

# Sulforaphane Protects Immature Hippocampal Neurons Against Death Caused by Exposure to Hemin or to Oxygen and Glucose Deprivation

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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 antioxidant 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 in 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 O<sub>2</sub> and glucose deprivation (OGD) or hemin. Treatment of immature neurons with SFP immediately after the OGD during reoxygenation was effective in protecting immature neurons from delayed cell death. Exposure of immature hippocampal neurons to hemin induced significant cell death, and both pre- and cotreatment with SFP were remarkably effective in 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), which is involved in glutathione biosynthesis, were up-regulated 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. © 2009 Wiley-Liss, Inc.

**Key words:** Nrf2; oxidative stress; ischemia/reperfusion; hemin; hippocampus

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. Under pathological conditions, oxidative stress can be mediated by different free radical species generated through multiple pathways, so effective neuroprotective therapies may have 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 AREs and induction of numerous cytoprotective genes, including NAD(P)H:quinone oxidoreductase 1 (NQO1; Riley and Workman, 1992; Li et al., 1995), heme oxygenase-1 (HO1; 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

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vegetables (*Brassica*; e.g., broccoli) and first became of interest because of its potential anticancer activity (Gamet-Payraastre, 2006). As an anticarcinogen, 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 AREs, inducing the expression of a large number of cytoprotective genes (Nioi et al., 2003; Hong et al., 2005; Lee and Surh, 2005).

Several recent *in vivo* studies have demonstrated the potential of SFP to protect against acute brain injury. In rodents, SFP administered 15 min after stroke led to an increase in HO1 expression in brain and reduced infarct volume (Zhao et al., 2006). Postinjury administration of SFP also reduced brain edema following TBI in rats (Zhao et al., 2005; Wang et al., 2007). 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 (Vannucci and Hagberg, 2004; Soane et al., 2008) and that neuroprotective interventions should be optimized according to age.

In addition, the cellular site of action in the brain for electrophilic compounds such as SFP that activate the ARE/Nrf2 pathway is not clear. By using an ARE reporter construct in mixed glial–neuron cocultures, a recent study indicated that activation of Nrf2 occurs predominantly in astrocytes and that neuroprotection was secondary to changes in glia (Shih et al., 2003; Kraft et al., 2004). 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 up-regulation of ARE-responsive genes (Lee et al., 2003b; Satoh et al., 2006). Whether this holds true 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 (Chang et al., 2005; Bayir et al., 2006). 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). After CNS hemorrhage, free heme can reach high micromolar concentrations in the extracellular space ( $EC_{50}$  about 10  $\mu$ M) and exert cytotoxic effects on both neurons and astrocytes (Goldstein et al., 2003; Chen-Roetling and

Regan, 2006). Heme and hemin (oxidized heme) are strong prooxidants and are normally metabolized by HO1 and HO2 enzymes into biliverdin, carbon monoxide, and iron, another prooxidant. Whether inhibiting or promoting heme catabolism is protective appears to be cell-type-specific, insofar 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), whereas HO2 may be neuroprotective against intracerebral hemorrhage (Wang et al., 2006). Inhibiting HO1 and HO2 protected SH-SY5Y cells from hemin toxicity via increased reactive oxygen species (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  $O_2$  and glucose deprivation (OGD).

## MATERIALS AND METHODS

### Materials

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Sulforaphane (catalog No. S4441) and hemin (catalog No. 51280; Biochemica, Fluka, Basel, Switzerland) were purchased from Sigma (St. Louis, MO), and all other reagents were from Invitrogen.

