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Sulforaphane protects immature hippocampal neurons against death caused by exposure to hemin or to oxygen and glucose-deprivation

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Abstract

Oxidative stress is a mediator of cell death following cerebral ischemia/reperfusion and heme toxicity which can be an important pathogenic factor in acute brain injury. Induced expression of Phase II detoxification enzymes through activation of the anti-oxidant response element (ARE)/Nrf2 pathway has emerged as a promising approach for neuroprotection. Little is known, however, about the neuroprotective potential of this strategy against injury immature brain cells. In this study, we tested the hypothesis that sulforaphane (SFP), a naturally occurring isothiocyanate that is also a known activator of the ARE/Nrf2 antioxidant pathway, can protect immature neurons from oxidative stress-induced death. The hypothesis was tested with primary mouse hippocampal neurons exposed to either O₂ and glucose deprivation (OGD) or hemin. Treatment of immature neurons with SFP immediately after the OGD during reoxygenation was effective at protecting immature neurons from delayed cell death. Exposure of immature hippocampal neurons to hemin induced significant cell death and both pre- and co-treatment with SFP were remarkably effective at blocking cytotoxicity. RT-PCR analysis indicated that several Nrf2-dependent cytoprotective genes, including NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO1) and glutamate-cysteine ligase modifier subunit (GCLM) that is involved in glutathione biosynthesis, were upregulated following SFP treatment both in control neurons and following exposure to OGD and hemin. These results indicate that SFP activates the ARE/Nrf2 pathway of antioxidant defense and protects immature neurons from death caused by stress paradigms relevant to those associated with ischemic and traumatic injury to the immature brain.

Keywords

Nrf2; oxidative stress; ischemia/reperfusion; hemin; hippocampus

Introduction

A net Increase in the generation of reactive oxygen species (ROS) and subsequent oxidative stress are well-established molecular mechanisms that promote neuronal death following acute brain injury, including ischemia/reperfusion injury and traumatic brain injury (TBI). A less well-studied factor that can contribute to acute brain injury is the accumulation of free heme as a consequence of hemorrhage and hemolysis, which also exerts cytotoxic effects on neurons through oxidative mechanisms. Since under pathological conditions, oxidative

stress can be mediated by different free radical species generated through multiple pathways, effective neuroprotective therapies may need to be broad-based.

One such neuroprotective approach is represented by the induction of Phase II detoxification enzymes, using pharmacologic or genetic strategies that promote activation of the antioxidant response element (ARE)/Nrf2 pathway. Nuclear factor erythroid 2-related factor 2 (Nrf2) belongs to the Cap 'n' Collar family of transcription factors that share a highly conserved basic leucine zipper structure (Motohashi et al., 1997). Its neuroprotective activity involves interaction with antioxidant response elements (ARE) and induction of numerous cytoprotective genes, including NAD(P)H:quinone oxidoreductase 1 (NQO1) (Li et al., 1995; Riley and Workman, 1992), heme oxygenase -1 (HO-1) (Choi and Alam, 1996), and the glutathione biosynthetic enzyme, glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits (Lu, 2009).

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, SFP), a known activator of the ARE/Nrf2 pathway, is an isothiocyanate compound naturally present in high concentrations in several varieties of cruciferous vegetables (*Brassica*; e.g. broccoli) and first became of interest due to its potential anti-cancer activity (Gamet-Payastre, 2006). As an anti-carcinogen, sulforaphane may have many actions, but there is a consensus that an important target of sulforaphane is the Keap1 protein, which normally binds and sequesters the nuclear transcription factor Nrf2 in the cytoplasm. Sulforaphane can directly act on Keap1 to release Nrf2 which then translocates to the nucleus where it heterodimerizes with small Maf proteins and binds to ARE's inducing the expression of a large number of cytoprotective genes (Hong et al., 2005; Lee and Surh, 2005; Nioi et al., 2003).

