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Targeting the Nrf2-Heme Oxygenase-1 Axis after Intracerebral Hemorrhage

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Abstract

Background—Injury to cells adjacent to an intracerebral hemorrhage (ICH) is likely mediated at least in part by toxins released from the hematoma that initiate complex and interacting injury cascades. Pharmacotherapies targeting a single toxin or pathway, even if consistently effective in controlled experimental models, have a high likelihood of failure in a variable clinical setting. Nuclear factor erythroid-2 related factor 2 (Nrf2) regulates the expression of heme oxygenase-1 (HO-1) and multiple other proteins with antioxidant and anti-inflammatory effects, and may be a target of interest after ICH.

Methods—Studies that tested the effect of HO and Nrf2 in models relevant to ICH are summarized, with an effort to reconcile conflicting data by consideration of methodological limitations.

Results—In vitro studies demonstrated that Nrf2 activators rapidly increased HO-1 expression in astrocytes, and reduced their vulnerability to hemoglobin or hemin. Modulating HO-1 expression via genetic approaches yielded similar results. Systemic treatment with small molecule Nrf2 activators increased HO-1 expression in perivascular cells, particularly astrocytes. When tested in mouse or rat ICH models, Nrf2 activators were consistently protective, improving barrier function and attenuating edema, inflammation, neuronal loss and neurological deficits. These effects were mimicked by selective astrocyte HO-1 overexpression in transgenic mice.

Conclusion—Systemic treatment with Nrf2 activators after ICH is protective in rodents. Two compounds, dimethyl fumarate and hemin, are currently approved for treatment of multiple sclerosis and acute porphyria, respectively, and have acceptable safety profiles over years of clinical use. Further development of these drugs as ICH therapeutics seems warranted.

Keywords

astrocyte; heme; heme oxygenase; intracerebral hemorrhage; Nrf2; Stroke

Scope of the problem

Intracerebral hemorrhage (ICH) is the primary event in 10–15% of the 15 million strokes occurring annually worldwide [1]. This percentage is significantly higher in Asians and

African Americans, and increases in all populations with aging. Compared with ischemic stroke, ICH has a higher mortality rate and greater lifetime cost [2]. About half of ICH patients will be dead at one month; only 10% will be living independently at this time point, and only 20% will be independent at six months [3]. Despite its severity, ICH has been less intensively investigated than ischemic stroke, and injury mechanisms remain largely undefined. No effective pharmacotherapies are currently available, and no improvement in outcome has been observed over the past two decades [4].

Pathophysiology

Reduced blood flow without ischemia

ICH is produced by rupture of small penetrating arteries or arterioles. The immediate impact, at least in animal models that attempt to replicate arterial rupture, is transmission of a pressure wave from the vessel lumen to the parenchyma. The resulting increase in tissue pressure reduces blood flow in a manner that varies inversely with distance from the hematoma and time, and likely reflects impaired flow through lower pressure capillaries and venules [5, 6]. Although tissue injury after clinical ICH has classically been attributed to compressive ischemia, early blood flow reduction may not be of sufficient magnitude and duration to produce ischemic cell death. Perfusion imaging studies conducted in the acute phase (< 22 hours) after symptom onset suggest that perihematomal ischemia is absent in most patients; any decrease in perfusion is likely due reduced metabolic demand [7]. Consistent with these observations, no significant changes in cerebral oxygen extraction, $CMRO_2$, regional cerebral blood flow, glucose utilization or lactate production were detected at five hours after a massive experimental ICH in dogs [8]. However, ischemia cannot be excluded in a subset of patients with a particularly poor prognosis, although protocols for rapidly identifying these patients have not been established [9, 10].

Toxicity of extravascular blood

Since most injury after ICH cannot be directly attributed to ischemia, delineation of alternative pathophysiological pathways remains a primary focus of preclinical investigation. One hypothesis is that toxins released from a hematoma initiate injury cascades that contribute to cell loss and poor outcome [11]. Unfortunately, a hematoma contains many potential cytotoxins, complicating mechanistic investigation and reducing the likelihood that a single selective agent will have any efficacy in a complex clinical environment. In vitro studies may provide some insight into the predominant injury mechanisms that are induced as a hematoma evolves in proximity to neural cells. In primary cell cultures containing both glial cells and neurons, most cell loss produced by co-culture with a blood clot can be attributed to heme-mediated oxidative stress and excitotoxicity [12, 13].

Hemoglobin Toxicity—Hemoglobin is a tetrameric protein containing four heme groups, and accounts for essentially all of the ~20 mM heme concentration in erythrocytes. The time course of erythrocyte lysis after experimental or clinical ICH has not been well-defined, nor has its predominant site, i.e. extracellular versus within microglia/macrophages after erythrophagocytosis. In the rat blood injection ICH model, Perls' staining demonstrated iron accumulation in neurons at 24 hours and in glial cells several days later [14], consistent with

some early extracellular hemolysis. The extraneous redox activity of the heme groups of ferrous hemoglobin is limited by their location within hydrophobic pockets [15]. In the extracellular space, this protective structure is rapidly compromised as hemoglobin autoxidizes to its ferric form (methemoglobin) via a reaction that generates equimolar superoxide [16]. Hemoglobin autoxidation occurs in a predictable fashion after clinical ICH, and is the basis for estimating hematoma age via magnetic resonance imaging [17]. The heme moieties of methemoglobin are less tightly bound than those of ferrous hemoglobin and are readily released [18]. Free hemin, an oxidized form of heme, accumulates in intracranial hematomas in concentrations that reach the high micromolar range [19]. Its lipophilicity accounts for its tendency to concentrate in cell membranes [20].

