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Sulforaphane Protects Astrocytes against Oxidative Stress and Delayed Death Caused by Oxygen and Glucose Deprivation

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Abstract

Oxidative stress is an important molecular mechanism of astrocyte injury and death following ischemia reperfusion and may be an effective target of intervention. One therapeutic strategy for detoxifying the many different reactive oxygen and nitrogen species that are produced under these conditions is induction of the Phase II gene response by the use of chemicals or conditions that promote the translocation of the transcriptional activating factor NRF2 from the cytosol to the nucleus, where it binds to genomic antioxidant response elements. This study tested the hypothesis that pre- or post-treatment of cultured cortical astrocytes with sulforaphane, an alkylating agent known to activate the NRF2 pathway of gene expression protects against death of astrocytes caused by transient exposure to O₂ and glucose deprivation (OGD). Rat cortical astrocytes were exposed to 5 μ M sulforaphane either 48 hr prior to, or for 48 hr after a 4 hr period of OGD. Both pre- and post-treatments significantly reduced cell death at 48 hr after OGD. Immunostaining for 8-hydroxy-2-deoxyguanosine, a marker of DNA/RNA oxidation, was reduced at 4 hr reoxygenation with sulforaphane pretreatment. Sulforaphane exposure was followed by an increase in cellular and nuclear NRF2 immunoreactivity. Moreover, sulforaphane also increased the mRNA, protein level, and enzyme activity of NADPH/Quinone Oxidoreductase 1, a known target of NRF2 transcriptional activation. We conclude that sulforaphane stimulates the NRF2 pathway of antioxidant gene expression in astrocytes and protects them from cell death in an *in vitro* model of ischemia/reperfusion.

Keywords

Ischemia/Reperfusion; NRF2; NQO1; Oxidative stress; 8-hydroxy-2-deoxyguanosine

INTRODUCTION

Cerebral ischemia followed by reperfusion is characterized by an increase in oxidative stress and a wide variety of cellular alterations including impaired energy metabolism, that lead to delayed neuronal and astrocyte cell death. Astrocytes undergo dysfunction and death following both focal and global cerebral ischemia (Li et al., 1995b; Sugawara et al.,

2002b;Petito et al., 1998), in some studies even prior to neuronal death (Liu et al., 1999) and display a spatial pattern of selective vulnerability (Ouyang et al., 2007). Many studies have demonstrated by using antioxidants or different ambient O₂ levels that a strong association exists between the extent of oxidative damage to macromolecules, e.g., proteins and DNA, and the extent of neuron and astrocyte cell death both *in vivo* and *in vitro* (Martin et al., 2000;Vereczki et al., 2006;Ouyang et al., 2007;Danilov and Fiskum, 2008). The extent of neuroprotection by antioxidants may be limited, however, by several factors, including blood brain barrier permeability and a limited ability to target the large number of different reactive oxygen or nitrogen species generated in these conditions (Yang et al., 2000). An alternative, combined therapeutic approach is the pharmacologic activation of multiple endogenous antioxidant defense systems.

Nuclear factor erythroid 2-related factor 2 (NRF2), first discovered by (Moi et al., 1994), is a transcription factor that belongs to the Cap 'n' Collar family of transcription factors that share a highly conserved basic leucine zipper (bZIP) structure (Motohashi et al., 1997). NRF2 activation can result in neuroprotection through its interaction with antioxidant response elements (ARE) and subsequent expression of numerous genes including NAD(P)H:quinone oxidoreductase 1 (NQO1) (Li et al., 1995a), heme oxygenase-1 (HO-1) (Choi and Alam, 1996), and glutathione S-transferase (GST) (Ikeda et al., 2002). NRF2 also stimulates the expression of genes coding for enzymes, e.g., glucose-6-phosphate dehydrogenase and malic enzyme (Thimmulappa et al., 2002) that generate NADPH, which maintains glutathione and protein sulfhydryl groups in a normal, relatively reduced redox state (Go and Jones, 2008). In response to an oxidized shift in cellular redox state or the presence of specific electrophiles, NRF2 is released from the Keap 1 protein that normally keeps NRF2 inactive in the cytosol. Unbound NRF2 translocates from the cytoplasm to the nucleus and transcriptionally activates ARE-dependent genes after recruiting Maf proteins (Nguyen et al., 2003). Phosphatidylinositol 3-kinase and protein kinase C signaling are also essential for this activity (Liu et al., 2007).

NRF2 knockout mice display relatively greater brain infarct volumes induced by focal ischemia, intracerebral hemorrhage, and head trauma (Shah et al., 2007;Shih et al., 2005;Wang et al., 2007;Zhao et al., 2007). Sulforaphane (4-methyl sulfinylbutyl isothiocyanate; SFP), a drug derived from glucosinolate found in cruciferous vegetables such as sprouts of broccoli, cabbage, cauliflower, has been identified as a potent inducer of genes involved in detoxifying ROS. *In vivo* studies have shown that systemically administered SFP after the onset of focal ischemia or head trauma reduces the total brain infarct volume (Zhao et al., 2006;Zhao et al., 2007). Sulforaphane also protects cortical neurons in mixed primary culture from non-excitotoxic glutamate toxicity (Kraft et al., 2004), and protects dopaminergic neurons from cytotoxicity of 6-hydroxydopamine (6-OHDA) and tetrahydrobiopterin (BH4) (Han et al., 2007). In one study using co-cultures of neurons and astrocytes, the neuroprotective effects of NRF2 activation appeared mediated primarily by modulation of astrocyte gene expression (Kraft et al., 2004). While little is known about the effects of NRF2 activators on astrocyte survival, inhibition of proteasomal NRF2 degradation protects astrocytes from hemin toxicity, possibly by increased expression of heme oxygenase 1, a well-established NRF2 target gene (Chen and Regan, 2005).