Several recent *in vivo* studies demonstrated the potential of SFP to protect against acute brain injury. In rodents, SFP administered 15 minutes after stroke led to an increase in HO1 expression in brain and reduced infarct volume (Zhao et al., 2006). Post-injury administration of SFP also reduced brain edema following TBI in rats (Wang et al., 2007; Zhao et al., 2005). Although these studies indicate that SFP is a candidate for treatment of adult brain injury, very little is known about the neuroprotective potential of SFP for immature brain injury. It is now well understood that developmental differences in energy metabolism, glutamate excitotoxicity, response to oxidative stress and susceptibility to apoptosis distinguish the immature brain response to injury from that of the adult (Soane et al., 2008; Vannucci and Hagberg, 2004), and that neuroprotective interventions should be optimized according to age.

In addition, the cellular site of action in the brain for electrophilic compounds like SFP that activate the ARE/Nrf2 pathway is not clear. Using an ARE reporter construct in mixed glial-neuron co-cultures, a recent study indicated that activation of Nrf2 occurs predominantly in astrocytes and that neuroprotection was secondary to changes in glia (Kraft et al., 2004; Shih et al., 2003). Subsequent reports have challenged the notion that the protective effects of Nrf2 activation occur exclusively in astrocytes and indicated that mature neurons may also respond to Nrf2 activators through upregulation of ARE-responsive genes (Lee et al., 2003b; Satoh et al., 2006). Whether this holds for immature neurons is not known. Therefore, the potential of SFP to directly protect neurons against insults such as ischemia/reperfusion injury is still not clear.

No studies have tested in neurons the protective potential of SFP against heme toxicity, another clinically relevant oxidative stress paradigm that can mediate neuronal death following acute injury to both immature and mature brain (Bayir et al., 2006; Chang et al., 2005). Extravasation of blood in TBI or hemorrhagic stroke leads to the release of hemoglobin from red blood cells, which in turn releases the iron-containing heme group

(Platt and Nath, 1998; Wagner et al., 2003). Following CNS hemorrhage, free heme can reach high micromolar concentrations in the extracellular space (EC_{50} about 10 μ M) and exert cytotoxic effects on both neurons and astrocytes (Chen-Roetling and Regan, 2006; Goldstein et al., 2003). Heme and hemin (oxidized heme) are strong pro-oxidants and are normally metabolized by the heme oxygenase (HO) 1 and HO2 enzymes into biliverdin, carbon monoxide and iron, another pro-oxidant. Whether inhibiting or promoting heme catabolism is protective, appears to be cell-type specific as genetic manipulation of the HO enzymes yielded opposing effects in neurons and astrocytes (Platt and Nath, 1998; Wagner et al., 2003). Overexpression of HO1 protected SN56 neuron-like cells from H_2O_2 -induced death (Le et al., 1999) but PC12 cells cultured on HO1 overexpressing astrocytes were more prone to oxidative injury (Song et al., 2007). HO1 induction has been shown to protect astrocytes but not neurons from heme injury (Chen-Roetling and Regan, 2006), while HO2 may be neuroprotective against intracerebral hemorrhage (Wang et al., 2006). Inhibiting HO1 and HO2 protected SH-SY5Y cells from hemin toxicity via increased ROS generation (Goldstein et al., 2003). Therefore, whether SFP, which can induce HO1 expression, protects or exacerbates hemin-induced injury in neurons is not known.

In this study, we used primary cultures of immature hippocampal neurons and tested the ability of SFP to induce the ARE/Nrf2 pathway and protect against oxidative stress-induced death by using two therapeutically relevant insults: hemin toxicity and OGD.

Methods

Materials

Cell culture reagents were obtained from Invitrogen. Sulforaphane (cat # S4441) and Hemin (catalog # 51280; Biochemica, Fluka) were purchased from Sigma and all other reagents were from Invitrogen (CA, USA).

