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Systemic hemin therapy attenuates blood–brain barrier disruption after intracerebral hemorrhage

Xiangping Lu, Jing Chen-Roetling, Raymond F. Regan *

Department of Emergency Medicine, Thomas Jefferson University, 1025 Walnut Street, College Building Room 813, Philadelphia, PA 19107, United States

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ABSTRACT

Injury to the blood–brain barrier (BBB) is a key feature of intracerebral hemorrhage (ICH) and may contribute to perihematomal cell injury. Pretreatment with the heme oxygenase (HO)-1 inducer hemin improves barrier function and neurological outcome in experimental models of traumatic and ischemic CNS injury. Since hemin is already in clinical use to treat acute porphyrias, this translational study was designed to test its effect on BBB function when initiated after ICH in two mouse models. At a dose similar to those used in most preconditioning studies (26 mg/kg i.p.), post-hemorrhage treatment with hemin reduced parenchymal extravasation of Evans blue by about three-quarters in both the blood injection and collagenase ICH models. Similar efficacy was observed when treatment was begun at 1 or 3 h. At the lower dose that is currently in clinical use (4 mg/kg beginning at 3 h), hemin also improved barrier function in both models, as assessed by both Evans blue and FITC-dextran leakage; however, it was somewhat less potent, reducing Evans blue leakage by about half. This dose was nevertheless sufficient to attenuate striatal cell loss and accelerate neurological recovery. Consistent with prior observations, striatal HO-1 expression was increased by hemin, and was localized to perivascular cells. These results suggest that hemin may be an effective therapy for ICH with a clinically relevant time window. Further study of the repurposing of this old drug seems warranted.

Introduction

Hemin, the ferric form of heme with a chloride ligand, accumulates in intracranial hematomas (Letarte et al., 1993). It may contribute to cell injury in adjacent tissue by a direct cytotoxic effect and also by release of redox-active iron with its breakdown (Robinson et al., 2009). However, at nontoxic concentrations, hemin delivers a potent preconditioning stimulus to cultured cells that protects them from subsequent exposure to toxic concentrations of hemin and other oxidants (Balla et al., 1992; Li et al., 2009; Regan et al., 2000). This effect, which requires a preconditioning interval of several hours (Balla et al., 1993), is mediated at least in part by induction of heme oxygenase (HO)-1 (Belcher et al., 2006; Li et al., 2009). In vivo, systemic pretreatment with hemin was protective in multiple acute injury models, including brain (Takizawa et al., 1998; Zhang et al., 2008), heart (Clark et al., 2000; Hangaishi et al., 2000), kidney (Demirogullari et al., 2006), liver (Xue et al., 2007), and gut (Attuwaybi et al., 2004) ischemia/reperfusion, spinal cord trauma (Yamauchi et al., 2004), colitis (Zhong et al., 2010), and pancreatitis (Habtezion et al., 2011). Hemin is excluded from most organs after parenteral injection due to rapid binding to hemopexin or albumin (Linden et al., 1987), and its primary site of action appears to be the microvasculature (Belcher et al., 2006; Holzen et al., 2008; Lindenblatt et al., 2004). In the CNS, this resulted in a significant attenuation of blood–spinal cord barrier permeability in a mouse weight-drop contusion model (Yamauchi et al., 2004).

Hematin, which is ferric heme with a hydroxide ligand rather than chloride, is currently FDA-approved to treat neurovisceral symptoms associated with the acute porphyrias. A related compound, hemin arginate, is marketed for the same indication in Europe. Although the putative mechanism of action in these diseases is feedback inhibition of δ-aminolevulinic acid synthase rather than HO-1 induction (Anderson et al., 2005), the safety of ferric heme has already been established by decades of clinical use. It is somewhat surprising, then, that the therapeutic potential of hemin or hematin postconditioning has not been addressed in translational studies to date. One possible explanation is an inadequate time window. Since hemin is not directly protective, but rather upregulates an endogenous defense against oxidative stress and inflammation, its onset of action is likely to be delayed for several hours. It therefore may not be an ideal agent for treating rapidly-progressing insults such as CNS ischemia or trauma.

Two factors suggest that parenteral hemin may be a particularly attractive drug therapy for intracerebral hemorrhage (ICH). First, the heme-mediated component of injury after ICH is delayed for at least 1–2 days in experimental models (Xi et al., 1998), providing an adequate time window to manifest the protective effects of hemin conditioning. This delay represents the interval required for both

* Corresponding author. Fax: +1 215 923 6225.
E-mail address: Raymond.Regan@jefferson.edu (R.F. Regan).
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erythrocyte lysis and hemoglobin oxidation to methemoglobin, which has a lower affinity for its heme moieties and releases them to lipid and protein binding sites (Bunn and Jandl, 1968). Second, blood–brain barrier breakdown is a prominent feature of ICH, is hypothesized to contribute to its poor neurological outcome, and is a likely target of systemic hemin therapy (Keep et al., 2008; Yamauchi et al., 2004). However, no publication to date has tested hemin in any model that is relevant to spontaneous ICH. The goal of this translational project was to assess the effect of hemin therapy when treatment was begun after ICH, specifically testing the hypotheses that it would improve blood–brain barrier function and neurological outcome.

Materials and methods

Experimental animals

All experiments were conducted using protocols that were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Swiss-Webster mice (138 male and 98 female) were purchased from Taconic. They were housed in a facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and were used for experiments when 11–13 weeks old. Animals were provided with food and water ad libitum throughout the course of all experiments.