In this study, we tested the hypothesis that sulforaphane protects astrocytes against early oxidative stress and delayed death caused by transient O₂ and glucose deprivation (OGD), as an *in vitro* model of ischemia/reperfusion. The results demonstrate that addition of sulforaphane both 48 h before and immediately after OGD results in activation of ARE-dependent genes, inhibition of DNA/RNA oxidation, and protection against delayed cell death.

III. MATERIALS AND METHODS

Chemicals and Reagents

DMEM/F12 50/50 culture medium was purchased from Cellgro (Manassas, VA, USA), Fetal bovine serum (FBS) from HyClone (Logan, UT, USA), penicillin-streptomycin solution from Gemini Bio-Products (West Sacramento, CA, USA), mounting medium from Vector Laboratories Inc. (Burlingame, CA, USA), phosphate buffered saline (PBS) from Cambrex BioScience (Walkersville, MD), trypsin EDTA from Cellgro (Manassas, VA, USA) protease inhibitor cocktail from Calbiochem (San Diego, CA, USA), RNA-Bee from TEL-TEST, Inc. (Friendswood, TX, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Primary Culture of Rat Cortical Astrocytes

Cortical astrocytes were prepared from brains of 1-day old Sprague-Dawley rat pups as described by (Zielke et al., 1990). The cell suspension enriched in astrocytes was seeded in 185 cm² Nunc tissue flasks, 30 mL cell suspension/flask. The cells were maintained in a culture incubator at 37°C in an atmosphere of 95% air/5% CO₂, with 90% humidity. The culture medium was replaced after 4 days and twice weekly thereafter. Experiments were performed on cultures with age between 18 and 21 days *in vitro* when they reach maximal sensitivity to cell death caused by the combination of O₂ and glucose deprivation (Juurlink et al., 1992). The purity of the astrocyte cultures was determined by immunocytochemical staining for glial fibrillary acidic protein (GFAP) (*DakoCytomation, Japan*), a marker for astrocytes, ionized calcium binding adaptor molecule (IBA 1) (*Wako, Richmond, VA, USA*), a marker for microglia, 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (*Wako, Saint Louis, MI, USA*) a marker for oligodendrocytes, and neuron nuclear antigen (NeuN) (*Chemicon, MD, USA*), a marker for neurons. Contamination of the astrocyte cell cultures was determined to be <5% microglia and < 2% oligodendrocytes or neurons.

Oxygen and Glucose Deprivation (OGD)

Rat cortical astrocytes were deprived of O₂ and glucose by changing the culture medium to glucose-free "ionic shift" solution (ISS) pH 6.55, containing, NaCl (39 mM), Na-gluconate (11 mM), K-gluconate (65 mM), NMDG-Cl (38 mM), NaH₂PO₄ (1 mM), CaCl₂ (0.13 mM), MgCl₂ (1.5 mM), Bis-Tris (10.5 mM), deoxygenated by a 24 hr preincubation in the anaerobic chamber as previously described by (Danilov and Fiskum, 2008). Compared to normal culture medium, ISS has lower pH, lower concentrations of Ca²⁺, Na⁺, Cl⁻, and increased K⁺, to model changes in the extracellular milieu during ischemia, as described by (Bondarenko and Chesler, 2001). The coverslips were placed in an anaerobic chamber (Forma Scientific Model 1025) under an atmosphere of 10% H₂, 5% CO₂ and 85% N₂. The levels of O₂ and H₂ inside the chamber were monitored by using a Monitor Analyzer (*Coy Laboratory Products, Inc*) and values of < 1 part O₂ per million and H₂ at between 5– 6% were considered acceptable. The dissolved O₂ concentration of the ISS solution was measured using CHEMet test (*CHEMetrics, VA, USA*) that employs the Rhodazine D method. The values of the dissolved oxygen in deoxygenated ISS were between 10 and 40 parts per billion, equivalent to 0.32– 1.28 μM. After washing the cells twice with deoxygenated ISS, they were incubated in the anaerobic chamber for 4 hr. At the end of this period, cells were removed from the anaerobic environment, the ISS was replaced with serum free medium (DMEM/F12) containing 5 mM glucose, and the cultures placed in an incubator under 95% air (20% O₂)/5% CO₂. Control experiments were performed with cells maintained under identical conditions before, during, and after OGD except that they were maintained during the sham OGD in serum free medium that contained 5 mM glucose.

For measurements of mRNA, protein immunoreactivity, and enzyme activity, rat cortical astrocytes were cultured in 60 mm Petri dishes in DMEM/F12 media with 10% serum and 1% Pen/Strep. For the preconditioning experiments, cells were exposed to sulforaphane (SFP) (5 μ M) (*Sigma*) or vehicle, 0.01% dimethyl sulfoxide (DMSO), for 48 hr. After 48 hr the media was removed and cells were incubated in ISS under anaerobic conditions (OGD insult) for 4 hr followed by 48 hr reoxygenation (REOX) in DMEM/F12 serum free medium containing 5 mM glucose.

Cell Death Measurement

Two days before the experiment, rat cortical astrocytes were replated at the density of $2-2.5 \times 10^5$ cells on 25 mm poly-L-lysine coated glass coverslips and maintained in DMEM/F12 media with 5 mM glucose, 10% serum, 1% Pen/Strep. Astrocyte cell death was assessed by using the membrane impermeable fluorescent dye propidium iodide (PI) at 50 μ g/ml to label dead cells, and the cell permeable fluorescent dye Hoechst 33258 (35 μ g/ml) to label all cells. Astrocytes were exposed to both dyes for 20 min at 37°C, fixed in ice-cold paraformaldehyde (PFA) for 10 min, washed with PBS, and mounted with VectaShield fluorescence mounting medium. Fluorescence was observed with a 20X objective lens using a Nikon Eclipse E800 fluorescence microscope and images were captured with a SPOT camera. Merged PI/Hoechst images were used for cell counting performed on three random fields per coverslip (between 500–700 cells/field). Nuclei of viable cells were observed as blue intact nuclei. Red round nuclei (PI-positive cells) and fragmented (or condensed) blue nuclei were considered as dead cells. The number of dead cells is expressed as percentage of the total Hoechst-stained cells.