Cell culture

Mouse hippocampal neuron cultures were prepared as previously described from embryonic day 15.5 fetuses. All animal use was in accordance with the University of Maryland IACUC protocols. Pregnant C57Bl6J mice were killed by cervical dislocation. Fetuses were removed to Petri dishes on ice then decapitated and the hippocampi dissected. Approximately 30,000 cells in 50 μ l Neurobasal medium containing glutamine (1mM), 1% Penicillin, Streptomycin (Pen/Strep) and 10% fetal bovine serum were seeded onto Poly-L-lysine/laminin coated 12 mm glass coverslips in 4 well plates. After 2 hours 0.5 ml of Neurobasal medium containing the serum-free B27 supplement (2%), Pen/Strep and glutamine was added in each well. At 2 days *in vitro* (DIV 2) 5 μ M cytosine arabinofuranoside was added to inhibit astrocyte proliferation. At 5 DIV the medium was changed to fresh Neurobasal medium containing B27, Pen/Strep and glutamine. Neurons were maintained in a humidified incubator, 5% CO_2 / balance air (result: 20% O_2). Astrocyte contamination was less than 5% in these cultures.

Drug treatments

To examine heme toxicity in neurons the cultures were exposed to hemin (oxidized form of heme) at the indicated concentrations or to vehicle. Before hemin treatment, cells were transferred to NBM and B27 (-) antioxidants (Neurobasal B27-Ao). Sulforaphane (SFP) or vehicle was added either 24 hours prior to or concomitant with the hemin. Hemin was added from a 3 mM stock (in ddH₂O and 1 N NaOH). SFP was added from a 100 mM stock in DMSO. The final concentration of DMSO in the media for 0.5 μ M SFP was 0.0005%. For the OGD experiments, SFP was added to the solution used for the reoxygenation.

Oxygen-glucose deprivation (OGD)

Neurons were transferred to an anaerobic chamber (ThermoForma model 1025) in a zero O₂/10% H₂/5% CO₂/85% N₂ atmosphere (atmospheric O₂ less than 1 ppm; Coy analyzer) and media changed to a 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) (Danilov and Fiskum, 2008) that was deoxygenated by preincubation for 48 hours in the anaerobic chamber (dissolved ISS oxygen between 5-30 ppb; measured using CHEMet test, Chemetrics). Neurons were exposed to OGD at 37°C for 60 minutes. At the end of the OGD, the cells were returned to the oxygenated environment and the media was replaced with NBM plus B27-Ao ± SFP or DMSO vehicle for 24 hours. Control cells were not exposed to OGD but were changed to the NBM plus B27-Ao with or without SFP or vehicle.

Analysis of cell death

At the end of the experiments, neurons were incubated in warmed PBS containing ethidium bromide and calcein green (Live/Dead kit; Invitrogen) and then incubated in the dark for 15 minutes before counting on a Nikon Eclipse E800 fluorescence microscope. Live, green cells, and dead, red cells, were counted in each field. The few astrocytes that were present were distinguishable by their flat morphology and were excluded from the analysis. Results obtained with astrocyte cultures also found that a period of 30-60 min OGD did not induce significant death by 24 hours (Danilov and Fiskum, 2008). Between 10 - 20 fields (for an average of 200 cells) were counted per each condition/experiment. The percent cell survival was calculated according to the formula: (calcein-positive cells/total cell number [calcein-positive + ethidium-positive]) × 100. Cells positive for both calcein and ethidium were not detected in these experiments.