### 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 and then decapitated, and the hippocampi were dissected. Approximately 30,000 cells in 50  $\mu$ l Neurobasal medium containing glutamine (1 mM), 1% penicillin, streptomycin (Pen/Strep), and 10% fetal bovine serum were seeded onto poly-L-lysine/laminin-coated 12-mm glass coverslips in four-well plates. After 2 hr, 0.5 ml Neurobasal medium containing the serum-free B27 supplement (2%), Pen/Strep, and glutamine was added to each well. At 2 days *in vitro* (DIV) 5  $\mu$ 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 at the indicated concentrations or to vehicle. Before hemin treatment, cells were transferred to Neurobasal medium plus glutamine and Pen-Strep (NBM) and B27 (-) antioxidants (Neurobasal B27-Ao). Sulforaphane or vehicle was added either 24 hr prior to or concomitant with the hemin. Hemin was added from a 3 mM stock (in ddH<sub>2</sub>O and 1 N NaOH). SFP was added from a 100 mM stock in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the media for 0.5 μM SFP was 0.0005%. For the OGD experiments, SFP was added to the solution used for the reoxygenation.

### Oxygen-Glucose Deprivation

Neurons were transferred to an anaerobic chamber (ThermoForma model 1025) in a zero-O<sub>2</sub>/10% H<sub>2</sub>/5% CO<sub>2</sub>/85% N<sub>2</sub> atmosphere (atmospheric O<sub>2</sub> less than 1 ppm; Coy analyzer), and media were changed to a glucose-free "ionic shift" solution (ISS), pH 6.5, containing NaCl (39 mM), Na-gluconate (11 mM), K-gluconate (65 mM), NMDG-Cl (38 mM), NaH<sub>2</sub>PO<sub>4</sub> (1 mM), CaCl<sub>2</sub> (0.13 mM), MgCl<sub>2</sub> (1.5 mM), and Bis-Tris (10.5 mM; Danilov and Fiskum, 2008) that was deoxygenated by preincubation for 48 hr in the anaerobic chamber (dissolved ISS oxygen between 5–30 ppb; measured using CHEMtest; Chemetrics). Neurons were exposed to oxygen-glucose deprivation (OGD) at 37°C for 60 min. At the end of the OGD, the cells were returned to the oxygenated environment, and the medium was replaced with NBM plus B27-Ao ± SFP or DMSO vehicle for 24 hr. 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) in the dark for 15 min 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 of OGD did not induce significant death by 24 hr (Danilov and Fiskum, 2008). Between 10 and 20 fields (for an average of 200 cells) were counted for each condition/experiment. The percentage 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-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, Valencia, CA). The iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) was used to analyze gene expression in a 50-μl reaction mixture containing 100 ng RNA template, gene specific primers, 2× 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 sec; annealing at 55°C, 30 sec; extension at 72°C, 40 sec), 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 (C<sub>T</sub>) was determined by using the IQ5 iCycler software, and -fold changes were calculated using the ΔΔCT 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'-GGTCGGT GAGCTGTGGGTGT-3'; and GCLC, 5'-ACAAGCACCC CCGCTTCGGT-3' and 5'-CTCCAGGCCTCTCTCCTCC C-3;. β-Actin primers designed as by Lee et al. (2003a; 5'-AGAGCATAGCCCTCGTAGAT-3' and 5'-CCCAGAG CAAGAGAGGTATC-3') were used for normalization of gene expression

### Data Analysis

Data are expressed as means and SEM for three to six 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. 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 hr. 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. 1), so 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 the study of Goldstein et al. (2003). As shown in Figure 2, exposure of hippo-

