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Astrocyte heme oxygenase-1 reduces mortality and improves outcome after collagenase-induced intracerebral hemorrhage

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ABSTRACT

Pharmacotherapies that increase CNS expression of heme oxygenase-1 (HO-1) and other antioxidant proteins have improved outcome in experimental models of spontaneous intracerebral hemorrhage (ICH). In order to more specifically investigate the relationship between HO-1 and ICH outcome, mice expressing human HO-1 driven by the glial fibrillary acidic protein (GFAP) promoter (GFAP·HMOX1 mice) were tested in a model of in situ parenchymal hemorrhage. Injection of collagenase into the striata of wild-type (WT) mice resulted in a 26.3% mortality rate, with deaths equally distributed between males and females. Mortality was reduced to 4.48% in GFAP·HMOX1 mice. Cell viability in the injected striata of surviving WT mice was reduced by about half at one week and was significantly increased in transgenics; this benefit persisted over a 22 day observation period. Cell counts guided by design-based stereology indicated loss of ~40% of neurons in WT hemorrhagic striata at one week, which was decreased by half in transgenics; no significant differences in microglia or astrocyte numbers were observed. Blood-brain barrier disruption and short-term neurological deficits were also mitigated in GFAP·HMOX1 mice, but long-term outcome did not differ from that of WT survivors. These results suggest that astrocyte HO-1 overexpression provides robust neuroprotection after acute intracerebral hemorrhage. Further investigation of drug or genetic therapies that selectively increase astrocyte HO-1 is warranted.

1. Introduction

Spontaneous intracerebral hemorrhage (ICH) accounts for about 10% of strokes, and continues to have a grim prognosis despite improvements in neurointensive care (Rincon and Mayer, 2013). One month mortality approximates 50%; recovery to independent living status is attained in only 20% of survivors (Broderick et al., 1999), and the vast majority report poor health-related quality of life (Christensen et al., 2009; Delcourt et al., 2017). The mechanisms mediating perihematomal cell loss have not been precisely defined, but considerable experimental evidence supports the participation of oxidative and inflammatory injury cascades that may be amenable to targeted pharmacotherapies. One promising target is heme oxygenase-1 (HO-1), which catalyzes the rate-limiting step of heme breakdown to carbon monoxide, iron and bilirubin. HO-1 robustly protects astrocytes from heme-mediated injury in vitro (Benvenisti-Zarom and Regan, 2007), and may have beneficial effects on heme clearance in vivo (Parfenova et al., 2005; Schallner et al., 2015). Conversely, an increase in the early inflammatory response has also been observed after ICH when expression is primarily localized to microglia and macrophages (Wang and Doré, 2007).

HO-1 is minimally expressed in the CNS under physiological conditions, but it is induced by oxidative stress via Nrf2-regulated transcriptional activation. In rodents, systemic treatment with low molecular weight Nrf2 activators increased CNS HO-1, with expression localized most prominently to perivascular astrocytes (Alfieri et al., 2013; Jazwa et al., 2011; Yamauchi et al., 2004). This treatment was sufficient to attenuate cell loss, blood-brain barrier breakdown, inflammation, and neurological deficits after ICH in wild-type (WT) mice, while Nrf2 knockout prevented any benefit and worsened injury (Iniaghe et al., 2015; King et al., 2011; Wang et al., 2007; Zhao et al., 2007). However, since Nrf2 regulates the expression of multiple antioxidant and anti-inflammatory proteins, the relationship between HO-1 induction and protection cannot be precisely defined by these experiments.

Abbreviations: GFAP, glial fibrillary acidic protein; HO, heme oxygenase; ICH, intracerebral hemorrhage; MTT, Methylthiazolyldiphenyl-tetrazolium; Nrf2, nuclear factor erythroid 2-related factor 2; Tg, transgenic.

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The use of transgenic mice provides a more specific method to test the effect of HO-1 per se on injury and outcome after ICH. In a prior study, HO-1 overexpression that was restricted to GFAP+ astrocytes markedly reduced mortality and behavioral deficits after striatal injection of autologous blood (Chen-Roetling et al., 2015). In light of these robust results, determination of the consistency of this effect across ICH models seemed warranted. In the present study, ICH was induced in WT and transgenic mice by striatal injection of bacterial collagenase, which disrupts multiple vessels and produces a more severe injury than stereotactic blood injection (MacLellan et al., 2008). In addition to mortality, perihematomal cell viability, neurological deficits, and histological endpoints were quantified.

2. Materials and methods

2.1. Animals

Mice expressing human HO-1 in GFAP+ cells (GFAP·HMOX1 mice, FVB background) were descended from founding pairs provided by the Schipper lab (Song et al., 2012b). They were bred and housed with control WT mice in the Thomas Jefferson University Laboratory Animal Facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All breeding and experimental protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023). Genotype was verified by PCR using previously-published primers (Chen-Roetling et al., 2015). HO-1 overexpression specifically in astrocytes has previously been confirmed by double immunofluorescence staining (Chen-Roetling et al., 2015). Mice were used for experiments when 3–6 months old.