At least three mechanisms contribute to the direct cytotoxicity of hemin. First, it directly and efficiently oxidizes membrane lipids by decomposing preformed lipid peroxides, thereby initiating free radical chain reactions [21, 22]. Second, it destabilizes membranes via an incompletely defined colloid osmotic mechanism that is not prevented by antioxidants [23]. Third, hemin breakdown by the heme oxygenase (HO) enzymes may produce an iron-dependent oxidative injury to cell populations with limited iron-sequestering capacity, such as neurons [24]. In addition, hemin also activates TLR4 and may thereby initiate an inflammatory response that indirectly contributes to secondary cell loss [25].

Excitotoxicity—Excessive activation of excitatory glutamate receptors contributes to ischemic CNS injury in multiple animal models [26], although clinical trials of receptor antagonists failed to demonstrate a significant benefit. Excitotoxicity has received less attention as a therapeutic target after ICH, particularly after experimental and clinical studies suggesting that perihematomal tissue was not ischemic [7, 8]. However, extravascular blood is a very likely source of excitotoxic stress [27]. Blood lysate has an excitatory amino acid content in the high micromolar range, due predominately to the high glutamate and aspartate concentrations in erythrocytes [28, 29]. Extravascular hemolysis in the days after ICH will release a constant supply of these receptor ligands, and their neurotoxicity may be enhanced in a hemorrhagic environment [12]. In vitro, the NMDA receptor antagonist MK-801 was moderately protective per se against blood clot toxicity [13]. In vivo, striatal injection of lysed blood produced a rapid increase in [C^{14}]-2-deoxyglucose utilization in adjacent tissue that was blocked by MK-801 and the AMPA receptor antagonist NBQX, indicating that the excitatory amino acid content in the lysate is sufficient to activate both receptor classes [30]. Studies testing the efficacy of glutamate receptor antagonists in ICH models have been relatively sparse. Memantine had some benefit as monotherapy after collagenase-induced ICH in rats, but was more effective when combined with the Cox-2 inhibitor celecoxib [31, 32]. The AMPA receptor antagonist YM872 had a modest benefit on neurological outcome in the rat collagenase ICH model, without affecting hematoma or lesion size [33].

The complexity of the injury initiated by an intracerebral hematoma is magnified in vivo by a secondary inflammatory response that is not prominent in simplified cell culture models. This response is activated at least in part by hemin, a TLR4 agonist [34], and thrombin, a serum protease generated by the coagulation cascade with multiple pro-inflammatory effects [35]. A selective pharmaceutical approach that targets a single mechanism, even if beneficial in a controlled experimental model, has a high probability of failure when tested in an

inherently variable clinical setting. A single therapy that modulates multiple injury mechanisms after ICH may be preferred. Heme oxygenase-1 (HO-1) attenuates oxidative [36], excitotoxic [37] and inflammatory injury [38] in experimental models, so agents that upregulate this enzyme are of particular interest.

Pleiotropic effects of the heme oxygenases

The heme oxygenase enzymes catalyze the rate-limiting step of heme/hemin breakdown. Two isoforms, designated HO-1 and HO-2, are expressed by mammalian cells; a third, HO-3, was initially described but was in fact a pseudogene derived from HO-2 transcripts [39]. HO-1 and HO-2 are the products of separate genes (HMOX1 and HMOX2) and catalyze the same reaction, consuming three molecules of oxygen and seven electrons donated by NADPH to yield equimolar carbon monoxide, biliverdin and ferrous iron. The biological activity of these products may account for the diverse and sometimes discordant effects of HO activity in CNS injury models. Carbon monoxide is toxic at high atmospheric concentrations because its affinity for the heme moieties of hemoglobin and cytochrome c oxidase interferes with oxygen exchange and mitochondrial electron transport [40]. However, at the much lower concentrations associated with heme breakdown, it has immunomodulating and vasodilating effects that appear to be beneficial after acute CNS insults [38]. Biliverdin is converted to bilirubin by ubiquitously-expressed biliverdin reductase; both pigments are potent antioxidants [41]. Ferrous iron is oxidized by hydrogen peroxide to yield the highly reactive hydroxyl radical, but also is a very efficient inducer of ferritin, the primary cellular iron storage protein. Each ferritin molecule is a 24-mer heteropolymer constructed as a nanocage surrounding a mineral core that has an enormous capacity for iron sequestration (up to 4000 atoms [42]). An inducing stimulus usually increases iron binding capacity in excess of immediate need, thereby increasing cellular resistance to subsequent iron challenge [43, 44].

Initial studies evaluating the relationship between HO and outcome in models relevant to hemorrhagic stroke consistently reported very significant effects. However, the direction of these effects was contradictory and a source of considerable confusion. In hindsight, it is apparent that these studies were limited by the methods available at the time to modulate HO activity and expression, which primarily involved the use of either unconditional knockouts or nonspecific pharmacological inhibitors.

Unconditional knockout of HO-1, the inducible isoform that is expressed predominantly by glial cells in the CNS, has a combined prenatal and perinatal mortality rate exceeding 90% (unpublished observations). Adult HO-1 KO mice are therefore highly selected animals that may have compensatory but as yet unidentified survival mechanisms that are very unlikely to be expressed to a similar extent in wild-type controls. At baseline, HO-1 knockouts suffer from chronic anemia and a marked dysregulation of heme catabolism, resulting in a sevenfold increase in plasma hemoglobin and a fourfold increase in plasma heme [45]. Bone marrow and splenic macrophages are depleted, likely due to their increased vulnerability to heme uptake during erythrophagocytosis and erythropoiesis. An experimental hematoma in an HO-1 KO mouse will therefore contain less heme than a wild-type hematoma of equal volume, and will be delivered to a macrophage-deficient mouse that has been preconditioned

by chronic elevation of extracellular heme. It is perhaps not surprising then, that unconditional HO-1 knockouts sustained less injury and inflammation after collagenase-induced ICH than their wild-type counterparts [46], despite the anti-inflammatory effects of HO-1 in wild-type animals [38, 47, 48].