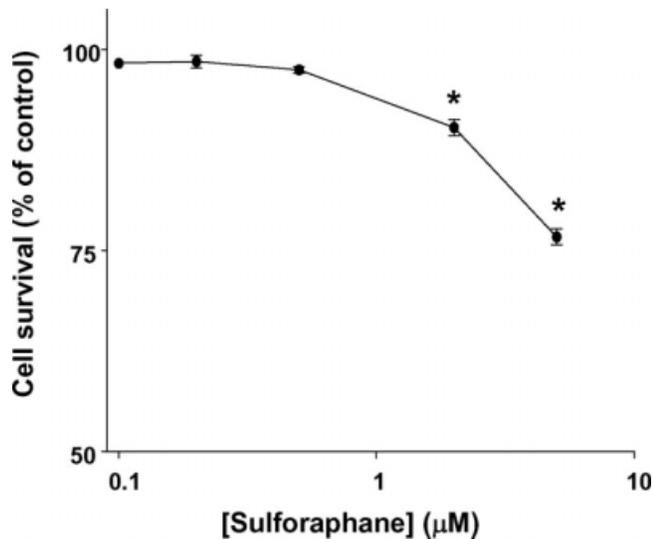


Fig. 1. Dose-dependent toxicity of sulforaphane in immature primary hippocampal neurons. Primary mouse hippocampal neurons (DIV 6–8) were treated with 0.1, 0.2, 0.5, 2, and 5  $\mu\text{M}$  SFP for 24 hr. Cell survival was analyzed using the Live/Dead assay and expressed as percentage of untreated cells.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

campal neurons to 10  $\mu\text{M}$  hemin caused significant death (~25%) at 24 hr. We then examined the effect of pretreatment with SFP on hemin toxicity. Immature neurons were incubated with 0.5  $\mu\text{M}$  SFP for 24 hr, and then the medium was changed and the cells were exposed to 10  $\mu\text{M}$  hemin. Cell death was analyzed 24 hr later as described above. As shown in Figure 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 coadministration 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 OGD-induced neuronal death. As shown in Figure 3, exposure of immature hippocampal neurons to 60 min of OGD induced significant cell death 24 hr after reperfusion. Because we had previously shown that both SFP pre- and posttreatment protects rat cortical astrocytes against delayed death after OGD, we decided to test the effect of SFP posttreatment on OGD-induced neuronal death. Figure 3 shows that, although 0.5  $\mu\text{M}$  SFP did not affect cell survival of control neurons, it significantly protected them against OGD-induced death. Note that

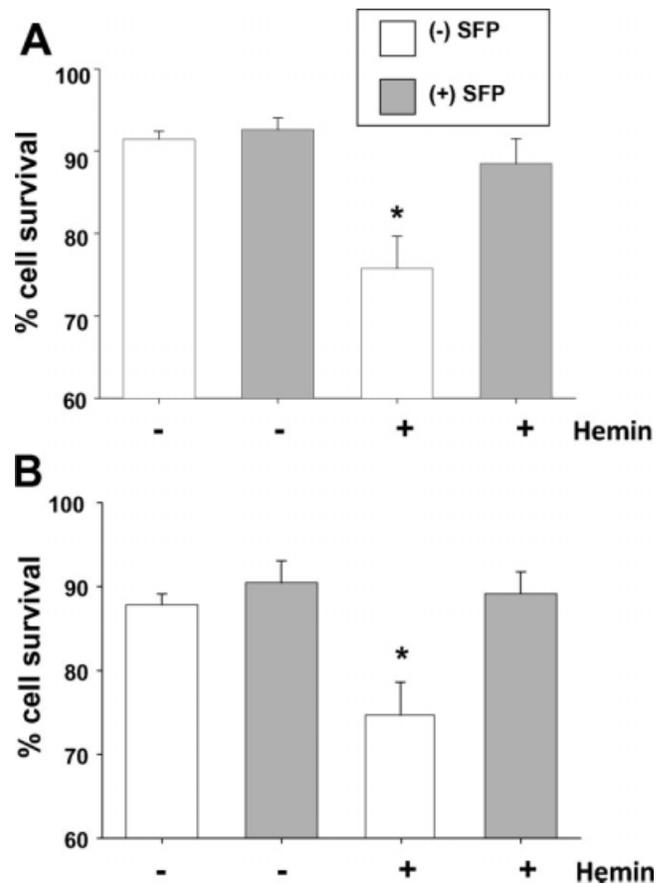


Fig. 2. Sulforaphane pretreatment or cotreatment protects immature hippocampal neurons against hemin-induced cell death. **A:** Primary mouse hippocampal neuronal cultures (DIV 5–8) were treated with SFP (0.5  $\mu\text{M}$ ) or vehicle (0.0005% DMSO) for 24 hr, and then the medium was replaced with NBM and B27-AO with or without hemin (10  $\mu\text{M}$ ). Cells were further incubated for 24 hr, 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  $\mu\text{M}$ ) and hemin (10  $\mu\text{M}$ ), and cell survival was measured after 24 hr using the Live/Dead assay. Data are shown as means  $\pm$  SEM ( $n = 4$ ). \* $P < 0.05$  vs. the other three groups by ANOVA and post hoc SNK.