Real-time reverse-transcription (RT)-PCR analysis

The expression of Nrf2-induced genes was examined by real-time reverse transcription PCR (qRT-PCR). Total cellular RNA was isolated using the RNeasy kit (Qiagen). The iScript One-Step RT-PCR Kit with SYBR Green™ (Bio-Rad) was used to analyze gene expression in a 50 µl reaction mixture containing 100 ng RNA template, gene specific primers, the 2X iQ SYBR Green Supermix and iScript Reverse Transcriptase. cDNA synthesis was performed for 10 min at 50°C in an Bio-Rad iCycler real-time PCR thermal cycler and 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. The fluorescence threshold value (CT) was determined using the IQ™5 iCycler software and fold changes were calculated using the $\Delta\Delta CT$ method. The following primers were used: HO1, 5'-GCCTGCTAGCCTGGTGCAAG-3' and 5'-AGCGGTGTCTGGGATGAGCTA-3' (Martin et al., 2004); NQO1, 5'-CATTCTGAAAGGCTGGTTTGA-3' and 5'-CTAGCTTTGATCTGGTTGTCAG-3'. GCLM, 5'-ACCTGGCCTCCTGCTGTGTG-3' and 5'-GGTCGGTGAGCTGTGGGTGT-3'; GCLC, 5'-ACAAGCACCCCGCTTCGGT-3' and 5'-CTCCAGGCCTCTCTCCTCCC-3; β -actin primers designed as in (Lee et al., 2003a) (5'-AGAGCATAGCCCTCGTAGAT-3' and 5'-CCCAGAGCAAGAGAGGTATC-3') were used for normalization of gene expression

Data Analysis

Data are expressed as means and SEM for 3 - 6 separate experiments with neurons prepared from different sets of animals. Data analysis was performed using either Student's *t*-test (for two groups) or one-way ANOVA (for multiple groups) followed by the *post-hoc* SNK test

as indicated in the figure legends. The SigmaStat 3.0 software was used and $p < 0.05$ was considered significant.

Results

Determination of safe concentrations of sulforaphane with immature hippocampal neurons

Primary neurons were isolated from E15.5 mouse hippocampi and were maintained for 5 - 8 days in culture (DIV 5 - 8) before they were used in the experiments. In order to establish an effective SFP dose for subsequent experiments, immature neurons were exposed to increasing concentrations of SFP (0.1 – 5.0 μM) for a period of 24 hours. The potential SFP toxicity was then examined by measuring cell death using the Live/Dead assay. Exposure of immature neurons to concentrations above 2 μM was found to be increasingly toxic (Fig. 1A), thus a dose of 0.5 μM SFP was selected for subsequent experiments. SFP in a DMSO stock was diluted in water and added to the medium, leading to a final concentration of DMSO of 0.0005%. This amount of DMSO had no toxic or protective effects (data not shown).

Sulforaphane protects hippocampal neurons against heme toxicity

Heme toxicity and potential protection by SFP were analyzed by exposing immature neurons to hemin, the oxidized form of heme, as in (Goldstein et al., 2003). As shown in Fig. 2, exposure of hippocampal neurons to 10 μM hemin caused significant death (~ 25%) at 24 hours. We then examined the effect of pretreatment with SFP on hemin toxicity. Immature neurons were incubated with 0.5 μM SFP for 24 hours and then the medium was changed and the cells exposed to 10 μM hemin. Cell death was analyzed 24 hours later as above. As shown in Fig 2A, SFP pretreatment resulted in complete protection of hippocampal neurons from hemin toxicity. We then examined whether SFP maintains its neuroprotective potential when administered concomitant with hemin exposure. Similarly to the pretreatment experiments, complete protection of neurons from hemin toxicity was obtained by co-administration of SFP with hemin (Fig. 2B).

Sulforaphane protects hippocampal neurons against OGD-induced death

The possibility that SFP can have a direct protective effect on immature neurons against ischemia/reperfusion injury was tested by analyzing the effect of SFP on O_2 and glucose deprivation (OGD)-induced neuronal death. As shown in Fig. 3, exposure of immature hippocampal neurons to a period of 60 min OGD induced significant cell death 24 hours after reperfusion. Since we had previously shown that both SFP pre- and post-treatment protects rat cortical astrocytes against delayed death after OGD, we decided to test the effect of SFP post-treatment on OGD-induced neuronal death. Fig. 3 shows that while 0.5 μM SFP did not affect cell survival of control neurons, it significantly protected them against OGD-induced death. Note that 10 μM hemin and 60 minutes OGD induced similar extents of neuron death (compare Figs 2 and 3); however, 0.5 μM SFP did not completely protect against the OGD-induced cell death, in contrast to the complete protection observed against hemin toxicity.