In contrast to HO-1, HO-2 knockouts breed well and are grossly indistinguishable from wild-type littermates; they do not differ from their wild-type counterparts in any hematological parameter [49]. However, while HO-2 is the predominant neuronal isoform, it is also constitutively expressed in glial and endothelial cells [50, 51], so an unconditional knockout will alter HO activity in multiple cell populations. In contrast to HO-1 KO, HO-2 knockout worsened outcome to a variable extent in the collagenase ICH model [52, 53], but was protective in the blood injection model [54], perhaps due to differences in the predominant injury mechanisms in these models. Collagenase disrupts multiple blood vessels near the injection site, and if sufficiently widespread may lead to ischemia. As mentioned above, generation of a hematoma by autologous blood injection does not reduce blood flow to ischemic levels [8]. The protective effect of HO-2 against collagenase-induced ischemia [55] may negate its deleterious effect against hemoglobin or hemin toxicity, and thereby account for the disparate impact of HO-2 knockout.

Diverging effects of HO on heme toxicity in different CNS cell populations are readily demonstrated in vitro. Primary neurons cultured from fetal mice express little HO-1, but uniformly express HO-2. Generation of reactive oxygen species and cell injury after hemin or hemoglobin treatment was markedly reduced by HO-2 knockout [56, 57], but HO-1 knockout had no effect [58]. However, when experiments were conducted in medium containing iron-poor transferrin (Neurobasal/B27), which protects against the iron-dependent component of heme toxicity [59], HO-2 knockout neurons were conversely more vulnerable to hemin [52]. The latter observation demonstrates that HO activity is beneficial as long as iron can be safely sequestered, likely due to the protective effects of other heme breakdown products, i.e. biliverdin/bilirubin and carbon monoxide. A deleterious effect of HO activity on heme-mediated injury appears to be limited to neurons, which express very little ferritin at baseline or with iron loading [60], and so are selectively vulnerable to low iron concentrations [61]. In contrast, knockout of either HO-1 or HO-2 increased the vulnerability of astrocytes to hemin or hemoglobin even in the absence of iron chelators [62, 63], reflecting their ability to rapidly increase ferritin expression and thereby detoxify iron [64]. HO-1 expression was also protective in models of pure excitotoxic [37] and inflammatory injury [65], which are also relevant injury mechanisms after ICH. The observed effect of unconditional HO knockout or nonselective HO inhibitors after ICH is likely a complex function of the local iron binding capacity and the injury mechanism that predominates in the model. The discordant results reported to date indicate that nonselective modulation of HO expression and activity is not an optimal therapeutic strategy after ICH; a more targeted approach is needed.

Overexpressing HO-1 protects cultured astrocytes

In mixed glial cultures containing >90% GFAP+ astrocytes, HO-1 expression and activity were increased four to six-fold by adenoviral gene transfer driven by either the CMV or

GFAP promoters, with a transfection efficiency exceeding 80% [36, 66]. These cells were then protected from toxic concentrations of hemin, with significant reduction in protein carbonylation and cell death. While these studies provided a useful proof of concept, CNS gene therapies present major and perhaps unsolvable challenges related to vector delivery, toxicity, and timely gene expression, and may be difficult to implement or even test within hours of hemorrhagic stroke. A low molecular weight compound with CNS bioavailability that increases endogenous HMOX1 transcription may be preferred.

Nrf2 activation increases HO-1 and protects against hemin

HO-1 expression is tightly regulated in cultured astrocytes. Basal levels are quite low but are increased within a few hours by hemin, hemoglobin or other oxidants, with rapid enzyme turnover [67–69]. Expression is positively regulated at the transcriptional level by nuclear factor erythroid-2 related factor 2 (Nrf2) (Fig. 1, see Baird and Dinkova-Kostova for review [70]). Under normal cell conditions, Nrf2 forms a complex with actin-bound Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3. This binding sequesters it in the cytoplasm, where it is ubiquitinated and rapidly degraded by the 26S proteasome, resulting in low steady-state Nrf2 levels. The sulfhydryl groups of Keap1 cysteine residues are very efficient sensors of oxidative and electrophilic stress, and their oxidation inhibits Nrf2 ubiquitination, permitting nuclear transport. After heterodimerization with small Maf proteins, the complex binds to antioxidant response elements (ARE) of HMOX1 and several other genes encoding proteins that may be beneficial after ICH (Table 1). This process is negatively regulated by Bach1, which competes with Nrf2 for MafK binding sites. Bach1-Maf heterodimers bind to HMOX1 enhancer sites and repress transcription. At least in some cell populations, release of Bach1 repression is a prerequisite for HMOX1 transcription [71].

Nrf2 is rapidly increased in cultured cells by selective proteasome inhibitors, but not by other protease inhibitors [72]. MG132 is a widely used peptide aldehyde proteasome inhibitor (Z-Leu-Leu-Leu-CHO) that reversibly binds to the chymotrypsin-like site in the β subunit of the 20S core of the 26S proteasome complex [73]. When added to the medium of cortical mixed glial cultures (>90% GFAP+), it increased Nrf2 levels with an onset time of one hour and a peak effect at two hours, to a level that was five-fold that of vehicle-treated control cultures. HO-1 expression was significantly increased by 3 hours and remained elevated through the subsequent 13 hour observation period, yielding a six-fold increase over controls (Fig. 2) [74]. Cultures pretreated with MG132 were significantly protected from toxic concentrations of hemin. These results provided the first evidence that Nrf2 activation was protective in a model that was relevant to intracerebral hemorrhage. However, since Nrf2 regulates the expression of multiple antioxidant proteins as noted above, selectivity for HO-1 was not demonstrated.

