striatal and cortical GSH levels in Nrf2-expressing mice (Nrf2+/− and Nrf2+/−) (Table I, tBHq-induced phase 2 enzyme levels). Importantly, the induction of brain GSH was not observed in Nrf2−/− mice, providing evidence that tBHq-induced changes in GSH are Nrf2 dependent. In addition, the tBHq-induced increase in liver GST and NQO1 activities was observed in Nrf2 expressing mice, but not Nrf2−/− mice, consistent with the effects of other Nrf2 inducers used in vivo (56, 57). However, no induction of GST or NQO1 was detected in brain. Levels of the negative control enzyme, LDH, was unaffected by tBHq in all tissues examined. Interestingly, we observed increased liver GSH levels in tBHq-fed Nrf2−/− mice. We believe that this Nrf2-independent change may reflect an inherent hepatic toxicity associated with tBHq feeding, perhaps because of inefficient detoxification from the body by the same pathways regulated by Nrf2 (phase 2 genes). Together, these results highlight the sensitivity of the Nrf2−/− phenotype to tBHq toxicity and suggest normal phase 2 induction in response to tBHq is abrogated in Nrf2−/− mice.

The tBHq diet was given 1 week prior to the 3-NP regimen and maintained until sacrifice. TBHq-fed Nrf2−/− mice had significantly attenuated 3-NP-induced motor deficits compared with mice receiving the control diet (Fig. 3E, **, p < 0.001 two-way ANOVA). In contrast, tBHq-fed Nrf2−/− mice exhibited an overall exacerbation of motor deficit (Fig. 3F, ***, p = 0.0002 two-way ANOVA). The average lesion volume of Nrf2−/− given control diet was similar to that measure in our initial experiments (0.65 ± 0.29 mm3, uncorrected for edema) (Fig. 3, G and H). However, consistent with reduced behavioral scores, no tBHq-fed Nrf2−/− mice exhibited detectable lesions indicating protection of neurons. tBHq feeding did not reduce lesion size in Nrf2−/− mice, confirming the protective effect of tBHq consumption was Nrf2-dependent (Fig. 3, G and H).

**Fig. 4.** Ad-Nrf2 infected animals developed smaller lesions than Ad-GFP control animals following 3-NP administration. A, immunostaining of GFP with the cell type-specific markers for astrocytes (GFAP, top panel), neurons (NeuN, middle panel), and oligodendrocytes (O4, bottom panel) was first performed to characterize the specificity of viral infection in rats in vivo. Scale bars = 10 μm. B, incidence of co-labeling between GFP and the different markers. N represents the total number of GFP+ cells counted for two animals. C, representative images of GFP expression in the striatum of Ad-GFP-infected animals at Bregma (clockwise from top left) 1.00 mm, 0.48 mm, −0.80 mm, and −0.26 mm. Scale bars, 500 μm. D, analysis of GFP fluorescence as a percentage of total striatal volume (as determined by the area outlined by a propidium iodide counterstain, n = 11 animals). E, quantification of 3-NP-induced lesion volumes in the ipsilateral (right) and contralateral (left) hemispheres. Data represent mean ± S.E. of n = 5 animals per group. *, p < 0.05, compared with Ad-GFP infected controls, two-tailed t test. F, representative cresyl violet-stained sections at Bregma 0.70 mm for all the indicated treatment groups. Arrow indicates hemisphere receiving the adenoviral vector. Scale bar = 2.5 mm.

