Targeting heme oxygenase after intracerebral hemorrhage

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Intracerebral hemorrhage (ICH) is the primary event in approximately 10% of strokes, and has higher rates of morbidity and mortality than ischemic stroke. Experimental evidence suggests that the toxicity of hemoglobin and its degradation products contributes to secondary injury that may be amenable to therapeutic intervention. Hemin, the oxidized form of heme, accumulates in intracranial hematomas to cytotoxic levels. The rate limiting step of its breakdown is catalyzed by the heme oxygenase (HO) enzymes, which consist of inducible HO-1 and constitutively-expressed HO-2. The effect of these enzymes on perihematomal injury and neurological outcome has been investigated in ICH models using both genetic and pharmacological approaches to alter their expression, with variable results reported. These findings are summarized and reconciled in this review; therapeutic strategies that may optimize HO expression and activity after ICH are described.

Keywords: intracerebral hemorrhage; iron; oxidative stress; stroke; subarachnoid hemorrhage

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Introduction

Intracerebral hemorrhage (ICH) deposits a ~10 mM suspension of hemoglobin-bound heme into the brain parenchyma. Although initially sequestered within erythrocytes, some will be released and account for the high micromolar concentration of oxidized heme (hemin) present in one week old intracranial hematomas [1]. The mechanisms responsible for this hemin accumulation to cytotoxic levels have not been precisely defined. However, brain tissue is clearly a poor microenvironment for erythrocyte survival. Whether due to complement activation [2], heme-mediated oxidative stress [3] or other mechanisms, they begin to lyse within a few days in rodent models and in vitro [4, 5]. Extracellular hemoglobin is unstable at physiological temperatures and spontaneously oxidizes at its heme groups to ferric methemoglobin, releasing a superoxide ion in the reaction [6]. The affinity of methemoglobin for its heme moieties is relatively weak, and it readily transfers them to higher-affinity lipid and protein binding sites [7]. In the CNS, the low concentration of the heme/hemin binding protein hemopexin (~5 µg/ml [8]) increases the likelihood of hemin uptake by vulnerable cell populations such as neurons and astrocytes. Some hemoglobin in the hematoma will be scavenged by microglia and infiltrating macrophages, which phagocytose intact erythrocytes and also take up hemoglobin and hemoglobin-haptoglobin complexes via the CD163 receptor [9, 10]. The fate of hemoglobin-bound heme after uptake by these cells remains undefined. However, when intracellular free heme or hemin is present in excess, it is very likely that at least some will be ejected into the extracellular space by the exporter protein FLVCR1 [11, 12], and will then be available to participate in free radical reactions.

The accumulation of hemin to toxic concentrations within a hematoma provides an obvious therapeutic target after ICH. The goal of one approach is optimization of hemin catabolism. The latter is catalyzed by the heme oxygenase (HO) enzymes, resulting in production of equimolar iron, carbon monoxide, and biliverdin, which is then converted to bilirubin via
biliverdin reductase \[^{13}\] . Two HO isoforms are present in mammalian cells. HO-1 is expressed at a very low level in the healthy CNS, but it is rapidly induced by hemin and a variety of other oxidants, as well as heat shock. HO-2 is constitutively expressed and is the predominant neuronal isoform; it accounts for most HO activity under normal conditions \[^{14}\] . Investigation of the effect of these enzymes on cell injury and outcome after ICH has been limited by a lack of specific and selective inhibitors, necessitating the use of genetically modified mice in mechanistic studies.

**Effect of HO-2 knockout.**

Mice lacking the HO-2 gene were initially produced two decades ago by Poss and Tonegawa \[^{15}\] . Their descendants have been used to evaluate the effect of HO-2 in studies that are directly relevant to ICH. HO-2 gene deletion per se has no significant effect on breeding, fetal viability, or postnatal growth, all of which are grossly indistinguishable from their wild-type counterparts. It has consistently altered outcome after experimental ICH, although in a direction that varies with the model used.