10  $\mu\text{M}$  hemin and 60 min of OGD induced similar extents of neuron death (compare Figs. 2, 3); however, 0.5  $\mu\text{M}$  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 Figure 4A, treatment with 0.5  $\mu\text{M}$  SFP induced NQO1 mRNA in control neurons. Exposure of immature hippocampal neurons to OGD and 24 hr of reperfusion in the absence of SFP had no effect on the expression of NQO1 compared with sham-treated control cells. Treatment with 0.5  $\mu\text{M}$  SFP immediately after

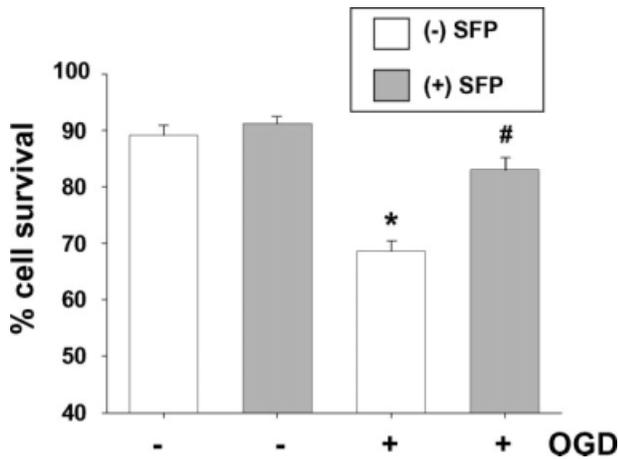


Fig. 3. Sulforaphane protects immature hippocampal neurons against delayed death after oxygen and glucose deprivation. Primary hippocampal neurons (DIV 5–8) were incubated in an anaerobic chamber in a glucose-free “ionic shift” solution (ISS) for 60 min (see Materials and Methods). At the end of the OGD, the medium was replaced with NBM B27-AO, and the cells were returned to 20% oxygen. Neurons were treated with 0.5  $\mu\text{M}$  SFP during the reoxygenation period, and cell survival was determined 24 hr 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). \* $P < 0.05$  vs. the other three groups by ANOVA and post hoc SNK. # $P < 0.05$  vs. SFP-treated control cells.

OGD exposure, during the 24-hr reperfusion period, resulted in a significant induction of NQO1 mRNA ( $\sim 4.5$ -fold,  $P < 0.005$  vs. untreated cells and vs. OGD alone). In contrast to the result obtained after OGD, 24 hr of treatment of immature neurons with hemin alone resulted in significant induction of NQO1 mRNA ( $\sim 2$ -fold induction,  $P < 0.05$  vs. sham-treated cells; Fig. 4B), to a degree comparable to that elicited by treatment of SFP-treated control neurons with SFP alone. Cotreatment of immature neurons with 10  $\mu\text{M}$  hemin and 0.5  $\mu\text{M}$  SFP exhibited a trend toward higher induction of NQO1 mRNA ( $\sim 3$ -fold increase) compared with hemin alone, although it did not reach statistical significance.

We next examined the effect of SFP on the expression of 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  $\mu\text{M}$ , 24 hr) resulted in induction of HO1 mRNA ( $\sim 2$ -fold,  $P < 0.05$  vs. untreated cells) but had no effect on HO2 mRNA expression (Fig. 5A,B). Similarly to NQO1, exposure of immature neurons to OGD alone did not result in HO1 induction (Fig. 5A), but SFP cotreatment 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 ( $\sim 2.5$ -fold,  $P < 0.05$  vs. untreated cells), and no additional increase was obtained by cotreating cells with SFP and hemin (Fig. 5A). This observation is consistent with previous studies indicating that heme exposure results in induction of the heme-catabolizing HO enzymes. Unlike the case 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).