Sulforaphane activates the ARE/NRF2 pathway in hippocampal neurons

To determine whether the observed protective effect of SFP was accompanied by activation of the Nrf2/ARE pathway of gene expression, the mRNA expression of several Nrf2-induced genes was analyzed by qRT-PCR in neurons exposed to OGD and hemin. As shown in Fig. 4A, exposure of immature hippocampal neurons to OGD and 24 hour reperfusion in the absence of SFP alone had no effect on the expression of NADPH/Coenzyme Q oxidoreductase 1 (NQO1) compared to sham-treated control cells. Treatment with 0.5 μM

SFP immediately after OGD exposure, during the 24 hour reperfusion period, resulted in a significant induction of NQO1 mRNA (~4.5 fold, $p < 0.005$ vs. untreated cells and vs. OGD alone). In contrast to the result obtained after OGD, 24 hour treatment of immature neurons with hemin alone resulted in significant induction of NQO1 mRNA (~2 fold induction, $p < 0.05$ vs. sham-treated cells; Fig 4B), to a degree comparable to that elicited by treatment of normal neurons with SFP alone (Fig. 1B). Co-treatment of immature neurons with 10 μ M hemin and 0.5 μ M SFP exhibited a trend toward higher induction of NQO1 mRNA (~3 fold increase), as compared to hemin alone, although it did not reach statistical significance.

We next examined the effect of SFP on the expression of heme oxygenase 1 (HO1), another well characterized Nrf2 target gene, and on HO2, which like HO1 is also involved in heme catabolism but is not typically induced via Nrf2, thus serving as a control gene. As expected, treatment of immature neurons with SFP (0.5 μ M, 24 hours) resulted in induction of HO1 mRNA (~ 2 fold, $p < 0.05$ vs. untreated cells) but had no effect on HO2 mRNA expression (Fig 5A and 5B). Similar to NQO1, exposure of immature neurons to OGD alone did not result in HO1 induction (Fig. 5A), but SFP co-treatment of OGD exposed neurons resulted in significant induction of HO1 mRNA (Fig. 5A). Unlike the OGD insult, hemin treatment alone resulted in induction of HO1 (~ 2.5 fold, $p < 0.05$ vs. untreated cells), and no additional increase was obtained by co-treating cells with SFP and hemin (Fig. 5A). This observation is consistent with previous studies indicating that heme exposure results in induction of the heme catabolyzing heme oxygenase enzymes. Unlike for HO1, no changes in expression of HO2 mRNA were noted in immature neurons in response to SFP in control or OGD and hemin exposed groups (Fig. 5B).

We also examined the expression of two other Nrf2-inducible genes that are involved in glutathione biosynthesis, the glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits. As shown in Fig. 6A, SFP treatment resulted in ~ 2 fold induction of GCLM in control neurons ($p < 0.05$ vs. untreated cells) and ~ 3 fold induction ($p < 0.05$ vs. untreated cells) in cells exposed to hemin. A similar trend of GCLM induction was noted in OGD and SFP treated cells, however it did not reach statistical significance (Fig. 6A). Similarly, a trend towards induction of GCLC by SFP treatment was noted but it was not statistically significant (Fig. 6B).

Discussion

While several previous studies have shown that activators of the ARE/Nrf2 pathway can protect neural cells against various *in vitro* oxidative stress cell death paradigms (e.g., H_2O_2 exposure), it remains unclear whether SFP, a naturally occurring electrophilic compound which activates the ARE/Nrf2 cytoprotective pathway, can effectively protect neurons against other, potentially more clinically relevant paradigms such as ischemia/reperfusion or heme toxicity. In addition, with the exception of one study reporting that the Nrf2 inducer tBHQ protects immature neurons against glutamate toxicity (Shih et al., 2005), these questions have not been addressed in immature neurons.