Oxidative Modification of Nucleic Acids

Fluorescence immunocytochemistry was performed on cell cultures on coverslips as previously described (Danilov and Fiskum, 2008). After OGD and 4 or 48 hr REOX, the coverslips were rinsed twice with 0.05 M potassium phosphate buffered saline (KPBS), pH 7.4, and fixed with 4% PFA for 10 min, rinsed twice followed by incubation with 1% Sodium Borohydride in phosphate buffer for 20 min. After being rinsed with KPBS multiple times until bubbles were eliminated cells were incubated with primary goat anti-8-hydroxydeoxyguanosine (8OHdG) antibody (*Chemicon International, Temecula, CA, USA*) at 1:10000 dilution in KPBS + 0.4% Triton at 4°C. After 48 hr, cells were rinsed with KPBS and incubated with a donkey secondary anti-goat antibody Alexa Fluor 546 (*Invitrogen, CA, USA*) at 1:600 dilution in KPBS + 0.4% Triton for 1 hr at RT. The coverslips were washed with KPBS 3 times, incubated for 2 min with Hoechst and mounted on glass slides using VectaShield mounting medium (*Vector laboratories, Inc., CA Cat # H-100*). The fluorescence was observed with a 20X objective lens, using a computer-assisted image analyzer consisting of a Nikon Eclipse E800 fluorescence microscope, a CCD digital camera (*Biovision Technologies*) and an IBM computer. Images were acquired on a Spot Advanced camera. The excitation wavelength range was 530 – 550 nm and fluorescence emission was measured at 565 nm.

For quantification, the coverslips double stained with antibodies against 8OHdG and Hoechst were analyzed with a computer-assisted image analyzer described above using the MetaMorph software. Three independent images were obtained from each individual coverslip, with 4 to 6 coverslips per experimental condition. The relative fluorescence for each image was calculated by dividing the integrated intensity by the number of cells present in the field. The value for each of the 4 to 6 experiments represents the average of the values for the three images.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of Nrf2 and of Nrf2-induced genes was examined by real-time reverse transcription PCR. For total RNA extraction the cells were washed twice with Dulbecco's Phosphate Buffer Saline solution (DPBS) and lysed in 1 mL RNA-Bee isolation buffer (a monophasic solution containing phenol and guanidine thiocyanate) then RNA extraction was performed based on the Manufacturer's protocol (TEL-TEST Inc.). The synthesis of cDNA and PCR amplification from the total RNA was performed by using the One-Step Real-time RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA, USA) using a iCycler thermal cycler and the IQTM5 software for data analysis (Bio-Rad). The PCR cycles were as follows: initial denaturation (95°C, 2 min), 50 cycles (94°C, 30 s; annealing at 55°C, 30 s; extension at 72°C, 40 s) and final extension (72°C, 10 min) followed by melting curve analysis. Specific amplification was confirmed by melting curve analysis and by analysis of amplified products by agarose gel electrophoresis. Rat NQO1 primers were: forward: 5'-GCCCGGATATTGTAGCTGAA-3' and reverse 5'-GTGGTGATGGAAAGCAAGGT-3'. Rat NRF-2 primers were: forward-5' CAGTCTTCACCACCCCTGAT-3' and reverse 5'-CAGTGAGGGGATCGATGAGT-3'. Rat β -actin primers were: forward 5'-AGCCATGTACGTAGCCATCC-3' and reverse 5'-CTCTCAGCTGTGGTGGTGAA-3'. The primers were designed using Primer3 software available online at <http://frodo.wi.mit.edu/>. Both NQO1 and β -actin amplified a ~200 base pairs fragment. The Ct values for NRF2 and NQO1 mRNAs were normalized with β -actin by subtracting the NRF2 and NQO1 Ct values from β -actin ones. The changes in NRF-2 and NQO1 mRNAs were expressed as fold-change using the formula $2^{-\Delta\Delta Ct}$.

Western Blotting Analysis

Samples of cortical astrocytes were analyzed for NRF2 and NQO1 protein levels. For the whole cellular lysate isolation, the samples were washed twice with ice-cold PBS and harvested in lysis buffer containing 0.15 M NaCl, 0.01 M Tris, 0.9 M EDTA, 1% Triton-X100, 0.5% Nonidet P-40 and protease inhibitors. For the nuclear fractions, the cells were extracted as previously described by (Calabrese et al., 2005). In brief, astrocytes were washed and harvested in PBS and then centrifuged at 3,000 rpm for 3 min at 4°C. The pellet was resuspended in 200 μ l buffer A consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μ M dithiothreitol (DTT) and protease inhibitor cocktail (EMD Biosciences, Inc., San Diego, CA, USA). The pellet was kept on ice for 15 min followed by addition of 15 μ l of 10% NP-40. The pellet was vortexed 10 sec, centrifuged at 3,000 rpm for 3 min at 4°C and resuspended in 30 μ l cold buffer B consisting of 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μ M DTT and protease inhibitors. The pellet was incubated on ice for 15 min and vortexed every 2 min. The nuclear extract was obtained by centrifugation at 13,000 rpm for 5 min at 4°C. The protein concentrations were measured using the Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards. The lysates and nuclear samples were treated with 50 mM dithiothreitol (DTT) and NuPage 4X LDS loading buffer (Invitrogen, CA, USA) prior to heating at 70°C for 10 min. The samples were separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with 30 μ g of total protein. Following the SDS-PAGE, the proteins were transferred onto a PVDF membrane. The membranes were washed in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBST) followed by blocking for 1 h using 5% non-fat milk in TBST at RT. This was followed by incubation overnight at 4°C with the primary goat polyclonal anti-NQO1 antibody (Santa Cruz Biotechnology, Cat # sc-16464), rabbit polyclonal anti-NRF-2 antibody (Santa Cruz Biotechnology, Cat # H-300) at 1:500 dilution, mouse monoclonal anti- β -actin antibody (Sigma, A2228) at 1:2000 dilution and rabbit monoclonal anti-histone H3 (AbCam, Cat # ab32151) at 1:5000 dilution. The membranes were then washed with TBST and incubated for 1 h at RT in HRP-conjugated anti-goat (PROSCI