Adenoviral Overexpression of Nrf2 Attenuates 3-NP-induced Striatal Lesioning in Vivo—To further address whether activation of Nrf2-dependent pathways is sufficient to protect the striatum during 3-NP toxicity, in parallel studies, we tested whether direct Nrf2 overexpression using adenovirus vectors (Ad-Nrf2 or Ad-GFP control) conferred neuroprotection to rats treated with 3-NP. Overexpression of Nrf2 causes its accumulation in the nucleus leading to even more robust ARE-dependent transcription than that observed with small molecule inducer treatment (43). In vivo intrastriatal adenovirus injection primarily led to infection of astrocytes (Fig. 4, A, top panel, and B), and almost no neurons or oligodendrocytes (Fig. 4A, middle and bottom panel, and B). Vector diffusion was observed throughout the striatum (Fig. 4C), infecting 0.48 ± 0.08% of the total striatal volume, as assessed by the presence of the GFP marker (Fig. 4D). After infection, protein expression was allowed to occur for 3 days before 3-NP treatment was initiated. Following 3-NP challenge, lesion volumes in the infected hemisphere were significantly smaller in animals receiving Ad-Nrf2, compared with Ad-GFP control virus (9.6 ± 2.6 mm3 versus 23.4 ± 4.2 mm3, p = 0.03, two-tailed t test, Fig. 4, E and F). Interestingly, a trend toward decreased lesion volume was also observed in the hemisphere contralateral to the virus injection (12.2 ± 3.8 mm3 compared with 25.9 ± 4.9 mm3, p = 0.06), although this difference did not attain statistical significance. This effect may be because of diffusion of secreted glial factors, possibly glutathione or glutathione precursors, by volume transmission along fiber bundles of the corpus callosum, or through the cerebral spinal fluid from the infected Nrf2 overexpressing astrocytes in the ipsilateral hemisphere (58, 59).

3-NP Activates ARE-dependent Gene Expression in Cultured Astrocytes—Our data show that both the Nrf2 inducer tBHq and Nrf2 overexpression can protect neurons by pre-activating the phase 2 response. Because increased susceptibility of Nrf2−/− mice may result from the inability to activate expres-
sion of protective genes necessary for reducing toxicity associated with 3-NP, we hypothesized that metabolic inhibition by 3-NP was acting as a signal for Nrf2 activation. To test this hypothesis, we examined the effect of 3-NP on Nrf2 activity in cultured astrocytes, the primary site of ARE-mediated gene expression in brain cultures (6, 41, 60).

To mimic the effects of bolus in vivo injection, cultured astrocytes were treated with a wide range of 3-NP concentrations (1–30 mM) for 2 h and then ARE-mediated gene expression was assessed 24 h later using a hPAP reporter assay regulated by an ARE-bearing *nqo1* promoter (Fig. 5A) (41). The astrocytes were then treated with varying concentrations of 3-NP (1–30 mM) for 2 h in a glucose-free basic salt solution (BSS vehicle), followed by washout. A doubling of hPAP activity was observed 24 h after 10 or 30 mM 3-NP treatment. B, expression of ARE-driven hPAP, with vehicle or 3-NP exposure, was reduced to sub-basal levels when astrocytes overexpressed a dominant-negative form of Nrf2 (Ad-Nrf2DN), but not with overexpression of a GFP control (Ad-GFP) (6). ARE-driven hPAP was induced by 24 h of tBHQ exposure and maximally increased with Ad-Nrf2 infection. Data represent the mean ± S.E. from three independent experiments. C, semi-quantitative RT-PCR confirmed that astrocytes treated with 30 mM 3-NP expressed more mRNAs from prototypic Nrf2 target genes including *nqo1*, *gclc*, and *Xct*. DNA templates were serially diluted to ensure that band intensities were observed within the linear range of the PCR (supplemental materials Fig. 2). As controls, Ad-Nrf2- and Ad-Nrf2DN-infected cultures were also evaluated with RT-PCR. In all cases, the mRNA levels were induced with Nrf2 overexpression and suppressed with Nrf2DN overexpression. RT-PCR experiments were reproduced three times with similar results. D, cultures treated with 30 mM 3-NP produced more total intracellular GSH than vehicle treated controls. GSH induction was blocked with Ad-Nrf2DN infection, but not Ad-GFP control. E, Western blot analysis shows that protein expression of HO-1 was induced with 30 mM 3-NP treatment in Ad-GFP-infected cultures. Both basal and induced expression of HO-1 was largely blocked with Ad-Nrf2DN infection. F, densitometric analyses of HO-1 protein levels. GSH and HO-1 data represent the mean ± S.E. from three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001, two-tailed paired t test.
lar extent as 3-NP treatment. Maximal hPAP activity was achieved with Nrf2 overexpression (Ad-Nrf2). Although the concentrations of 3-NP we used to activate Nrf2 mediated gene expression were high, the ability of dominant negative Nrf2 to block the induction indicates that it is nonetheless mediated by the ARE. Perhaps these high concentrations reflect the activated and toxin-resistant nature of astrocytes in vitro (61).