SFP toxicity towards neurons has not been explicitly addressed in previous studies. Our results (Fig. 1A) clearly indicate that this should be considered since 24 hour exposure to increasing concentrations of SFP above 1 μ M resulted in progressive cell death in immature hippocampal neurons (Fig. 1A). This finding is in contrast to results we obtained with primary rat astrocytes for which doses up to 5 μ M had no toxic effects (Danilov et al., 2009). In general, neurons are more sensitive than astrocytes to many toxins, due to their relatively high energy demand and relatively greater dependence on mitochondrial ATP production, which is highly sensitive to chemical inhibition. Exposure of human bladder cancer cells to SFP was reported to cause drop in mitochondrial membrane potential in a small fraction

(<20%) of cells, albeit at a concentration of 15 μ M. Studies are in progress to determine if neuronal aerobic energy metabolism is inhibited by SFP at the lower concentrations found to be toxic in our experiments.

Free heme released from extravasated red blood cell hemoglobin is known to be a potent inducer of neuronal and astrocyte cell death following injury to the brains of both mature and immature animals (Bayir et al., 2006; Chang et al., 2005). Heme toxicity results from its catalysis of oxidative alterations to proteins, membrane lipids, and DNA. Oxidative damage to these molecules is also promoted by heme catabolites (Fe, biliverdin, CO) (Bayir et al., 2006; Platt and Nath, 1998). Due to its broad-based antioxidant effects resulting from induction of multiple antioxidant systems through the ARE/Nrf2 pathway, SFP might offer an effective approach toward protection against heme toxicity. Indeed, Zhao et al., showed recently that SFP-mediated activation of Nrf2 protected against damage produced by intracerebral hemorrhage, an injury in which heme toxicity is involved (Zhao et al., 2007). Whether Nrf2 activators such as SFP can directly protect neurons against heme toxicity has not been tested, however. In this study we tested this possibility and found that pretreatment with SFP is remarkably effective at protecting immature hippocampal neurons against hemin-induced death (Fig. 2A). Concomitant treatment of neurons with both SFP and hemin without pretreatment was equally protective (Fig. 2B). A similar protective effect of both pre- and co-treatment with SFP against hemin toxicity was also observed in mature hippocampal neuronal cultures (data not shown), suggesting that SFP could have therapeutic potential against heme toxicity in both the immature and adult injured brain.

We also examined the potential protective effect of SFP against another clinically relevant oxidative stress cell death paradigm, ischemia/reperfusion injury, which was modeled *in vitro* by exposing primary cultures of immature hippocampal neurons to oxygen/glucose deprivation (OGD). Using this model, we showed previously that pre- and post-treatment with SFP reduces oxidative DNA/RNA damage and protects primary cultures of astrocytes against OGD-induced cell death through activation of the ARE/Nrf2 pathway (Danilov et al., 2009). Here we show that post-OGD treatment with SFP also reduces OGD-induced cell death of immature hippocampal neurons (Fig. 3), although SFP protection was less complete than in the case of hemin-induced cell death. One likely explanation for the difference in effectiveness of SFP for hemin toxicity compared to cell death caused by OGD is that these pathogenic paradigms exhibit different cell death pathways. The toxicity of hemin is primarily based on its induction of oxidative stress, which secondarily causes apoptosis (Levy et al., 2002). Cell death caused by OGD is triggered initially by metabolic failure and loss of ATP, which is followed by oxidative stress during re-oxygenation, eventually culminating in some combination of necrosis and apoptosis (Kalda et al., 1998). It is therefore possible that the greater cytoprotection observed by SFP with hemin toxicity compared to OGD is due to hemin working primarily through oxidative stress, which is a major target of the genes induced by the ARE/Nrf2 pathway. Another possible explanation relates to the fact that SFP was added either prior to or during exposure to hemin whereas it was added immediately after OGD. Although not tested, it is highly unlikely that addition of SFP during the OGD would be protective since gene transcription and translation is precluded by cellular de-energization. In contrast, exposure to SFP for 24 hours prior to OGD would likely be protective based on our previous results with astrocytes and OGD. As with post-treatment of neurons, only partial protection was observed with pre- or post-treatment of astrocytes. We therefore conclude that while SFP is protective against delayed death of both neurons and astrocytes caused by OGD, the extent of protection is less than that of a more pure oxidative stress insult, e.g., hemin toxicity, due to the involvement of additional injury mechanisms in OGD, including metabolic dysfunction.