Incorporated, Cat # XR-9320), anti-rabbit (*Amersham Biosciences NA934V*), or anti-mouse antibodies (*Amersham Biosciences*) at 1:5000 dilution for 1 h at RT. The washed blots were then treated with enhanced chemiluminescence detection reagent (*Amersham Bioscience, UK, Cat # RPN2106*). Optical densities of individual bands were quantified after subtraction of background levels of film exposure using the Image J software. Ratios of NRF2/Histone H3 and NQO1/ β -actin were calculated and fold changes were expressed relative to control.

NAD (P) H: Quinone Oxidoreductase 1 (NQO1) Enzyme Activity

Endogenous enzyme activity (nmole/min/mg protein) was determined by calculating the dicumarol-sensitive fraction of 2, 6-dichlorophenol-indo-phenol reduction as previously described (Benson et al., 1980). A 1 ml volume of reaction mixture containing 25 mM Tris-HCl buffer (pH 7.4), 0.7 mg/mL bovine serum albumin (BSA), 5 μ M FAD, 300 μ M NADH, 25 μ g protein/mL, with and without 20 μ M dicumarol was incubated for 10 min at RT. 2, 6-dichlorophenol-indo-phenol was added to a final concentration of 75 μ M and its reduction was measured spectrophotometrically at a wavelength of 560 nm. The NQO1 activity (nmole/min/mg protein) was calculated based on the reduction of dicumarol-sensitive fraction: the slope and intercept were calculated for each measurement and multiplied with the specific protein concentration of each sample and the value obtained was divided by the extinction coefficient of 2, 6-dichlorophenol-indo-phenol (Benson, Hunkeler, and Talalay, 1980). The extinction coefficient for 2, 6-dichlorophenol-indo-phenol was $2.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

Data Analysis

Results are expressed as means $\pm$ SEM for three to nine independent experiments. Where indicated, statistical analyses used analysis of variance (ANOVA) followed by Student-Newman-Keuls or Holm-Sidak tests for multiple comparisons and Student's *t*-test for two groups' comparison. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

A. Sulforaphane pre-treatment inhibits early DNA/RNA oxidation and subsequent death of astrocytes after transient oxygen and glucose deprivation

To evaluate whether sulforaphane (SFP) protects against oxidative stress and death of astrocytes induced by transient O_2 and glucose deprivation (OGD), we exposed primary cultures of rat cortical astrocytes to 5 μ M SFP for 48 h prior to the OGD insult, as described in Methods. Pilot experiments that compared results obtained with 0.1, 0.5, 1.0, 5.0, and 10 μ M SFP concentrations indicated that maximal cytoprotection is obtained at 5 μ M SFP (Fig. 1). As shown in Fig. 2, the number of dead cells observed after exposure to 5 μ M SFP was not different than that observed after exposure to the DMSO vehicle ($7.6 \pm 1.1\%$ compared to $6.0 \pm 1.1\%$) and neither were different from background cell death in cells that underwent time-controlled changes of normal medium but no OGD ($6.2 \pm 0.8\%$; not shown). At 48 h reoxygenation (REOX), pretreatment of cells with SFP conferred significant protection against delayed cell death ($16.4 \pm 2\%$ compared to vehicle $31.4 \pm 2.8\%$; $p \leq 0.01$).

Previous experiments using this OGD model demonstrated that astrocytes undergo protein and DNA/RNA oxidation within 4 h REOX after OGD, which precedes delayed cell death (Danilov and Fiskum, 2008). We therefore tested for the ability of SFP-pretreatment to inhibit DNA/RNA oxidation at this early period of REOX, which precedes delayed cell death. Fig. 3 provides representative fluorescent images (A) and quantification (B) of 8-hydroxy 2-deoxyguanosine (8-OHdG) immunostaining of astrocytes after 4 h REOX. Relative fluorescence for SFP-treated cells at 4 h REOX was significantly lower than that observed with vehicle-treated cells ($1185 \pm 92 \text{ U}$ compared to $1702 \pm 176 \text{ U}$; $p < 0.05$) but

not significantly greater than cells not exposed to OGD. Oxidative modifications to DNA/RNA therefore occur early during REOX in this model and are inhibited by pretreatment of astrocytes with SFP.

B. Sulforaphane pre-treatment increases NRF-2 and NQO1 mRNA and protein expression

To determine whether the observed protection by SFP pretreatment was accompanied by activation of the NRF-2 pathway of gene expression, astrocyte cultures were exposed to SFP or the vehicle for 48 h and the transcript and protein levels of NRF2 and of the ARE-target gene NAD(P)H: Quinone Oxidoreductase 1 (NQO1) were analyzed. As shown in Fig. 4A, SFP pretreatment increased NRF-2 mRNA expression by 3.44 ± 0.651 fold compared to 1.46 ± 0.4 fold in vehicle treated cells ($p < 0.05$). In all SFP-pretreated astrocytes, the NRF2 immunoreactivity in nuclear fractions showed a double band around 90 kDa (Fig. 4B and 4C). The discrepancy between the predicted molecular weight (66.9 kDa) and the observed molecular weight has been previously reported and is likely due to the abundance of acidic residues in NRF2 that leads to abnormal migration in SDS/PAGE gels (Chan et al., 1993; Moi et al., 1994). The upper band, which likely represents the phosphorylated form of NRF2, was almost undetectable under untreated conditions (control) or in vehicle-treated cells.