Use of proteasome inhibitors in critically ill stroke patients may be limited by toxicity, which proceeds via multiple mechanisms and produces apoptotic, necrotic and autophagic cell death. This toxicity has led to development of these compounds as antitumor agents, with FDA approval of first-in-class bortezomib in 2008 to treat multiple myeloma [75, 76]. The search for alternate Nrf2 activators that are safe and effective has focused on compounds with a prior history of human use as pharmaceuticals for other indications or

Retinoic acid receptor-alpha (RAR α) agonists decrease transcription of ARE-regulated genes in an oxidative environment by facilitating the formation of Nrf2-RAR α complexes that cannot activate the ARE enhancer [89]. Consistent with the effect of Nrf2 knockout, rats treated with retinoic acid sustained greater neurological deficits after blood injection ICH, associated with reduced HO-1 expression [90, 91]. The multiple independent observations that ICH outcome was inversely related to Nrf2 expression and binding activity provided the rational basis for further testing of small molecule Nrf2 activators in ICH models.

Nrf2 activator testing in vivo

Protection by pre or post-injury treatment with Nrf2 activators has been reported in several acute CNS injury models, including both the collagenase and blood injection ICH models (Table 2). Of particular interest are the two compounds already in clinical use for other indications that may be repurposed for treatment of hemorrhagic stroke.

Hemin

Hemin has been in clinical use for several decades to treat attacks of acute porphyria [92]. It is usually administered at a dose of 3–4 mg/kg i.v. daily for four consecutive days, although longer courses have been described. The major adverse effects are phlebitis and a transient coagulopathy, which are likely not due to hemin itself but to degradation products that spontaneously form when the lyophilized compound is dissolved in water [93]. Both phlebitis and coagulopathy are attenuated or prevented when hemin is stabilized by reconstitution in an albumin solution. A single case of acute but reversible kidney injury has also been reported when a large dose (1000 mg) was rapidly infused [94].

Initial preclinical studies of hemin for treatment of acute CNS injury tested its effects in models of acute spinal cord injury and global ischemia after cardiac arrest. Yamauchi and colleagues [95] pretreated mice with 0.45 μ moles/kg (0.29 mg/kg) hemin i.p. 24 hours before a moderate spinal cord contusion injury. This single injection alone was sufficient to increase spinal cord HO-1 expression, which was localized to the vicinity of blood vessels; the specific cell populations upregulating HO-1 were not defined. At 24 hours after trauma, blood-spinal cord barrier disruption and neutrophil infiltration were both significantly reduced in hemin-pretreated animals compared with vehicle controls. Zhang et al. [96] subsequently pretreated rats with 23 μ moles/kg (15 mg/kg) hemin i.p. 12 hours before asphyxial cardiac arrest and resuscitation. Brain water content 1 hour after return of spontaneous circulation was significantly reduced in hemin-pretreated animals compared with untreated controls. Hemin pretreatment also increased CA1 neuronal survival at 4 and 14 days and improved neurological deficit scores.

These key studies demonstrated that peripheral administration of hemin before an acute traumatic or ischemic CNS insult reduced cell injury while improving barrier function and behavioral outcome. The clinical utility of hemin pretreatment is obviously quite limited, although it may be relevant to ischemia and trauma associated with vascular and neurosurgical procedures. Two subsequent studies suggest that initiating hemin therapy after acute traumatic spinal cord injury or ischemic stroke may offer benefit, although the time windows tested were brief. Diaz-Ruiz et al. treated rats with 0.45 μ moles/kg hemin i.p. at 2

and 8 hours after spinal cord contusion. This was sufficient to increase spinal cord HO activity 7.2-fold compared with vehicle-treated animals, and was associated with reduced tissue injury and improved motor scores [97]. Zhang et al. reported that hemin administered as a single 50 mg/kg dose one hour after permanent middle cerebral artery occlusion reduced mean infarct volume and neurological deficit scores compared with untreated controls [98].

Since a hematoma contains millimolar concentrations of heme, the benefit of administering hemin after ICH may not be apparent. Timing is likely the critical factor that determines its efficacy. After hemorrhage, heme is initially sequestered within the hydrophobic pockets of hemoglobin, which itself is sequestered within erythrocytes [15]. This heme is not available to provide a conditioning stimulus until erythrocytes lyse and cell-free hemoglobin oxidizes to methemoglobin, which has a lower affinity for its heme moieties and releases them to protein and lipid binding sites [18]. The entire process does not initiate for at least several hours and probably requires 2–3 days [99]. However, once it begins, toxic quantities of extracellular heme accumulate due to its very high concentration (~20 mM) within erythrocytes [19]. Delivery of a low, nontoxic concentration of hemin to perihematomal tissue prior to hemolysis provides a preconditioning stimulus, increasing cellular resistance to subsequent toxic hemin challenge. Since clinical ICH is rapidly symptomatic (headache, nausea, hemiparesis, altered mental status) and prompts patients to seek medical attention, this disease process may be particularly well-suited to a hemin conditioning approach.

Consistent with observations in spinal cord injury studies, mice injected on consecutive days with a single daily dose of hemin (26 mg/kg) increased brain HO-1 expression in perivascular cells [100]. Initiating treatment with hemin at 1–3 hours after experimental ICH modeled by either blood or collagenase injection improved barrier function as quantified by Evans blue and low molecular weight FITC dextran assays. Improvement was also noted in perihematomal cell viability, brain water content and neurological function. Hemin therapy therefore has consistent efficacy in hemorrhagic, ischemic, and traumatic CNS injury models.