Semi-quantitative RT-PCR confirmed that astrocytes treated with 30 mM 3-NP for 2 h expressed higher levels of prototypic Nrf2 target gene mRNAs including npo1, γ-glutamylcysteine synthetase (gclc), and the cystine/glutamate exchanger (Xct) (Fig. 5C). The expression of these mRNAs was increased by Ad-Nrf2 and suppressed by Ad-Nrf2DN infection, verifying the specificity of these target genes. For all RT-PCR experiments, DNA templates were serially diluted to ensure that band intensities were observed within the linear range of the PCR (supplemental materials Fig. 2). Because a number of Nrf2 target genes are involved in GSH synthesis (i.e. Xct, gclc, and glutathione synthase), treatment with 30 mM 3-NP also increased intracellular GSH levels in cultured astrocytes (Fig. 5D) (5, 6, 54, 55). Consistent with ARE-driven hPAP expression, 3-NP-mediated GSH induction was blocked with Ad-Nrf2DN infection, but not Ad-GFP control (Fig. 5D). Protein levels of heme oxygenase-1 (HO-1), a well characterized Nrf2 target, were also induced by 3-NP treatment (Fig. 5, E and F) (1). Both basal and induced expression of HO-1 was suppressed with Ad-Nrf2DN infection. Although this analysis does not encompass the full scope of Nrf2 gene targets, it nevertheless indicates the phase 2 response is activated as a dose-dependent effect of 3-NP.

We further evaluated nuclear translocation of GFP-tagged Nrf2 (Nrf2-GFP) in response to 3-NP exposure. In initial experiments using astrocytes, Nrf2-GFP fluorescence was almost undetectable when co-expressed with its cytoplasmic regulator Keap1, possibly because of a faster rate of Nrf2 turnover by proteasome-dependent pathways (62). As an alternative, COS-1 cells were used because of their high level of transgene expression and well defined morphology for evaluating nuclear localization (5, 6, 54, 55). Consistent with previous studies, overexpressed Nrf2-GFP was almost exclusively localized to the nucleus and initiates phase 2 gene induction in response to 3-NP and Keap1-FLAG constructs were functional, as assessed by their ability to augment and suppress ARE-driven hPAP expression, respectively. Data represents three separate experiments scored by a researcher blinded to the treatment conditions (total cells evaluated: n = 119 and n = 158, BSS and 3-NP-treated cells, respectively). *, p < 0.05, two-tailed paired t test.

Fig. 6. 3-NP promotes nuclear translocation of Nrf2-GFP. A, COS-1 cells were transiently transfected with Nrf2-GFP (N-terminal fusion to Nrf2) with and without Keap1-FLAG. Consistent with previous studies, overexpressed Nrf2-GFP was almost exclusively localized to the nucleus unless co-expressed with its cytoplasmic regulator Keap1 (top row) (13, 43). COS-1 cells co-expressing Nrf2-GFP and Keap1-FLAG were evaluated after treatment with 3-NP or BSS vehicle control. The proportion of cells exhibiting nuclear-enriched Nrf2-GFP was significantly higher in 3-NP-treated cultures, when compared with vehicle control. 3-NP also appeared to cause a transient aggregation of some Keap1-FLAG and Nrf2-GFP in the cytoplasm (bottom row). Treatment with 20 μM tBHQ for 4 h, as a positive control, also robustly increased nuclear localization of Nrf2-GFP (scale bar = 10 μm). B, summary of Nrf2-GFP localization in response to tBHQ and 3-NP. C, both Nrf2-GFP and Keap1-FLAG constructs were functional, as assessed by their ability to augment and suppress ARE-driven hPAP expression, respectively. Data represents three separate experiments scored by a researcher blinded to the treatment conditions (total cells evaluated: n = 119 and n = 158, BSS and 3-NP-treated cells, respectively). *, p < 0.05, two-tailed paired t test.