The effect of sulforaphane on NQO1 mRNA and protein levels were measured both prior to and after OGD and REOX. β -actin mRNA and protein were used as internal controls. No significant change in β -actin mRNA was observed in any of the groups compared to control cells. The ratio of NQO1 to β -actin mRNA was calculated and changes in NQO1 mRNA were expressed as fold-change relative to vehicle before OGD. As illustrated in Fig. 5A, SFP treatment prior to the OGD insult increased the level of NQO1 mRNA by 13.7 ± 3.4 fold compared to untreated cells. At the end of OGD, the NQO1 mRNA remains significantly high (9.14 ± 0.78) in SFP-treated cells compared to vehicle treated cells (0.77 ± 0.23 , $p < 0.01$). At 48h REOX the level of NQO1 mRNA was still higher in SFP-treated cells (2.1 ± 1.3) than in vehicle-treated cells (1.033 ± 0.5).

The effect of SFP on NQO1 total protein level was determined in astrocyte cultures at the same time points as for mRNA message assessment. As shown in the representative immunoblots in Fig. 5B (upper panel) and by the densitometric analysis in Fig. 5B (lower panel), SFP-pretreatment induced a statistically significant increase (4.5 ± 0.34 fold) in NQO1 total protein level compared to 0.8 ± 0.25 fold increase in vehicle treated cells ($p < 0.001$). At the end of OGD the fold change in NQO1 protein level was 9.4 ± 0.89 compared to 1.22 ± 0.05 in vehicle treated cells ($p < 0.001$) and after 48h REOX NQO1 expression is still high (7.0 ± 2.5), compared to the in vehicle-treated group (1.4 ± 0.35 , $p < 0.05$) relative to control.

Consistent with the increase in NQO1 protein level, SFP-pretreatment also induced a 3.5 fold increase in NQO1 enzyme activity compared to vehicle treated cells, as shown in Fig. 5C. At the end of OGD and at 48h REOX the NQO1 enzyme activity was 2.5 ($p < 0.05$) respectively 2.3 fold higher ($p < 0.05$) than in vehicle treated cells. The results shown in Fig 5 demonstrate that exposure to SFP induces activation of NRF2/ARE pathway that results in increased transcription of ARE-containing genes such as NQO1, increased levels of NQO1 protein and increased enzyme activity. These results also suggest that the mechanism by which exposure to SFP protects astrocytes against OGD-induced delayed cell death is likely to involve activation of NRF2/ARE pathway.

C. Post-treatment with sulforaphane during reoxygenation protects astrocytes against OGD insult

Since we observed that SFP treatment prior to the OGD insult protects astrocytes from delayed cell death, we tested whether post-treatment of cells with SFP during the REOX would confer protection against delayed astrocyte death. As described in Methods, after 4h OGD, the astrocyte cultures were exposed to SFP (2.5 μ M) during the REOX. Pilot experiments indicated that exposure of control astrocytes to 2.5 μ M SFP for 48h in DMEM/F12 medium without serum shows less cytotoxicity than the previously used dose of 5.0 μ M SFP ($p < 0.01$) (data not shown). Therefore, a 2.5 μ M concentration of SFP was used in all post-treatments experiments.

As shown in Fig. 6 exposure of cells to SFP or vehicle alone had no effect on cell death ($3.4 \pm 0.5\%$ respectively $2.3 \pm 0.5\%$). However, SFP post-treatment significantly reduced the cell death to $25.6 \pm 2.5\%$ compared with $37.5 \pm 1.4\%$ in vehicle-treated cells ($p < 0.001$).

D. Sulforaphane post-treatment induces NRF2 mRNA expression and nuclear accumulation of NRF2

Further experiments were conducted to determine whether the effects of SFP on NRF2 activation observed prior the OGD insult were also present after SFP treatment during the REOX time. The astrocyte cultures were exposed to SFP (2.5 μ M) during the REOX and at 48h REOX total cellular RNA and proteins were extracted. RT-PCR analysis with NRF2 gene primers showed that SFP induced NRF2 mRNA message (3.5 ± 0.7 fold compared with 1.3 ± 0.24 fold in vehicle control; $p \leq 0.01$), as shown in Fig. 7. The total NRF2 protein expression in nuclear fractions detected by western blot analysis was increased by 1.7 fold in SFP-treated cells compared with vehicle treated cells detected by western blot analysis as shown in Fig. 8. These results indicate that administration of SFP after the OGD insult is efficient in inducing NRF2 mRNA expression and nuclear accumulation of NRF2 protein in astrocytes.

E. Sulforaphane post-treatment induces up-regulation of NQO1 mRNA and protein and increases NQO1 enzyme activity

The effect of SFP post-treatment on NQO1 message, protein expression and enzyme activity was determined in cultures of cortical astrocytes after 48h REOX. SFP (2.5 μ M) was added to cultures after the OGD insult and at 48h REOX the total cellular RNA was isolated and analyzed for NQO1 mRNA expression by RT-PCR. As shown in Fig. 9A SFP post-treatment increased the NQO1 mRNA message to 8.94 ± 2.36 fold compared with 0.69 ± 0.1 fold in vehicle control ($p < 0.01$).