Dimethyl fumarate

A mixture of fumaric acid esters including dimethyl fumarate (DMF) has been in clinical use since 1994 as an oral treatment for psoriasis (Fumaderm®), and an acceptable safety profile has been established with long-term use [101]. Purified DMF administered in a delayed-release formulation was subsequently demonstrated in Phase 3 clinical trials to reduce the relapse rate and MRI lesion frequency in patients with relapsing-remitting multiple sclerosis, while also increasing time to disability progression [102, 103]. Like Fumaderm®, adverse effects have been largely limited to flushing and mild to moderate gastrointestinal symptoms. However, fatal progressive multifocal leukoencephalopathy associated with moderate to severe lymphopenia has been reported in multiple sclerosis and psoriasis patients after long-term treatment with DMF [104, 105].

When administered orally, DMF is rapidly converted in the small bowel to monomethyl fumarate (MMF), its active metabolite [106]. DMF has similar efficacy when administered to rodents by either oral gavage or intraperitoneal injection [107], but safety of percutaneous

administration of either DMF or MMF has not been established. Fumaric acid esters are electrophilic compounds that covalently modify cysteine residues of Keap 1 via a Michael addition, particularly Cys 151 [108]. This modification stabilizes Nrf2 and promotes its nuclear translocation and transcription of HMOX1 and other antioxidant genes relevant to heme-mediated injury, including haptoglobin, hemopexin and H-ferritin [109]. Although the benefits of DMF have been linked to Nrf2 activation in several models, it also has potent immunomodulatory effects that are not mediated by Nrf2 activation, and it was equally protective in wild-type and Nrf2 knockout mice in an experimental autoimmune encephalomyelitis (EAE) model [110]. DMF regulates transcription via both Nrf2-dependent and Nrf-2 independent mechanisms, which may be tissue-specific [111].

DMF has been investigated in rodent ICH models by two research groups, with largely concordant results. Zhao et al. reported that i.p injection of 15 mg/kg DMF administered at two hours after striatal blood injection and then orally twice daily for three days reduced early neurological deficits and brain edema in rats, while increasing hematoma resolution [112]. In order to mechanistically link this effect with Nrf2 activation, additional experiments were conducted in wild-type and Nrf2 knockout mice. In wild-type mice, 15 mg/kg i.p. beginning 24 hours after blood injection and repeated daily for 3 days was sufficient to increase brain expression of several Nrf2 target genes at 48 hours after ICH, including HO-1, catalase, haptoglobin, and CD163, the receptor for hemoglobin and the hemoglobin-haptoglobin complex. Upregulation of these proteins was attenuated in Nrf2 knockout mice. Consistent with mitigation of the inflammatory response initiated by blood injection, DMF also reduced brain expression of iNOS and IL-1 β while increasing IL-10. Surprisingly, the latter effects were also observed in Nrf2 knockouts, again indicating a mechanism independent of Nrf2 activation. However, Nrf2 activation mediated the improvement in functional outcome in DMF-treated mice in the week after hemorrhage, since it was not observed in knockouts. The lengthy therapeutic window (24 hours) identifies DMF as an excellent candidate for further clinical development, if confirmed in other models.

Iniaqhe et al. evaluated low and high dose (10 mg/kg and 100 mg/kg) DMF in mice using both the collagenase and blood injection ICH models [113]. At the lower dose it was ineffective. However, 100 mg/kg i.p. administered one hour after striatal injection decreased neurological deficits and brain water content at 24–72 in both models. In the collagenase model, perihematomal microglial activation and expression of the adhesion molecule ICAM1 were reduced, and phosphorylated Nrf2 and MAFG were increased. However, in contrast to observations by Zhao et al., DMF did not accelerate hematoma resolution, which was evaluated only in the collagenase model. Casein kinase-2 inhibitors or MAFG siRNA pretreatment attenuated the effect of DMF on brain edema and neurological deficits, suggesting that it may be acting via casein kinase 2 phosphorylation of Nrf2.

Other Nrf2 activators

Beneficial effects of several Nrf2 activators in rodent ICH models have been reported. Some of these compounds were isolated from commonly consumed foods, and are currently in human use in an uncontrolled fashion as over-the-counter dietary supplements. However,

their safety after parenteral administration at the relatively high doses needed in rodent studies has not been established, and they do not offer the efficiencies inherent in repurposing of drugs that are approved for other indications, which are already manufactured in pharmaceutical grade.

Sulforaphane (SFN) is the electrophilic isothiocyanate breakdown product of glucoraphanin, which is found in cruciferous vegetables such as broccoli, cauliflower and Brussels sprouts. The physical damage produced by chewing mixes glucoraphanin with myrosinase, which catalyzes its hydrolysis. SFN is available in microgram quantities in health food stores and online, without any proven efficacy for any indication. When administered to cultured cells, it reacts with cysteine 151 of Keap1 and thereby blocks Keap1-mediated Nrf2 degradation [114]. Rats receiving 5 mg/kg i.p. at 30 min after striatal blood injection had significantly lower neurological deficit scores at 10 days, associated with increased Phase II enzyme expression and reduced protein and lipid oxidation [88]. Neurological deficits were also mitigated in wild-type but not Nrf2 KO mice. The efficacy of SFN was independently confirmed using a lower dose (2 mg/kg/day i.p.) and a rat blood injection ICH model [91].