Collectively, this in vitro data confirms Nrf2 translocates to the nucleus and initiates phase 2 gene induction in response to 3-NP-mediated metabolic inhibition. This effect can be mim-
icked using the Nrf2 inducer, tBHQ, and by adeno viral Nrf2 overexpression.

**DISCUSSION**

This study provides evidence to support the neuroprotective function of Nrf2 during metabolic compromise in the *in vivo* brain. First, loss of Nrf2 function led to an increased susceptibility to 3-NP because Nrf2−/− mice developed motor deficits and striatal lesions more rapidly than Nrf2 expressing controls. Second, pre-activation of endogenous Nrf2 using the small molecule inducer, tBHQ, attenuated 3-NP toxicity in Nrf2−/− mice, but not Nrf2−/−, confirming both the neuroprotective action and Nrf2 dependence of tBHQ *in vivo*. Third, direct overexpression of Nrf2 in the striatum is sufficient to reduce lesion size caused by 3-NP. Fourth, ARE-dependent gene expression in cultured astrocytes was activated by 3-NP, and could be completely suppressed by overexpression of a dominant-negative form of Nrf2.

**Nrf2−/− Mice Are Hypersensitive to 3-NP Toxicity**—The increased sensitivity of Nrf2−/− mice may result from an inability to induce phase 2 genes in response to 3-NP toxicity. Indeed, a recent study by Calc瞌 et al. (64) (published while this work was submitted) showed using transgenic reporter mice, that ARE-dependent gene expression occurred in the immediate border of 3-NP induced striatal lesions, perhaps in reactive astrocytes commonly found within this region (64). Because Nrf2 is generally thought to respond to oxidative stress or signal transduction pathways associated with it (65, 66), we speculate that 3-NP activates this protective pathway by indirectly generating ROS/RNS. 3-NP may increase brain oxidative stress through multiple mechanisms that may be implicated in our *in vitro* and *in vivo* experiments (6, 27, 29, 30). Also supported by our data, an alternative possibility for the increased sensitivity of Nrf2−/− mice may be the lower basal levels of phase 2 enzymes expressed in this genotype (Table I, basal phase 2 enzyme levels) (83).

Given that systemic 3-NP undergoes first pass metabolism before it reaches the striatum, it was possible that increased striatal damage in Nrf2−/− mice was in part because of impaired clearance of 3-NP from the body by peripheral detoxification. Indeed previous studies of Nrf2−/− mice have shown decreased phase 2 enzyme activity in the liver and intestines (53, 67), organs that may affect the removal of systemically injected 3-NP. However, we have shown that after a single high *in vivo* dose of 3-NP, striatal SDH was inhibited to an equal extent between Nrf2+/+ and Nrf2−/− genotypes, suggesting that 3-NP produces similar metabolic inhibition on a dose-wise basis regardless of genotype. Thus, it appears that the brains of Nrf2−/− mice are intrinsically more sensitive to direct damage. This conclusion is also supported by a number of other findings. First, neurons and glia isolated from Nrf2−/− mice are more sensitive to *in vitro* toxicity paradigms involving oxidative stress or metabolic compromise (8, 27, 28). Second, direct injection of malonate (reversible SDH inhibitor) into the striatum produced larger lesions in Nrf2−/− mice, compared with Nrf2 expressing controls (64). Third, Nrf2−/− mice develop larger cortical infarcts than Nrf2+/+ mice after experimental stroke (permanent distal middle cerebral artery occlusion), a model that also involves local brain metabolic inhibition (31).