The effect of SFP on NQO1 total protein level was determined at the same time points as for mRNA message assessment. As shown in Fig. 9B, SFP post-treatment induced an increase in NQO1 protein level after 48h REOX. Quantification of the NQO1 immunoreactivity (Fig. 9B lower panel), indicated that NQO1 protein induction in SFP post-treated cells (10.25 ± 1.83 fold increase) is a significantly higher than in vehicle treated cells (4.5 ± 0.56 fold; $p < 0.01$) at 48h REOX. Consistent with the increase in total protein level, a 2.4-fold increase in NQO1 enzyme activity was also found in SFP-treated cells compared to vehicle treated cells ($p < 0.05$) as shown in Fig 9C. These results indicate that even when administered after OGD, SFP is still effective at activating NRF2/ARE-target genes as shown by the increase in NQO1 mRNA and protein levels and NQO1 enzyme activity.

DISCUSSION

In the present study, we demonstrated that oxidative stress contributes to delayed cell death in astrocytes after OGD/reoxygenation and that protection against cell death can be conferred by activating the NRF2-mediated anti-oxidant response (ARE) pathway by the use of sulforaphane. While *in vivo* studies using focal ischemia models such as common carotid artery/middle cerebral artery (CCA/MCA) occlusion (Zhao et al., 2006) or a traumatic brain injury model in rodents (Zhao et al., 2005) have shown reduction in cerebral infarct volume and decrease in brain edema after post-injury administration of SFP, the effect of SFP on astrocyte survival using an *in vitro* model of ischemia/reperfusion has not been studied.

Activation of NRF2 by SFP involves posttranslational modifications that result in its release from Keap 1 which normally targets NRF2 for degradation, NRF2 protein accumulation and translocation to the nucleus. In our model activation of the NRF2/ARE pathway by SFP is indicated by the finding that SFP but not vehicle pre-treatment induced a significant increase in the level of phosphorylated NRF2 protein in the nuclear fraction of astrocytes. This finding is consistent with reports indicating that several cytosolic kinases such as protein kinase C, mitogen-activated protein kinase, p38, and phosphatidylinositol 3-kinase are involved in transducing signals from antioxidants to the ARE through NRF2 phosphorylation (Bloom and Jaiswal, 2003; Yu et al., 2000)). In addition, we found that in astrocytes pretreatment with 5 μ M SFP for 48h prior to the OGD insult also results in up-regulation of the steady-state level of NRF2 mRNA. Unlike other cell systems where activation of NRF2 has been shown to involve mainly post-transcriptional mechanisms, it appears that in astrocytes SFP-mediated NRF2 activation involves both transcriptional and posttranscriptional mechanisms.

We further investigated the involvement of NRF2/ARE pathway in the activity of SFP in astrocytes by analyzing the expression of endogenous ARE-regulated genes. NAD (P) H: quinone oxidoreductase 1 (NQO1) is generally considered a detoxifying enzyme because of its ability to reduce reactive quinones and quinone-imines to less reactive and less toxic hydroquinones (Lind et al., 1990). NQO1 a FAD⁺-containing protein, is an obligate two-electron reductase characterized by its unique ability to use either NADH or NADPH as reducing cofactors (Hollander et al., 1975). NQO1 is highly inducible by many stimuli including electrophilic metabolites and oxidative stress (Prochaska et al., 1987) and its induction is considered to be transcriptionally regulated by ARE (Jaiswal, 2000; Ross et al., 2000). In this study we demonstrated that 48h pre-treatment with SFP increased the transcript level of NQO1 and this remained high even at the end of OGD. Furthermore, the assessment of total protein level and the enzyme activity in whole cell lysates after 48h SFP-treatment showed an up-regulation of NQO1 protein and an increase in NQO1 enzyme activity at the end of OGD and at 48h REOX. We also found that in addition to NQO1 another well known NRF2-2 induced gene, malic enzyme 1 (ME1) is also upregulated in astrocytes following SFP pre-treatment (data not shown).

A primary factor in initiation of the pathological response to the ischemia/reoxygenation injury is the generation of reactive oxygen species (ROS). The increase in the levels of ROS produced upon REOX appears to be essential for the development of astrocyte dysfunction and delayed death as shown by others for neurons (Sugawara et al., 2002a). In our *in vitro* model, astrocytes pre-treated with SFP and then exposed to 4h OGD showed no difference in 8OHdG immunoreactivity at the end of OGD when compared to vehicle treated cultures indicating that no oxidative DNA or RNA damage was present at this time point (data not shown). This data is consistent with our cell death measurements at the end of OGD when there was no difference between SFP-treated and control cells. However, a significant increase in the oxidative DNA/RNA damage was noted after 4h REOX as indicated by the

increased 8OHdG immunoreactivity and this was significantly lower in SFP-treated astrocytes compared with vehicle controls. Consistent with the SFP-effect in reducing oxidative damage, the cell death assessment at 48h REOX showed a reduction in delayed death after SFP-pretreatment as identified by PI/Hoechst staining. No statistical difference was observed in cell death in SFP-control ($5.58 \pm 1.533\%$), vehicle-control ($4.6 \pm 1.56\%$) or control alone ($3.76 \pm 1.48\%$) at 48h REOX after 48h pre-treatment. Activation of NRF2, induction of NRF2-dependent genes (e.g. NQO1, ME1) and the decrease oxidative damage observed following SFP pre-treatment are consistent with involvement of NRF2-mediated anti-oxidant response (ARE) pathway in SFP-mediated astrocyte protection against OGD/REOX-induced oxidative stress and delayed death.

Studies have shown that NRF2 activation following alterations in redox state of the cells can be caused by the increased amount of electrophiles or ROS. In our OGD model, we found that 48h REOX alone, without any SFP treatment was able to up-regulate the NQO1 mRNA expression to 2.23-fold when compared to the end of OGD. However, it appears that expression of NRF2-induced genes above a certain threshold is necessary in order for the cells to be protected. Therefore, pharmacological intervention with agents known to up-regulate the ARE dependent gene expression during the REOX was also tested.