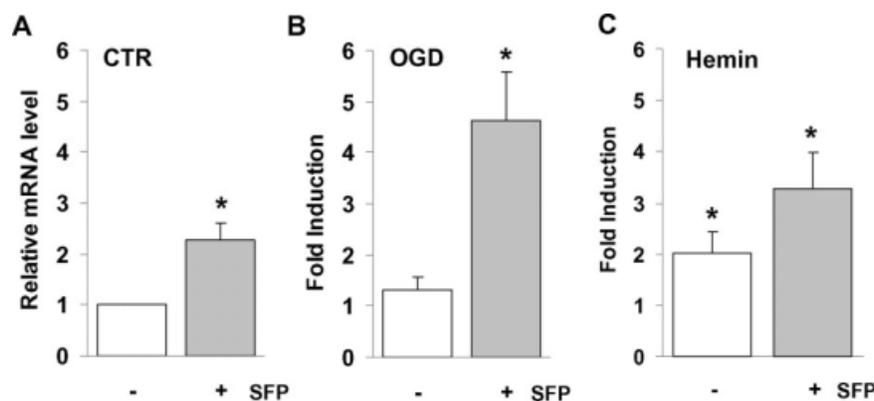


Fig. 4. Sulforaphane induces the expression of NADPH/coenzyme Q oxidoreductase 1 mRNA in immature hippocampal neurons. Primary hippocampal neurons (DIV 5–8) were exposed to sham-OGD treatment (A) or OGD (B) in the absence or presence of SFP (0.5  $\mu\text{M}$ ) present during the 24-hr reoxygenation. Neurons were also exposed to 10  $\mu\text{M}$  hemin in the absence or presence of 0.5  $\mu\text{M}$  SFP for 24 hr (C). After 24 hr, total mRNA was purified as described in

Materials and Methods, and the NQO1 mRNA expression was analyzed by quantitative RT-PCR. NQO1 mRNA expression was normalized to  $\beta$ -actin and was expressed as -fold induction relative to untreated cells. Data are expressed as mean  $\pm$  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).