**Experimental models of ICH.** Two ICH models are currently in use, but their relevance to clinical ICH is a source of ongoing controversy. ICH is induced by stereotactic injection of either autologous blood or clostridial collagenase, which disrupts local blood vessels. The former model allows for more precise control of hematoma volumes. However, backflow of blood through the needle entry site is a potential source of variability, particularly with injection of more than 15 µl in mice. The major criticism of this model is that it fails to simulate clinical ICH, since the hematoma is not produced by hemorrhage in situ \[^{16}\] . The collagenase model addresses that concern, but raises others. Since a bacterial protein is injected, a local inflammatory response is possible, although published data quantifying inflammation in the absence of hemorrhage (e.g. by injecting an inactivated enzyme) are lacking. Another concern is that disruption of the microvascular network in the vicinity of the injection site by collagenase digestion may reduce local blood flow sufficiently to produce ischemia, which does not appear to be a prominent feature of clinical ICH and has not been observed when ICH was modeled by blood injection\[^{17, 18}\] . MacLellan et al. \[^{19}\] reported that the collagenase model produced more rapid and pronounced breakdown of the blood-brain barrier, greater injury to adjacent tissue, and more severe and persistent neurological deficits than the blood injection model.

**HO-2 KO reduces cell injury in the blood injection ICH model.** Stereotactic injection of blood into the mouse striatum is followed by reduced viability of perihematomal cells over the following four days, as detected by ex vivo MTT assay or quantification of striatal fluorescence in mice expressing the red fluorescent protein dTomato in central neurons. The validity of these methods has been established by their close agreement with cell counts of histological sections guided by design-based stereology \[^{20}\] . Compared with wild-type mice, HO-2 knockouts sustained significantly less cell loss at four days, a discrepancy that remained constant when the observation period was extended to 8 days\[^{21}\] . HO-2 KO also significantly reduced striatal protein and lipid oxidation, as well as striatal expression of HO-1, which is induced by oxidative stress due to the presence of antioxidant response elements in its promoter region \[^{22}\] . Very similar results were observed when mice were injected with an autologous solution of stroma-free hemoglobin rather than blood, suggesting that similar mechanisms mediate cell injury in these two models \[^{23}\] . However, the protection provided by HO-2 was associated with only a weak and inconsistent improvement in neurological outcome\[^{21}\] .

In *vitro* studies. HO-2 KO also protected neurons from hemoglobin or hemin in cell culture \[^{24, 25}\] . This effect could be mimicked by treatment with deferoxamine or apotransferrin, which bind iron with very high affinity and in a manner that reduces or prevents its participation in free radical reactions \[^{26, 27}\] . These observations support the hypothesis that HO-2 is deleterious to neurons exposed low micromolar hemoglobin or hemin concentrations due to iron release as a consequence of heme/hemin breakdown. This effect may be specific to neurons, which are very sensitive to iron due at least in part to low expression of the iron sequestering protein ferritin \[^{28, 29}\] . HO-2 KO astrocytes, which are capable of rapid ferritin upregulation, are conversely more vulnerable to hemin than their wild-type counterparts \[^{30}\] . Cultured HO-2 KO neurons are more vulnerable to inorganic iron, perhaps due to the protective effect of the other products of heme breakdown, i.e. biliverdin/bilirubin and carbon monoxide, when exposure to iron was equalized.

In addition to toxicity mediated by iron release, hemin can directly injure cells by oxidative and membrane destabilizing effects \[^{31, 32}\] . These mechanisms will predominate if iron toxicity is mitigated by concomitant chelator administration. When Wang et al. treated cultured neurons with hemin in medium containing iron-poor transferrin, they observed that HO-2 KO increased cell death \[^{33}\] . The net effect of HO-2 therefore appears to be dependent on the iron binding capacity of the cellular microenvironment. When cells are exposed to equal concentrations of redox-active iron, then HO-2 is protective.

**HO-2 KO worsens outcome in the collagenase ICH model.**
In contrast to its protective effect in the blood injection ICH model, HO-2 KO increased perihematomal lesion volume, neuroinflammation, and edema as measured by hemisphere volume after striatal collagenase injection; however, tissue water content was not altered \cite{33, 34}. Neurological deficits were also more severe in the first three days after hemorrhage. Recent studies in our laboratory have confirmed the latter finding, although the effect was somewhat less robust \cite{20}. Also in contrast to its effect in the blood injection ICH model, HO-2 KO had no effect on striatal neuron loss or tissue viability in the collagenase ICH model \cite{20}. These disparate results suggest the predominance of different injury mechanisms in the collagenase and blood injection ICH models. A plausible but still speculative explanation for this discrepancy is that the protective effect of HO-2 KO on heme-mediated neurotoxicity is counteracted in the collagenase model by a deleterious effect on other injury mechanisms, such as inflammation, ischemia, or iron neurotoxicity.