2.2. Striatal collagenase injection

Collagenase (Sigma-Aldrich C9572) was reconstituted from the lyophilized product (0.1 mg/33 μl), aliquoted, and stored at −70 °C for up to one month prior to use. Careful handling of collagenase dilutions (0.04 units/μl) was necessary to avoid loss of activity, as previously described in detail (Chen-Roetling et al., 2013). Mice were anesthetized with 1.5% isoflurane in oxygen and were placed into a stereotaxic frame. Temperature was continuously monitored using a rectal probe and maintained at 37 ± 0.5 °C with a heating lamp. Using aseptic technique, the skin over the skull was incised and bregma was identified. A burr hole was then made 2.5 mm to the right and 0.5 mm anterior to bregma, and 30 gauge needle with attached glass syringe was inserted to a depth of 3 mm. Collagenase (0.04 units in 1 μl) was then injected at a rate of 0.6 μl per minute. The needle was withdrawn 1 mm 4 min later, and was removed completely after another 4 minute pause. Surgical control mice were subjected to anesthesia and needle trauma only. After wound repair, mice recovered in a warm environment.

2.3. Hematoma volume

In order to determine if hemorrhage volume after collagenase injection differed in WT and GFAP·HMOX1 mice, hematoma volume was quantified by high frequency ultrasound imaging, using the Vevo 2100 high frequency ultrasound imaging scanner (FujiFilm VisualSonics, Toronto, Ontario) as previously described (Stanczak et al., 2015).

2.3.1. Outcome assessment

All testing was conducted by observers blinded to mouse genotype.

2.4. Methylthiazolyldiphenyl-tetrazolium (MTT) assay

Striatal cell viability was quantified by MTT assay, as previously described and validated (Chen-Roetling et al., 2013). At 7 and 22 days after collagenase injection, mice were euthanized under deep isoflurane anesthesia. Injected and contralateral striata were rapidly excised under a dissecting microscope and dissociated by trituration in 1 ml Hanks Balanced Salt Solution supplemented with 27.5 mM glucose, 20.5 mM sucrose and 4.2 mM sodium bicarbonate. MTT solution (0.25 mg in 1 ml) was added and the cell suspension was incubated for 4 min at 37 °C. After low speed centrifugation, the supernatant was discarded and formazan was extracted in 2 ml isopropanol. Formazan absorbance (562 nm) was quantified and expressed as a percentage of that in contralateral striata.

2.5. Histology

Under isoflurane anesthesia, mice were perfused with 5 ml isotonic saline followed by 50 ml 4% paraformaldehyde, followed by postfixation and cryopreservation in 20% sucrose. Sectioning and immunostaining were conducted by FD Neurotechnologies, Inc., Columbia, MD, USA as previously described (Chen-Roetling et al., 2013), using the following antibodies: anti-NeuN, Millipore, Billerica, MA; Cat. No. MAB377B, 1:1000 dilution; anti-Iba1, Wako Chemicals USA, Richmond, VA, Cat. No. 019-19741, 1:10,000; anti-GFAP, Invitrogen, Carlsbad, CA, Cat. No. 13-0300, 1:30,000. Unbiased counting of immunoreactive cells was guided by the *Stereologer* system (Stereology Resource Center, Chester, MD) (Long et al., 1998), using the optical fractionator method (Chen-Roetling et al., 2013; West, 1993).

2.6. Blood-brain barrier permeability assay

Mice were injected with 2% Evans blue as previously described (Lu et al., 2014). Evans blue binds to plasma albumin and is a marker of protein leakage across the blood-brain barrier. After saline perfusion, striata were excised and Evans blue was extracted following an established protocol (Uyama et al., 1988). Fluorescence (ex: 620 nm, em: 680 nm) of the solution was then measured.

2.7. Neurological deficit testing

A neurological deficit score was calculated daily for the first week after hemorrhage and weekly thereafter via the method of Huang et al. (1994), which minimizes animal disturbance while assigning scores as follows: 0: nl; 1: contralateral flexion when mice lifted by tail; 2: circling right but normal rest posture; 3: leftward leaning at rest; 4: no spontaneous movement. Neurological deficits were also assessed weekly with the adhesive removal and corner tests, as previously described (Chen et al., 2011).

2.8. HO-1 expression assays

Striata were removed and placed into 500 μl RIPA Lysis and Extraction Buffer (Thermo Fisher Cat. No. 89900) containing protease inhibitor cocktail (Sigma Aldrich P8340). Human and mouse HO-1 expression were quantified using species-specific ELISA kits (Enzo Life Sciences, Cat. No. ADI-960-071 and ADI-EKS-800).

2.9. HO activity assay

Striata were excised, sonicated in ice-cold potassium phosphate buffer, and centrifuged (13,000 ×*g*, 2 min). Supernatant samples (40 μg protein) were immediately placed into septum-sealed vials containing 25 μM hemin and 1.5 mM NADPH (total volume 120 μl). Vial headspace air was purged and replaced with air that had passed through a catalytic converter to remove ambient CO; reaction was run at 37 °C for 15 min and then was quickly stopped by freezing on dry ice. Headspace CO was quantified by gas chromatography (Peak Laboratories, Mountain View, CA, USA).