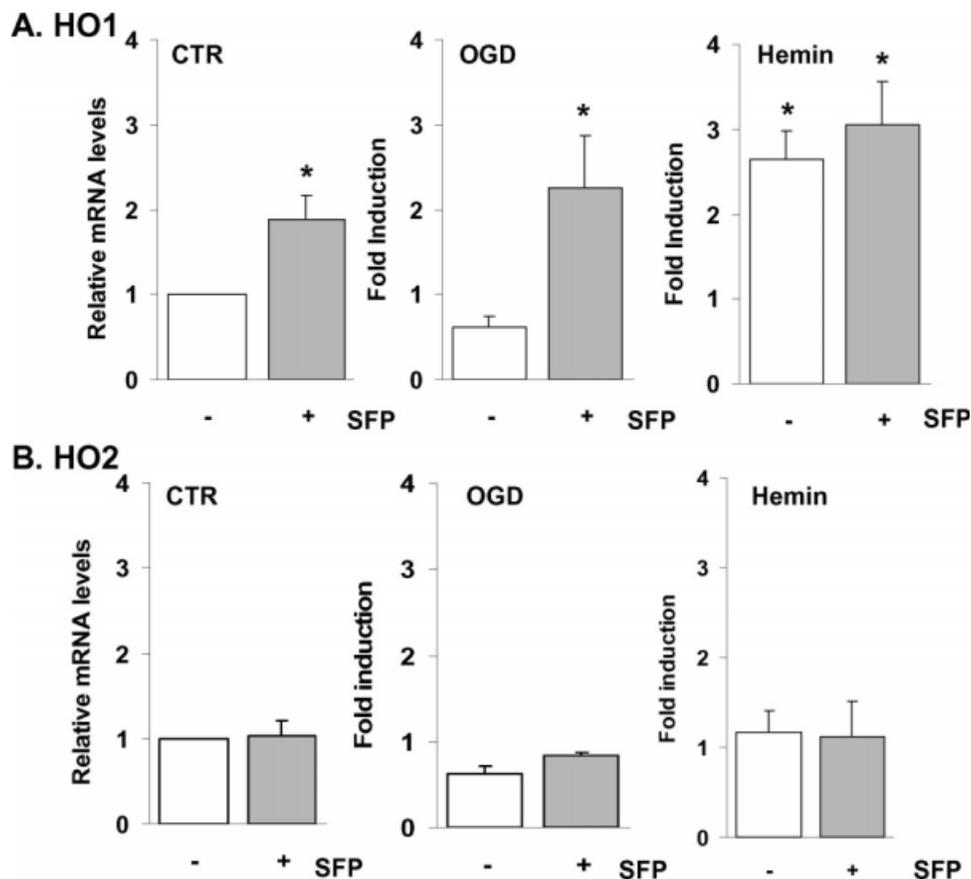


Fig. 5. Sulforaphane induces the expression of HO1 but not HO2 mRNA in immature hippocampal neurons. Control (CTR), OGD-exposed, or hemin-treated neurons were treated with 0.5  $\mu$ M SFP or vehicle for 24 hr, then the expression of both HO1 (A) and HO2 mRNA (B) was analyzed by quantitative RT-PCR. HO1 and HO2 expression was normalized to  $\beta$ -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.

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 Figure 6A, SFP treatment resulted in  $\sim 2$ -fold induction of GCLM in control neurons ( $P < 0.05$  vs. untreated cells) and  $\sim 3$ -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, but it did not reach statistical significance (Fig. 6A). Similarly, a trend toward induction of GCLC by SFP treatment was noted but was not statistically significant (Fig. 6B).

## DISCUSSION

Although 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 com-

pound that 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 toward neurons has not been explicitly addressed in previous studies. Our results (Fig. 1) clearly indicate that this should be considered, insofar as 24-hr exposure to increasing concentrations of SFP above 1  $\mu$ M resulted in progressive cell death in immature hippocampal neurons. This finding is in contrast to results we obtained with primary rat astrocytes, for which doses up to 5  $\mu$ M had no toxic effects (Danilov et al., 2009). In general, neurons are more sensitive than astrocytes to many toxins, because of their relatively high energy demand and relatively greater dependence on mitochondrial ATP production, which is highly sensitive to chemical inhibition. Exposure of human bladder