**The Small Molecule Inducer, tBHQ, Provided Nrf2-dependent Amelioration of 3-NP Toxicity in Vivo**—Although studies indicate that loss of Nrf2 potentiates neurotoxicity (64), whether the *in vivo* neuroprotective effects of small molecule inducers are lost in Nrf2 knock-out mice has not been previously addressed. Recent *in vitro* studies have shown that tBHQ treatment augments ARE-dependent gene expression in cultured astrocytes and protects neurons from toxicity paradigms involving oxidative stress (6, 28, 68, 69). We extend these findings to the *in vivo* situation because dietary consumption of tBHQ significantly reduced motor deficits and lesion developments in Nrf2−/− mice. Importantly, the protective effect of tBHQ was abrogated in Nrf2−/− mice. Furthermore, dietary tBHQ administration increased brain GSH levels in Nrf2−/− and Nrf2+/− mice, but not Nrf2−/− mice (Table I, tBHQ-induced phase 2 enzyme levels).

As expected from previous studies (70), dietary tBHQ treatment also increased phase 2 enzyme activity (GST and NQO1) in the liver. Thus, it remains possible that attenuation of 3-NP toxicity in tBHQ-fed mice could be in part because of enhanced removal of 3-NP from the body by increased liver detoxification or conceivably liver-derived antioxidants such as GSH released into the blood. However, our observed increase in brain GSH would be expected to contribute to local neutralization of ROS/RNS, leading to tissue preservation. In preliminary studies, we have found that even local infusion of tBHQ in brain ventricles using osmotic pumps can lead to increases in liver phase 2 enzyme levels suggesting that it is difficult to completely dissociate brain and peripheral organ detoxification. Future studies could more directly address the role of brain Nrf2 by developing tissue and organ-specific Nrf2 knock-out. However, in support of the brain being the site of tBHQ action, overexpression of Nrf2 within the striatum using adenovirus was sufficient to reduce the effect of systemically administered 3-NP. Although some uncertainty remains as to the site of Nrf2 induction mediated by tBHQ *in vivo*, our study nonetheless indicates that striatal-specific damage because of the systemic administration of a metabolic stressor can be strongly attenuated by this dietary strategy. Given that many neurodegenerative diseases may be triggered by exposure to diet or lung-derived toxins (71–73), it is conceivable that a dietary strategy to increase antioxidant function in multiple organs may be an ideal prophylactic strategy. To our knowledge, this is the first study to show that a protective effect of a phase 2 inducer on brain function *in vivo* is dependent on Nrf2. Although tBHQ is widely used as a food antioxidant, and is well tolerated by the body (74, 75), it is also important to consider the detrimental action of tBHQ and other Nrf2 inducers. As observed in this study, Nrf2−/− mice do not tolerate tBHQ administration as well as controls (as evidenced by weight loss and reduced food consumption) perhaps because of inefficient detoxification of tBHQ through Nrf2-regulated pathways (Fig. 3, A and B). Other groups have also found that Nrf2−/− mice are less tolerant to dietary administration of known Nrf2 inducers (67). Recently, increased susceptibility to hypoxia has been linked to polymorphisms in the Nrf2 gene (76). The question arises as to whether such mutations affect Nrf2 function in humans, and could underlie sensitivity to human disease. Because many Nrf2 inducers are essentially reactive electrophiles, the inability to properly detoxify Nrf2 inducers from the body could be harmful to individuals with such a genetic predisposition.

With regard to practical application, recent studies have elucidated a variety of structurally related small molecules that are able to activate Nrf2 by reacting with thiols/disulfide groups on the cytoplasmic regulatory protein Keap1 (4, 48, 50, 77–79). Some of these inducers are found within our diet. For example, the isothiocyanate sulforaphane, a potent Nrf2 inducer abundant in cruciferous plants (i.e. broccoli sprouts), was found to inhibit gastric tumor formation induced benzo[a]pyrene (78). Juurlink and colleagues (51, 80) have recently demonstrated a significant improvement of cardiovascular fit-