As SFP cytoprotective activity is known to occur mainly through activation of the ARE/Nrf2 cytoprotective pathway, the activity of SFP in immature neurons was further investigated by analyzing the expression of several ARE-regulated genes, including NQO1, HO1 and HO2 and the glutathione biosynthetic enzymes GCLM and GCLC. NQO1 is a detoxifying enzyme that reduces reactive quinones and quinone-imines to non-toxic, free radical scavenging hydroquinones (Lind et al., 1990). NQO1 is highly inducible and its induction is considered to be transcriptionally regulated by ARE (Jaiswal, 2000; Prochaska et al., 1987; Ross et al., 2000). HO1, the inducible heme metabolizing enzyme, is another well characterized target of the ARE/Nrf2 pathway. HO2 is considered to be a constitutive enzyme and appears to be expressed mainly in neurons in the brain and unlike HO1 is not induced by Nrf2. Interestingly, although HO1 has been previously reported to be predominantly glial (Bayir et al., 2006; Platt and Nath, 1998), our data show that it is expressed in immature neurons and its expression is inducible by SFP.

As expected, SFP treatment of control neurons resulted in induction of Nrf2-dependent genes, (NQO1, HO1, and GCLM; Fig 1A, Fig 5 CTR, Fig. 6 CTR) with the exception of GCLC for which only a trend towards increased expression following SFP was observed (Fig. 6B CTR). The finding that the genes analyzed in this study were not all induced by SFP to the same extent is consistent with previous reports using other cell types (Marrot et al., 2008). Lack of induction of HO2 confirmed the ARE/Nrf2-selective upregulation of gene expression by SFP treatment. These results show that SFP is able to induce the ARE/Nrf2 antioxidant pathway in immature cortical neurons.

Reoxygenation after ischemic brain injury is known to cause oxidative stress-related death in both astrocytes and neurons. Despite clear *in vivo* evidence for the neuroprotective potential of SFP against ischemia/reperfusion, the exact cellular targets of SFP are not clearly established. Studies using neuron and astrocyte co-cultures exposed to ARE/Nrf2 pathway activators suggested that neuronal protection against oxidative stress (i.e. H₂O₂ exposure (Kraft et al., 2004)), in this particular setting, is secondary to activation of ARE/Nrf2 pathway in astrocytes. Using the more clinically relevant OGD paradigm with pure primary astrocyte cultures, we previously demonstrated SFP protects astrocytes against ischemia/reperfusion injury (Danilov et al., 2009). We now also demonstrate protection by SFP against delayed death of immature neurons caused by OGD. Taken together, these studies support the hypothesis that pharmacologic activation of the ARE/Nrf2 pathway can provide neuroprotection against ischemia/reperfusion both by direct effects on neurons and by protection against the damage or death of astrocytes, which are a critical metabolic and trophic support system for neurons under both normal and pathologic conditions.

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Fig. 1. (A) Dose-dependent toxicity of sulforaphane in immature primary hippocampal neurons Primary mouse hippocampal neurons (DIV 6-8) were treated with 0.1, 0.2, 0.5, 2 and 5 μM SFP for 24 hours. Cell survival was analyzed using the Live/Dead assay and expressed as percent of untreated cells. DMSO (0.0005%) treatment was not significantly different from control (untreated cells). SFP caused a significant decrease in neuronal survival at doses equal or greater than 2 μM . * $p < 0.05$ vs. untreated or vehicle treated cells.