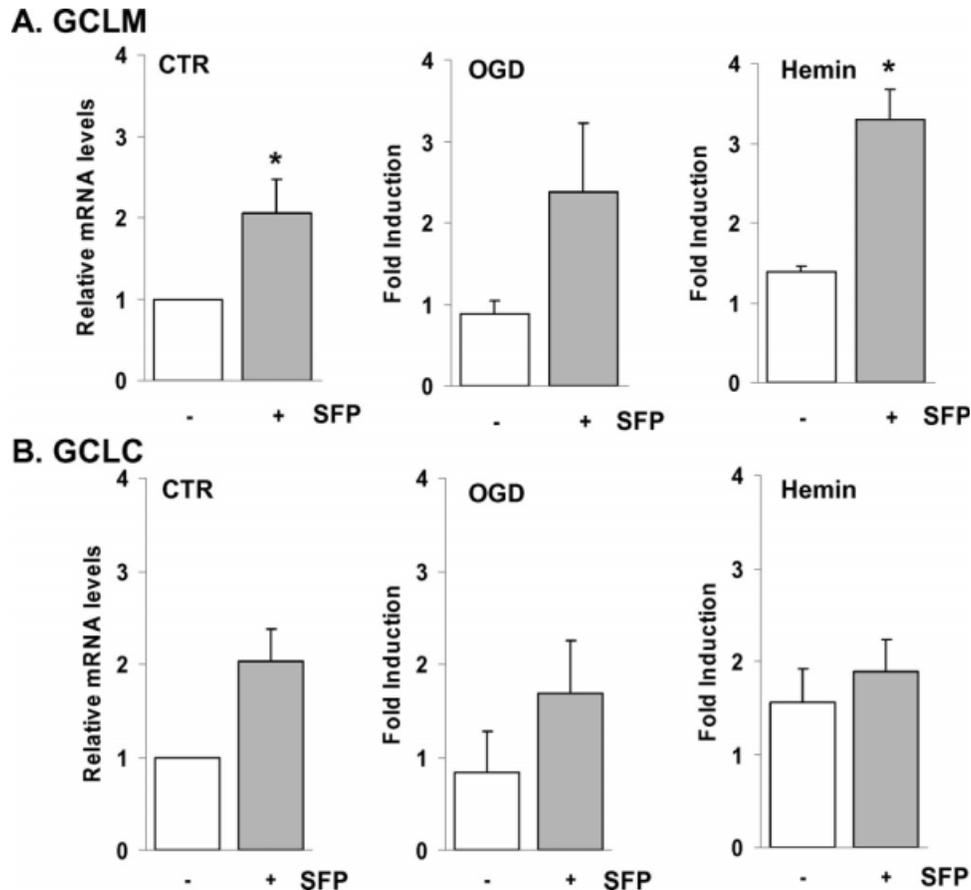


Fig. 6. Sulforaphane induces the expression of glutamate-cysteine ligase subunit mRNAs in immature hippocampal neurons. OGD-exposed, hemin-treated, or control neurons (CTR) were treated with 0.5  $\mu$ M SFP or vehicle for 24 hr, and then the expression of both GCLM (A) and GCLC mRNA (B) was analyzed by quantitative RT-PCR as described above. The GCLM and GCLC expres-

sion was normalized to  $\beta$ -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.

cancer cells to SFP was reported to cause a drop in mitochondrial membrane potential in a small fraction (<20%) of cells, albeit at a concentration of 15  $\mu$ M. Studies are in progress to determine whether 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 (Chang et al., 2005; Bayir et al., 2006). 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; Platt and Nath, 1998; Bayir et al., 2006). Because of 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. (2007) showed recently that SFP-mediated activation of Nrf2 protected against damage produced by intracerebral hemorrhage, an injury in which heme toxicity is involved. Whether Nrf2 activators such as SFP can directly protect neurons against heme toxicity has not been tested, however. Here we tested this possibility and found that pretreatment with SFP is remarkably effective in 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 cotreatment 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 the 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 OGD. Using this model, we showed previously that pre- and posttreatment 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 with cell death caused by OGD is that these pathogenic paradigms exhibit different cell death pathways. The toxicity of hemin is based primarily 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 reoxygenation, 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 with 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 this was not tested, it is highly unlikely that addition of SFP during the OGD would be protective, because gene transcription and translation are precluded by cellular deenergization. In contrast, exposure to SFP for 24 hr prior to OGD would likely be protective based on our previous results with astrocytes and OGD. As with posttreatment of neurons, only partial protection was observed with pre- or posttreatment of astrocytes. We therefore conclude that, although 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, because of the involvement of additional injury mechanisms in OGD, including metabolic dysfunction.

SFP cytoprotective activity is known to occur mainly through activation of the ARE/Nrf2 cytoprotective pathway, so 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 nontoxic, free-radical-scavenging hydroquinones (Lind et al., 1990). NQO1 is highly inducible, and its induction is considered to be transcriptionally regulated by ARE (Prochaska et al., 1987; Jaiswal, 2000; 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 that its expression is inducible by SFP.

As expected, SFP treatment of control neurons resulted in induction of Nrf2-dependent genes (NQO1, HO1, and GCLM, Figs. 5,6), with the exception of GCLC, for which only a trend toward increased expression following SFP was observed (CTR, Fig. 6B). The finding that the genes analyzed in this study were not all induced by SFP to the same extent is consistent with previous studies using other cell types (Marrot et al., 2008). Lack of induction of HO2 confirmed the ARE/Nrf2-selective up-regulation of gene expression by SFP treatment. These results show that SFP is able to induce the ARE/Nrf2 antioxidant pathway in immature hippocampal 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 cocultures exposed to ARE/Nrf2 pathway activators suggested that neuronal protection against oxidative stress [i.e., H<sub>2</sub>O<sub>2</sub> 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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