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2 A. Y. Shih and T. H. Murphy, unpublished data.
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62%. Adenovirus was found to mainly infect astrocytes (from culture) directly into the striatum prior to injection of the complex II inhibitor, malonate. In addition, many factors contributing to oxidative stress (i.e., ROS/RNS) are cell permeable allowing them to be metabolized in a sub-population of cells (82). Another factor to consider is the actual spatial localization of the astrocytes infected in situ. The patchy nature of the infection (Fig. 4C) suggests a wider area can be protected within the volume of the brain. Furthermore, there is precedent for a small number of cells playing a major role in striatal function. One such example are the cholinergic interneurons of the striatum that only make up 1–2% of all striatal neurons yet have profound impact on the functional output of the striatum influencing such diverse behaviors as sensorimotor function, sleep and arousal states, learning and memory, anxiety, and pain sensations (83).

3-NP Exposure Activated ARE-mediated Gene Expression in Vitro—Our in vitro experiments show that 3-NP exposure up-regulated phase 2 gene expression in cultured astrocytes, providing a mechanistic link between 3-NP and Nrf2 activation. The effect of 3-NP was attributed to Nrf2 because a dominant negative version of Nrf2 was able to block the effect of 3-NP. However, further experiments are necessary to determine which component of 3-NP toxicity acts as a signal for Nrf2 activation. For example, Nrf2 activation may result from 3-NP-induced ROS/RNS generation, loss of ATP, or even as a direct response to SDH inhibition. In preliminary studies using cultured astrocytes, we examined the level of SDH inhibition caused by the range of 3-NP concentrations used in vitro. After 2 h of exposure to non-Nrf2 inducing 3-NP concentrations (0.3–1 mM), SDH activity was reduced by ~90%. However, at Nrf2 inducing concentrations (10–30 mM), SDH activity was almost completely inhibited (~98%). Given this difference, it is possible that a non-linear relationship exists between SDH activity and activation of ARE-mediated gene expression. Consequently, the requirement for high concentrations of 3-NP to induce Nrf2-mediated gene expression may in part be due to the high intrinsic resistance of cultured astrocytes that show an activated phenotype to agents that induce metabolic inhibition and oxidative stress (61).

CONCLUSION

Nrf2 plays an important role in regulating neuronal survival during metabolic compromise in vitro. The absence of Nrf2 function in Nrf2−/− mice exacerbates neurodegeneration caused by 3-NP administration. Augmentation of ARE-mediated gene expression in Nrf2-expressing animals using the small molecule inducer, tBHQ, attenuated tissue damage and preserved motor function. Dietary administration of Nrf2 inducers may have profound effects for neuronal viability in neurodegenerative disease, stroke, and related forms of energy deprivation.

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REFERENCES

Supplementary Figure 1. Behavioural scores and weight are highly correlated with lesion volume during 3-NP challenge. A B.) Lesion volume was plotted against either behavioural scores obtained 1.5 days after the last 3-NP injection or percentage start weight 1.5 days after the last 3-NP injection. Strong correlations were found with both comparisons. Data represent the mean + SEM (animals per genotype: Nrf2+/+ = 5, Nrf2+/− = 7, Nrf2−/− = 5). *** p < 0.0001, r = 0.867 (A), ** p < 0.001, r = -0.693 (B), Spearman test.

Supplementary Figure 2. RT-PCR images were collected within the linear range of the PCR reaction. RNA extracted from vehicle, 3-NP treated glia (3 and 30 mM) was used for standard semi-quantitative RT-PCR. Appropriate primers were used to determine relative quantities of Nrf2, NQO1, GCLC, xCT transcripts, and these values were normalized with actin density (A-D). E.) 3-NP treatment did not affect actin mRNA levels. cDNA templates were serially diluted to ensure that band intensities were observed within the linear range of the PCR reaction. Densitometric analyses were performed with ImageJ Software (NIH). Data shown is representative of 3 independent experiments with similar results.
Supplementary Figure 1

A

B

Behavioural Score

Lesion Volume (mm$^3$)

Animal Weight (% start weight)

Lesion Volume (mm$^3$)