Fig. 2. Sulforaphane pretreatment or co-treatment protects immature hippocampal neurons against hemin-induced cell death

A. Primary mouse hippocampal neuronal cultures (DIV 5-8) were treated with SFP (0.5 μ M) or vehicle (0.0005% DMSO) for 24 hours and then the medium was replaced with NBM and B27-AO with or without hemin (10 μ M). Cells were further incubated for 24 hours and then cell survival was measured by using the Live/dead assay. Data are expressed as mean $\pm$ SEM (n= 3 - 4 experiments with neurons prepared from separate groups of animals). B. Primary hippocampal neurons (DIV 5 - 8) were concomitantly treated with SFP (0.5 μ M) and hemin (10 μ M) and cell survival was measured after 24 hours using the Live/Dead assay. Data are shown as means $\pm$ SEM (n=4). * indicates significant differences, $p < 0.05$, from the other three groups by ANOVA and *post-hoc* SNK.

Fig. 3. Sulforaphane protects immature hippocampal neurons against delayed death after oxygen and glucose deprivation

Primary hippocampal neurons (DIV 5-8) were incubated in an anaerobic chamber in a glucose-free “ionic shift” solution (ISS) for 60 min (see Methods). At the end of the OGD the media was replaced with NBM B27-AO and the cells were returned to 20% oxygen. Neurons were treated with 0.5 μ M SFP during the reoxygenation period and cell survival was determined 24 hours later. Control cells were not exposed to OGD but were changed to NBM plus B27-AO with or without SFP. Data are expressed as mean $\pm$ SEM (n= 3 - 4 experiments with neurons prepared from separate groups of animals). * indicates significant differences, $p < 0.05$, from the other three groups by ANOVA and *post-hoc* SNK. # indicates significantly different, $p < 0.05$, from SFP treated control cells.

Fig. 4. Sulforaphane induces the expression of NADPH/Coenzyme Q oxidoreductase 1 mRNA in immature hippocampal neurons

Primary hippocampal neurons (DIV 5-8) were exposed to sham-OGD treatment (A) or OGD (B) in the absence or presence of SFP (0.5 μM) present during the 24 hour reoxygenation. Neurons were also exposed to 10 μM hemin in the absence or presence of 0.5 μM SFP for 24 hours (C). After 24 hours, total mRNA was purified as described in Methods and the NQO1 mRNA expression was analyzed by quantitative RT-PCR. NQO1 mRNA expression was normalized to β-actin and was expressed as fold induction relative to untreated cells. Data are expressed as mean ± SEM (n= 3 - 4 experiments with neurons prepared from separate groups of animals). * $p < 0.005$ vs. untreated cells; # $p < 0.005$ vs. OGD alone (ANOVA with SNK post-hoc test).

Fig. 5. Sulforaphane induces the expression of heme oxygenase 1 but not heme oxygenase 2 mRNA in immature hippocampal neurons

Control (CTR), OGD exposed or hemin treated neurons were treated with 0.5 μ M SFP or vehicle for 24 hours then the expression of both HO1 (A) and HO2 mRNA (B) was analyzed by quantitative RT-PCR. HO1 and HO2 expression was normalized to β -actin and expressed relative to untreated cells. Data were analyzed by Student's *t* test for CTR group ($n = 5$ for HO1; $n=4$ for HO2) and by one way ANOVA with SNK post-hoc test for OGD ($n = 3$ for both HO1 and HO2) and hemin group (HO1, $n = 3$; HO2, $n=4$). * $p < 0.05$ vs untreated cells.

Fig. 6. Sulforaphane induces the expression of glutamate-cysteine ligase subunit mRNAs in immature hippocampal neurons

OGD exposed, hemin treated or control neurons (CTR) were treated with 0.5 μ M SFP or vehicle for 24 hours then the expression of both GCLM (A) and GCLC mRNA (B) was analyzed by quantitative RT-PCR as above. The GCLM and GCLC expression was normalized to β -actin and expressed relative to untreated cells. Data were analyzed by Student's *t* test for control (CTR) groups (GCLM, *n*=4; GCLC, *n*=6) and by one way ANOVA with SNK post-hoc test for OGD (GCLM, *n*=3; GCLC, *n*=4) and hemin groups (GCLM, *n*=3; GCLC, *n*=5). * *p* < 0.05 vs untreated cells.