ICH models

Mice were anesthetized with 2% isoflurane in oxygen and were placed into a stereotactic frame (David Kopf Instruments). Collagenase (Type VIIS, Sigma-Aldrich, St. Louis, MO) and blood injections followed previously described procedures (Chen et al., 2011). A 28–33 g needle attached to a glass Hamilton syringe was inserted into the right striatum at the following stereotaxic coordinates relative to bregma: 0.5 mm rostral, 2.5 mm lateral, and 3.5 mm below the skull surface. After 10 min, collagenase was injected in 1 μl artificial CSF (NaCl 148 mmol/l, KCl 3 mmol/l, $CaCL_2$ 1 mmol/l, $MgCL_2$ 0.8 mmol/l, Na_2HPO_4 0.8 mmol/l, and NaH_2PO_4 0.2 mmol/l) over 1 min. Due to variability in the potency of different collagenase lots, the dose of each lot was adjusted to that which produced ~50% reduction in striatal cell viability via MTT assay (Chen-Roetling et al., 2013). Dose range was 0.014–0.074 units, and all direct comparisons were made between groups receiving the same collagenase dose. For striatal autologous blood injection, blood was collected from a tail vein and injected at the above coordinates at a rate of 1 μl/min. Ten minutes after completion of collagenase or blood injection, the needle was slowly removed and the wound was closed with sutures. Surgical control mice were subjected to needle trauma only. Mice were recovered in a warm environment.

Hemin injection

Hemin (Frontier Scientific, Logan, UT) was prepared under reduced light immediately prior to use as a 2 mM solution in sodium phosphate buffer, pH 7.4. It was further diluted in sterile saline and was injected i.p. at 1 or 3 h after striatal collagenase or blood injection, at the following doses: 1) 26 mg/kg, repeated 24 h later; 2) 4 mg/kg, repeated 24 and 48 h later. Pilot studies demonstrated that a single repeat dose of 4 mg/kg at 24 h was sufficient to increase perihematomal cell viability in the blood injection model as quantified by MTT assay, so the third dose was omitted in those experiments to minimize animal discomfort. Control mice were injected with an equal volume of vehicle. In order to control for reduced edema and behavioral deficits in female mice as previously reported by Nakamura et al. (2004), gender ratios were balanced in hemin and vehicle groups.

Assessment of blood–brain barrier permeability

Protein permeability was quantified by Evans blue, which binds to albumin. Mice (5–8/condition) were injected with 2% Evans blue in sterile saline, 4 ml/kg i.p. Three hours later, under isoflurane anesthesia, they were perfused with 50 ml PBS through the left ventricle to remove intravascular dye, and were euthanized by cervical dislocation. Striata were dissected free and weighed; Evans blue extraction and assay followed the method of Uyama et al. (1988). Tissue was homogenized in 200 μl 50% trichloracetic acid. After debris removal by centrifugation, the supernatant was collected and diluted 1:3 in 100% ethanol. Evans blue fluorescence (ex: 620 nm, em: 680 nm) was then measured using a Perkin Elmer fluorescence spectrometer. In order to quantify leakage of lower molecular weight molecules, additional mice (4–7/condition) were injected with 2% fluorescein isothiocyanate (FITC)-dextran (MW 3–5 kDa, 4 mg/kg, Sigma-Aldrich) rather than Evans blue. Five minutes later, mice were euthanized and striata were removed, homogenized in PBS, and centrifuged. The supernatant fluorescence (ex: 490 nm, em: 520 nm) was then measured. Mean fluorescence intensity (MFI) was normalized to that in vehicle-treated controls injected with collagenase (= 100).

Striatal water content

At three days after ICH, injected and contralateral striata ($n = 10$) were excised and weighed. After drying in an oven at 95 °C for 24 h, tissue samples were weighed again. Water content was calculated by subtracting dry weight from wet weight. The water content of the contralateral striatum was subtracted from that of the corresponding injected striatum to yield the value produced by ICH-mediated injury.

MTT striatal viability assay

The close correlation of this method with stereology-based cell counts of tissue sections has recently been described (Chen-Roetling et al., 2013). At 5 days after striatal blood or collagenase injection, mice (6–8/condition) were deeply anesthetized with isoflurane and euthanized by cervical dislocation. Injected and contralateral striata were rapidly removed, minced with forceps, and dissociated by trituration in Hanks Balanced Salt Solution supplemented with 27.8 mM glucose, 20.5 mM sucrose, and 4.2 mM sodium bicarbonate. One milliliter of 0.25 mg/ml MTT was added, and the cell suspension was incubated at 37 °C for 4 min. After centrifugation (1380 ×g, 2 min), the supernatant was discarded, and formazan was immediately extracted in 2 ml isopropanol. The absorbance of this solution was then quantified at 562 nm, and was normalized to the absorbance of the solution obtained from the contralateral striatum.

Behavioral testing

Neurological outcome was assessed in mice (11–12/condition) receiving striatal collagenase injections followed by treatment with vehicle or hemin (4 mg/kg i.p.) at 3 h, 27 h, and 51 h. Testing was conducted on the same mice at 3, 5, 10, 14, 21 and 28 days after ICH, using a previously described protocol (Chen et al., 2011; Chen-Roetling et al., 2013).

Adhesive removal test

Adhesive dots (3 mm diameter) were cut from electrical tape and attached to the left or right forepaw. The interval until the mouse noticed the dot and the subsequent time needed for removal were recorded by a blinded observer. Mean times from the right (ipsilateral to hemorrhage) forepaw were subtracted from that of the left to calculate an asymmetry score (MacLellan et al., 2006). Four training sessions were conducted prior to data collection.