**Effect of HO-1 KO.**

In contrast the grossly healthy phenotype of HO-2 KO mice, unconditional HO-1 knockouts breed poorly and have a very high pre- and perinatal mortality. Due primarily to this limited availability, only one published study has evaluated the effect of HO-1 gene deletion on outcome after experimental ICH. Using the collagenase model, Wang and Doré reported smaller lesion volumes and reduced neuroinflammation in knockouts than in wild-type mice \cite{35}. Early neurological deficits were also mitigated, but this effect was statistically significant only on day 1. The effect of HO-1 in the collagenase model was therefore the opposite of HO-2. Although these enzymes catalyze the same reaction, their expression differs markedly in both location and timing after ICH, which may account for their divergent effects. As noted above, neurons constitutively express HO-2 but little HO-1, with little change after ICH. Astrocytes, microglia, and endothelial cells robustly increase HO-1 expression after hemoglobin or hemin exposure. Delineation of the effect of HO-1 and HO-2 knockout or overexpression in specific cell populations after ICH seems a worthy topic for further investigation.

**Pharmacological Studies**

**HO Inhibitors.** A variety of non-iron heme analogs that competitively inhibit the activity of both HO-1 and HO-2 are commercially available. These are reactive compounds with significant off-target effects, including inhibition of nitric oxide synthase and soluble guanylyl cyclase inhibition, albeit at higher concentrations than that needed for HO inhibition\cite{46}. Another drawback to the use of these compounds as mechanistic probes is that they may induce HO-1 \cite{37}, raising the possibility that post-treatment HO activity may be increased compared with vehicle-treated controls as local drug levels decline. Furthermore, HO-1 protein may be protective even if catalytically inactive by upregulating antioxidant gene expression \cite{38}. With the caveat that the relationship between brain or systemic administration of these compounds and perihematomal HO activity has never been established, porphyrin HO inhibitors have been consistently protective in blood injection ICH models. Wagner et al. reported that tin mesoporphyrin injected with autologous blood into pig frontal white matter reduced edema at 24 hours compared with DMSO vehicle controls \cite{39}. A similar effect was produced in rats receiving striatal infusions of hemoglobin containing tin protoporphyrin \cite{40}. Using a more translational approach, Gong et al. observed that zine protoporphyrin, administered as a continuous i.p. infusion via osmotic pump, reduced brain edema, atrophy, ventricular enlargement and neurological deficits in a rat autologous blood ICH model, with a time window of 6 hours \cite{41}. Efficacy in a collagenase ICH model has not yet been described.

**Perivascular HO-1 induction.** Hemin in a sodium carbonate buffer for intravenous infusion (hematin) has been in clinical use in the USA for decades to treat attacks of acute porphyria\cite{42}; hemin arginate has been marketed in Europe for the same indication. After injection, hemin binds with extraordinary affinity to hemopexin \cite{43}, or to albumin when hemopexin binding sites are saturated \cite{44}. In vitro, both complexes are capable of inducing HO-1 at a somewhat reduced rate than hemin per se and with markedly less toxicity \cite{45, 46}. Systemically administered hemin has little or no access to the CNS parenchyma, but induces HO-1 in perivascular cells \cite{47}. We have recently reported that mice treated with hemin i.p. after striatal collagenase or blood injection sustained less blood-brain barrier breakdown, perihematomal cell loss, and neurological deficits than mice receiving vehicle, with a time window of at least 3 hours \cite{48}. Similar protection has also been provided by hemin treatment before spinal cord trauma \cite{47}, as well as focal and global brain ischemia \cite{49, 50}, although it is unknown if post-injury therapy has any benefit in these models.

**Conclusions**

The variable effects of HO-1 and HO-2 in models relevant to ICH suggest that they are challenging therapeutic targets. Given the multiple injury cascades initiated by clinical ICH \cite{51}, demonstration of efficacy in only one ICH model is an insufficient recommendation for further development of drug or genetic therapies. Administration of HO inhibitors, for example, may attenuate neuron loss due to iron release as heme/hemin is catabolized, while also increasing the
vulnerability of neurons and other cell populations to the toxicity of hemin itself and enhancing the inflammatory response. Maximal benefit may require a combinatorial approach using two or more agents to increase HO activity while detoxifying iron with chelators or antioxidants. Therapies focused on increasing HO-1 expression in perivascular cells appear to be particularly promising given the consistent benefit of systemic hemin after striatal blood or collagenase injection, and the protective effect that has already been demonstrated in other acute CNS injury models.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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References

28. Kress GJ, Dineley KE, Reynolds J. The relationship between intracellular free iron and cell injury in cultured neurons,