Curcumin, an electrophilic polyphenol isolated from the rhizomes of *Curcuma longa* (turmeric), has been consumed in Asia for millennia as a curry spice and herbal medicine. Like SFN, it is available in capsule form as a dietary supplement, although bioavailability after oral intake is very poor [115]. Curcumin is a pleiotropic molecule that modulates multiple signaling pathways, accounting for its putative anti-inflammatory, antioxidant, antineoplastic and antimicrobial properties [116]. However, its induction of HO-1 appears to be primarily mediated by releasing Keap1 inhibition of Nrf2 nuclear import, likely by direct interaction with Keap1 cysteine residues [117]. King et al. reported that 150 mg/kg curcumin administered i.p. coincident with striatal collagenase injection attenuated edema, blood-brain barrier disruption, and neurological deficits in the first three days after ICH [118]. Hematoma volume was also reduced in both the collagenase and blood injection models, with a time window of three hours in the former. Using exclusively the blood injection ICH model, Sun et al. reported that curcumin administration at 15 minutes or 2 hours improved gait and neurological scores at 1–3 days, and also decreased blood-brain barrier injury and edema [119]. Subsequent studies independently confirmed these observations, and also demonstrated that curcumin reduced microglial activation, inflammatory cytokine production, lymphocyte infiltration and expression of aquaporin 4 and aquaporin 9 after experimental ICH [120–122].

Tert-butylhydroquinone (TBHQ) becomes electrophilic only when oxidized to tert-butylbenzoquinone, which then covalently binds to multiple cysteine residues of Keap1, activates Nrf2, and potently induces Phase II enzymes [123]. Unlike electrophilic Nrf2 activators, TBHQ does not interact with thiol groups or glutathione per se. It may therefore offer the advantage of less systemic toxicity, particularly if activated only or predominantly in the oxidative environment of its hemorrhagic target [124]. TBHQ 50 mg/kg i.p. divided into three doses over 24 hours beginning one hour after collagenase injection was sufficient to increase the DNA binding activity of Nrf2 in the mouse striatum [125]. Compared with vehicle, TBHQ treatment reduced perihematomal protein oxidation, microglial activation, IL-1 β expression, and neuronal degeneration, and improved neurological outcome.

Cellular targets of Nrf2 activators

SFN is a lipophilic, low molecular weight compound that is widely distributed after systemic administration, but low brain tissue concentrations indicate a limited ability to cross an intact blood-brain barrier [126]. Although it is capable of inducing a Phase II response in the rodent brain after i.p. administration, it appears to be selective for astrocytes. At 16 hours after injection of high dose (50 mg/kg i.p.) SFN, Jazwa et al. [127] reported a modest ~1.5 fold increase in striatal HO-1 protein expression in mice by immunoblot analysis. However, immunostaining demonstrated that this increase was completely limited to GFAP+ astrocytes, with no change in expression in endothelial cells, microglia, or dopaminergic neurons. Approximately 30% of striatal astrocytes expressed HO-1 at this time point, compared with < 2% of saline-treated controls. Using a lower SFN dose (5 mg/kg i.p.) administered 1 hour before middle cerebral artery occlusion (MCAo)-induced stroke in rats, Alfieri et al. reported that SFN increased HO-1 expression in perivascular astrocytes in tissue surrounding the infarction, enhancing the effect of ischemia alone [128]. SFN-treated rats sustained less blood-brain barrier disruption, lesion progression, and neurological deficits. Perivascular astrocyte expression of HO-1 after SFN 5 mg/kg was also observed by Zhao et al., although cortical neuron expression was also reported [129].

After parenteral administration, hemin binds to hemopexin or albumin and is largely cleared by the liver; only a minimal fraction is recovered from the brain [130]. However, this is apparently sufficient to induce HO-1 in the CNS, but expression is localized to the microvasculature [95, 100]. The latter studies did not determine the cell population expressing HO-1. We have recently observed that mice injected with 4 mg/kg hemin i.p. daily for 3 days overexpress HO-1 in perivascular astrocytes, mimicking the effect of SFN (Fig. 3).

The selectivity of Nrf2 activators for astrocytes is consistent with observations that the Nrf2 pathway is substantially more active in this cell population than in neurons (see Baxter and Hardingham [131] for recent review). In cortical cell cultures under normal conditions, Nrf2 expression in astrocytes exceeds that in neurons by 2–3 orders of magnitude [132]. It is therefore not surprising that astrocytes robustly induce HO-1 and ferritin after hemin or hemoglobin treatment, while weaker expression is observed in neurons [44, 69, 133]. In vivo, even the profound oxidative stress produced by CNS hemorrhage induces HO-1 that is largely limited to glial cells [134, 135]. Nevertheless, Nrf2 activators clearly protect neurons in a variety of stroke and neurodegenerative disease models (Table 2), see also [136, 137]. Nrf2 overexpression in astrocytes via GFAP promoter-driven transgene expression provides greater specificity than treatment with activators, and is likewise sufficient to protect neurons [138, 139]. The precise signaling pathways that mediate the neuroprotective effect of astrocytes on neurons have not yet been defined. Localization of HO-1 expression to perivascular astrocytes after parenteral administration of Nrf2 activators suggests a primary benefit to barrier or microvascular function after acute injury [95, 100, 128, 140, 141].

Astrocyte HO-1 overexpression improves ICH outcome

In addition to activating Nrf2-regulated signaling, all activators have multiple off-target effects. Both agents currently in clinical use, hemin and DMF, bind nonselectively to thiol

groups, a property which has been utilized in experimental models to deplete functional glutathione [142–144]. Adding to this nonspecificity, Nrf2 activation per se may regulate the expression of up to 600 target genes, based on ChIP-Seq and microarray analyses [145]. Consistent with these analyses, administration of hemin or DMF to mice alters the transcription of a plethora of genes via both Nrf2-dependent and independent mechanisms [111, 146]. Although astrocyte HO-1 overexpression and Nrf2 activators have similar effects on heme-mediated injury in vitro [36, 74], other proteins may contribute or predominate in a more complex in vivo environment.