In this study, we provide experimental evidence that treatment of astrocyte cultures with SFP immediately after exposure to OGD significantly decreased delayed cell death compared to vehicle control. Our preliminary dose response experiments showed that at 2.5 μM concentration SFP is less toxic than at 5.0 μM (the dose used for the pretreatment studies) in post-treatment conditions. The higher sensitivity of astrocytes to SFP in post-versus pre-treatment conditions might be explained by the fact that for the post-treatment experiments the cells were maintained in serum-free medium while the pre-treatment was performed in medium supplemented with 10% serum. Serum deprivation and the pro-oxidant activity of SFP might both contribute to the higher toxicity observed at 5 μM compared to 2.5 μM .

Our results indicate that astrocyte protection following post-treatment with SFP also involves activation of the NRF2/ARE pathway. Similar to the results obtained in the pretreatment experiments, we found that SFP post-treatment results in induction of both NRF2 mRNA and an increase in total NRF2 protein in nuclear fractions when compared to control and vehicle-treated cells. As we observed in SFP-pretreated experiments, the NRF2 phosphorylated form was detected only in the SFP-treated samples. The data suggests that NRF2 activation by SFP involves NRF2 phosphorylation in astrocytes. We also found that SFP treatment after the OGD injury is efficient in up-regulating the NRF2-dependent gene NQO1 at both transcription and translation levels.

Although the increase in NQO1 protein was used as an indicator of SFP action in rat cortical astrocytes it does not imply that the protective effect was mediated by one single enzyme. Studies by (Hu et al., 2004) have shown that SFP can activate multiple NRF-2 response genes whose protein products participate in cellular defense. As noted above we found that other NRF2-induced genes (i.e. ME1) are also induced by SFP treatment in our system.

In summary, our results support the concept of pre- and post-treatment against oxidative stress and delayed death by the use of prooxidants that activate the NRF2/ARE pathway of antioxidant gene expression. SFP was effective at increasing NQO1 gene and protein expression and protecting against astrocyte cell death when administered before and after OGD. Therefore, pharmacologic stimulation of antioxidant gene expression is a promising approach to neuroprotection after cerebral ischemia.

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Figure 1. Dose-dependent effect of Sulforaphane pretreatment in astrocytes

Rat cortical astrocyte cultures were pretreated with either vehicle (DMSO 0.01%) or with 0.1, 0.5, 1, 5 and 10 μM SFP for 48h. The cells were then exposed to OGD in a deoxygenated glucose-free ionic shift solution (ISS) as described in Methods. OGD was terminated by removing the cultures from the anaerobic chamber and replacing the ISS with serum-free DMEM/F12 medium containing 5 mM glucose. At 48h REOX cell death was analyzed by staining with PI/Hoechst. The number of dead cells was expressed as percentage of the total Hoechst-stained cells. Results are expressed as mean ± SEM (n = 4).

Figure 2. Sulforaphane pre-treatment protects astrocytes against cell death

Rat cortical astrocyte cultures pretreated with SFP (5 μ M) or vehicle (DMSO 0.01%) for 48h were exposed to OGD and reoxygenation, as in Fig. 1. OGD was terminated by removing the cultures from the anaerobic chamber and replacing the ISS with serum-free DMEM/F12 medium containing 5 mM glucose. Controls cells were incubated 48h with either SFP or vehicle in medium plus 10% FBS, then cultured in serum-free medium for the duration of REOX. At 48h REOX cell death was analyzed by staining with PI/Hoechst. The number of dead cells was expressed as percentage of the total Hoechst-stained cells. Results are expressed as mean $\pm$ SEM (n = 4–7). Data were analyzed using one-way ANOVA and Holm-Sidak post-hoc test. **p<0.001 vs. OGD/REOX Vehicle and # p<0.05 vs. OGD/REOX SFP

Figure 3. Immunocytochemical staining of 8-hydroxy-2-deoxy-guanosine (8 OHdG) in cortical astrocytes after 4h REOX

Rat cortical astrocytes pretreated for 48h with SFP or vehicle were exposed to OGD for 4h and stained for 8OHdG at 4h REOX. **A:** Representative images of 8OHdG immunoreactivity. **B:** Quantitative comparison of immunocytochemical fluorescence. Relative fluorescence values were obtained as described in Methods and are expressed as mean $\pm$ SEM (n = 4–6). Data were analyzed using One Way ANOVA with Holm-Sidak post hoc test. # p<0.05 vs. VEH/REOX

Figure 4. Sulforaphane pre-treatment increases NRF2 mRNA and protein expression in rat cortical astrocytes

A: Cortical rat astrocytes were pre-treated for 48h with SFP or vehicle and total RNA was extracted as described in Methods. The NRF2 mRNA fold change was calculated relative to control. Control is represented by cells maintained in DMEM/F12 medium supplemented with 10% FBS. NRF2 mRNA values were normalized to β -actin. The results are represented as mean $\pm$ SEM ($n = 4$). Data were analyzed using Student's t-test. [#] $p < 0.05$ vs. vehicle. **B:** Astrocytes were pretreated for 48h with SFP, vehicle (VEH) or maintained in DMEM/F12 medium supplemented with 10% FBS (control: CTR). At the end of 48h the nuclear fractions were isolated as described in Methods. The immunoblots were probed for NRF2 then stripped and re-probed for histone H3 as loading control and marker of nuclear fractions ($n = 3-4$). As illustrated in the representative blot, in contrast to VEH or CTR cells, two immunoreactivity bands were detected at ~ 90 kDa in SFP-pretreated cells. **C:** Quantitative analysis of NRF2 immunoreactivity in nuclear fractions isolated from astrocytes pre-treated with SFP and VEH: lower band (left) and upper band (right). NRF2 band intensities were normalized to histone H3 and expressed as fold change relative to control (untreated cells). The values were squared transformed and the data were analyzed using one-way ANOVA followed by Tukey Test. [#] $p < 0.05$