In order to determine if selective astrocyte HO-1 per se is protective after ICH, transgenic mice expressing human HO-1 driven by the GFAP promoter were tested in the blood injection ICH model [147]. HO-1 expression in ipsilateral striata at 7 hours after blood injection was increased over sevenfold in transgenics, and was localized predominantly to perivascular astrocytes. A dramatic reduction in mortality (34.8% v. 0%) was observed after experimental ICH in transgenics, associated with increased striatal cell viability and reduced blood-brain barrier disruption and neurological deficits. These observations demonstrate that astrocyte HO-1 overexpression per se is robustly protective after ICH, and highlight the therapeutic potential of drug therapies that activate the Nrf2-HO-1 axis in this cell population.

Conclusion

Considerable experimental evidence indicates that systemic treatment with Nrf2 activators after ICH is sufficient to increase expression of HO-1 and other Phase II proteins that may attenuate perihematomal injury cascades (Fig. 4). Nrf2 activators have been tested in eight preclinical ICH trials to date, using both the blood injection and collagenase models in rats and mice. All eight have reported positive effects, providing a compelling proof of concept. Two of the agents used in these studies, dimethyl fumarate and hemin, are already approved for use in other clinical conditions, and pharmaceutical-grade products are available to facilitate the repurposing of these compounds. Future studies should focus on establishing their optimal dose, time window, and treatment duration in rodent and large animal ICH models. Given the poor prognosis and limited therapeutic options available to ICH patients, and the acceptable safety profile of both DMF and hemin established over many years of use, progression to clinical trials should receive the highest priority.

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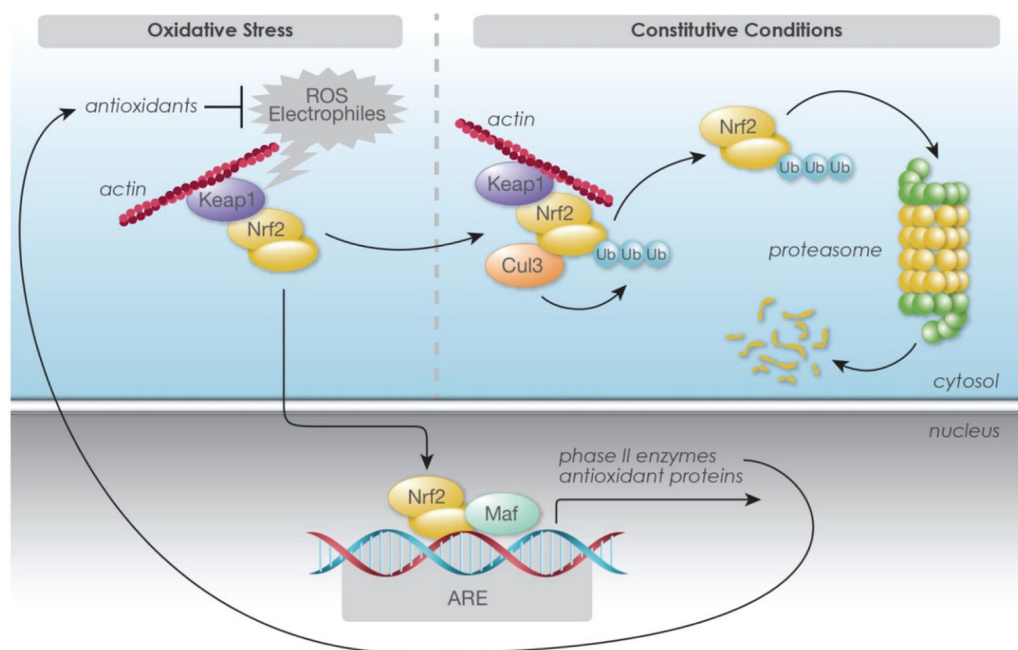


Figure 1. Schematic representation of Nrf2 activation by oxidative or electrophilic stress. Under constitutive conditions, Nrf2 binds to kelch-like ECH-associated protein 1 (Keap1) and is sequestered in the cytoplasm, where it is ubiquitinated and degraded. Reactive oxygen species (ROS) or electrophiles interact with Keap1 cysteine residues and mediate dissociation of Nrf2-Keap1 complex. This results in ubiquitination and proteasomal degradation of Keap1, while Nrf2 is stabilized and translocated to the nucleus. After heterodimerization with other transcription factors such as Maf, it binds to antioxidant response elements (ARE) in the promoter regions of heme oxygenase-1 and other target genes and activates transcription. ©Cayman Chemical, with permission.

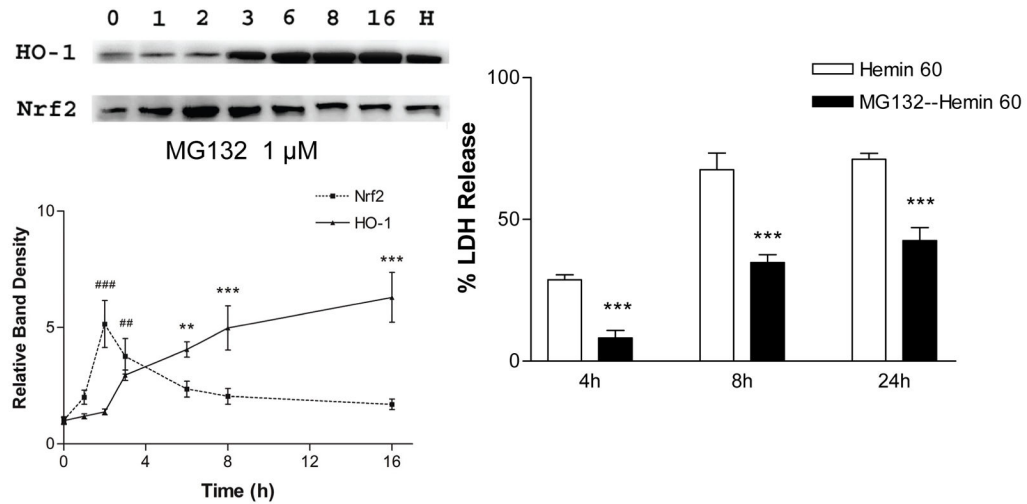


Figure 2.