A

B

C

Figure 5. Sulforaphane pre-treatment increases NQO1 expression and NQO1 enzyme activity in rat cortical astrocytes

A: Cortical rat astrocytes were pretreated for 48h with SFP then exposed to OGD (4h) and 48h REOX as described in Methods. The samples were collected after 48h pre-treatment, at

the end of OGD and after 48h REOX and total RNA was extracted. NQO1 mRNA values were normalized to β -actin. The NQO1 mRNA fold change was calculated relative to vehicle at the end of pretreatment. The results are expressed as mean $\pm$ SEM (n = 3–4). Data were analyzed using one-way ANOVA with Holm-Sidak post hoc test. **p<0.001 vs. SFP before OGD; *p<0.001 vs. SFP OGD; # p<0.05 vs. SFP OGD. **B: Upper panel** - Representative Immunoblots of NQO1 in total cellular lysates. The immunoblots were probed with anti-NQO1 antibody then stripped and re-probed for β -actin as a loading control (n = 3–4). NQO1 immunoreactivity appears at ~ 36 kDa. Controls (CTR) represent the following: control-before OGD, astrocytes maintained in DMEM/F12 supplemented with 10% FBS; control-end of OGD, astrocytes maintained 4h in serum-free DMEM/F12; control-48h REOX, astrocytes maintained 48h in serum-free DMEM/F12. **Lower panel** - Quantitative densitometric analysis of NQO1 immunoreactivity. The ratio of the NQO1/ β -actin was calculated and the results were expressed as fold change relative to control at each different time point. Data were analyzed using one-way ANOVA with Holm-Sidak post hoc test. **p<0.001 vs. SFP and #p<0.05 vs. SFP **C:** NQO1 enzyme activity in total cell lysates was determined at the same time points as for measuring the NQO1 total protein level. The results are expressed as mean $\pm$ SEM (n = 3–4). Data were analyzed using one-way ANOVA with Holm-Sidak post hoc test. # p< 0.05 vs. SFP and * p< 0.05 vs. control

Figure 6. Sulforaphane post-treatment protects astrocytes against cell death

Rat cortical astrocyte cultures were exposed to OGD in a deoxygenated, glucose-free ionic shift solution (ISS). Sulforaphane (2.5 μ M) was added to the culture at the beginning of REOX and maintained 48h. After 48h REOX the cells were stained with PI/Hoechst. The number of dead cells was expressed as percentage of the total Hoechst-stained cells (n= 8–9). Data were analyzed using one-way ANOVA and Student Newman-Keuls post-hoc test. **p<0.001 vs. OGD/REOX Vehicle and #p<0.001 vs. OGD/REOX SFP

Figure 7. Sulforaphane post-treatment increases NRF2 mRNA expression in rat cortical astrocytes

Cortical rat astrocytes were exposed to OGD for 4h followed by 48h REOX in serum-free DMEM/F12 medium plus 5 mM glucose. The SFP (2.5 μ M) or vehicle (DMSO 0.01%) was added to the culture at the beginning of REOX. At 48h REOX total cellular RNA was extracted and the NRF2 mRNA expression was analyzed by RT-PCR. The results are expressed as mean $\pm$ SEM (n = 8). Data were analyzed using Student's t-test. [#]p<0.05 vs. vehicle

Figure 8. Representative Immunoblots of nuclear fractions probed for NRF2

Upper panel - Astrocytes were exposed to SFP or vehicle at the beginning of REOX. Controls were maintained in serum-free DMEM/F12 medium. At the end of 48h the nuclear fractions were isolated as described in Methods. The immunoblots were probed with anti-NRF2 antibody then stripped and re-probed for histone H3 as loading control (n = 3–4). NRF2 was detected as 2 immunoreactive bands at ~ 90 kDa in the SFP post-treatment groups. *Lower panel* - Quantitative analysis of NRF2 immunoreactivity in nuclear fractions isolated from astrocytes post-treated with SFP and VEH: quantification of the lower band (left) and upper band (right). The ratio of NRF2/Histone H3 was calculated and expressed as fold change relative to control. The values were square transformed and the data were analyzed using one-way ANOVA with Tukey post hoc test. #p<0.05

Figure 9. Sulforaphane post-treatment increases NQO1 expression in rat cortical astrocytes

A: Astrocytes were exposed to SFP or vehicle at the beginning of REOX. Controls were maintained in serum-free DMEM/F12 medium. At 48h REOX total RNA was extracted. NQO1 mRNA values were normalized by β -actin values and fold change was calculated relative to control. The results are expressed as mean $\pm$ SEM ($n = 6-7$). Data were analyzed using Student's t-test. $**p < 0.001$ vs. vehicle **B:** Representative immunoblots of total cellular lysates probed for NQO1. **Upper panel** - Astrocytes were exposed to SFP or vehicle at the beginning of REOX as in (A). At the end of 48h REOX total protein lysates were prepared as described in Methods. Immunoblots were probed with anti-NQO1 antibody then stripped and re-probed for β -actin ($n = 3$). **Lower panel** - Densitometric analysis of NQO1 immunoreactivity in total lysates of astrocytes post-treated with SFP and VEH. The ratio of NQO1/ β -actin was calculated and the results expressed as fold change relative to control. Data were analyzed by one-way ANOVA using the Holm-Sidak post hoc test. $*p < 0.05$ vs. SFP **C:** Sulforaphane post-treatment increases NQO1 enzyme activity at 48h REOX. Cortical rat astrocytes were exposed to SFP or vehicle at the beginning of REOX as in (A). Controls were maintained in serum-free DMEM/F12. At 48h REOX the NQO1 enzyme activity was determined from total cell lysates as described in Methods. The results are expressed as mean $\pm$ SEM ($n = 4-6$). Data were analyzed by one-way ANOVA with Holm-Sidak post hoc test. $p < 0.05$ vs. control