Proteasome inhibitor MG132 increases HO-1 expression and protects astrocytes from hemin. Glial cultures (>90% GFAP+) were treated with 1 μM MG-132 for indicated intervals; HO-1 and Nrf2 protein levels in lysates were then detected by Western blot analysis. “H” is protein from culture exposed to hemin (positive control). Glia pretreated with MG132 for 16 hours sustained less cell death after 4–24 h exposure to 60 μM hemin. From Chen and Regan, *Current Neurovascular Research* 2(3):189–96, with permission.

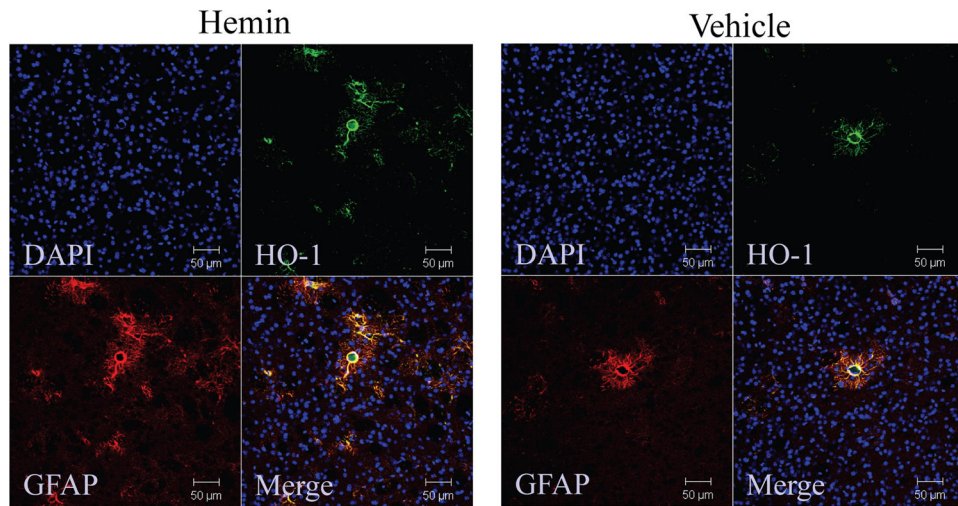


Figure 3. Systemic hemin treatment increases astrocyte HO-1 expression. Sections from mice treated with 4 mg/kg hemin or vehicle i.p. daily for 3 days, stained with antibodies to HO-1 and GFAP.

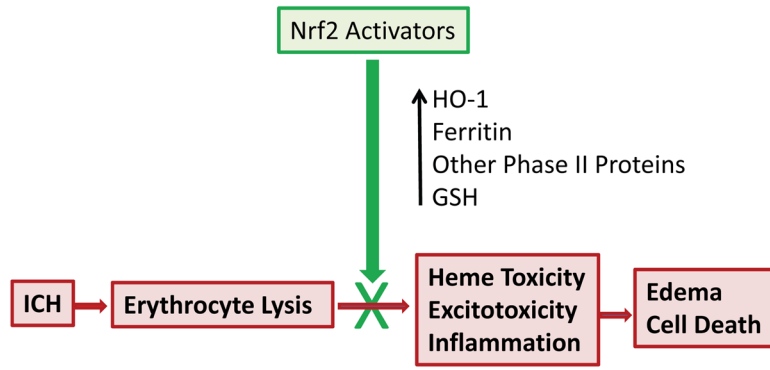


Figure 4. Summary of effects of Nrf2 activators on injury mediated by ICH and subsequent erythrocyte lysis.

Table 1

Partial list of proteins upregulated by Nrf2 activation that may be relevant to intracerebral hemorrhage.

Protein	Activity
Heme oxygenase-1	Heme breakdown, CO and bilirubin production
H and L-Ferritin	Sequester and detoxify free iron
Haptoglobin	Binds hemoglobin
Metallothioneins	Metal-binding and detoxification
Thioredoxins	Direct antioxidants
Glutamate-cysteine ligase	Glutathione synthesis
Glutathione reductase	Reduces oxidized glutathione
Glucose-6-P dehydrogenase	NADPH generation
Catalase	Direct antioxidant
Superoxide dismutase	Direct antioxidant

Table 2

Studies testing Nrf2 activators in rodent blood injection or collagenase ICH models, demonstrating consistent benefit. TBHQ: tert-butylhydroquinone

Drug	Species	Model	Reference
Sulforaphane	Rat, Mouse	Blood Inj	Zhao et al, Stroke 38:3280–6, 2007
Curcumin	Mouse	Both	King et al., J Neurosurg 115:116–23, 2011
Curcumin	Mouse	Blood Inj	Sun et al, J Neurotrauma 28:2513–21, 2011
Hemin	Mouse	Both	Lu et al, Neurobiol Dis 70:245–51, 2014
Dimethyl fumarate	Rat, Mouse	Blood Inj	Zhao et al, Stroke 46:1923–8, 2015
Dimethyl fumarate	Mouse	Both	Iniaghe et al., Neurobiol Dis 82:349–58, 2015
Sulforaphane	Rat	Blood Inj	Yin et al, Drug Dis Dev Ther 9:5973–86, 2015
TBHQ	Mouse	Collagenase	Sukumari-Ramesh et al, J Mol Neurosci 58:525–531, 2016

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