Protease-Activator Receptor 1 and 4 Signal Inhibition Reduces Preterm Neonatal Hemorrhagic Brain Injury

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Background and Purpose—This study examines the role of thrombin’s protease-activated receptor (PAR)-1, PAR-4 in mediating cyclooxygenase-2 and mammalian target of rapamycin after germinal matrix hemorrhage.

Methods—Germinal matrix hemorrhage was induced by intraparenchymal infusion of bacterial collagenase into the right ganglionic eminence of P7 rat pups. Animals were treated with PAR-1, PAR-4, cyclooxygenase-2, or mammalian target of rapamycin inhibitors by 1 hour, and ≤5 days.

Results—We found increased thrombin activity 6 to 24 hours after germinal matrix hemorrhage, and PAR-1, PAR-4, inhibition normalized cyclooxygenase-2, and mammalian target of rapamycin by 72 hours. Early treatment with NS398 or rapamycin substantially improved long-term outcomes in juvenile animals.

Conclusions—Suppressing early PAR signal transduction, and postnatal NS398 or rapamycin treatment, may help reduce germinatal matrix hemorrhage severity in susceptible preterm infants. (Stroke. 2015;46:1710-1713. DOI: 10.1161/STROKEAHA.114.007889.)

Key Words: hydrocephalus ■ stroke

Germinal matrix hemorrhage (GMH) is the leading cause of mortality and morbidity from prematurity because this brain region is selectively vulnerable to spontaneous bleeding within the first 72 hours of preterm life.1 Cerebroventricular expansion contributes to long-term injury through mechanical compression of surrounding brain tissues.2 Neurological outcomes include hydrocephalus, mental retardation, and cerebral palsy.1,3 Current neonatal intensive care treatments are ineffective at preventing GMH, and neurosurgical shunts are prone to devastating complications.4 Importantly, the blood constituent thrombin has been identified as a causative factor in hydrocephalus formation.5 Thrombin activates a subfamily of G-protein-coupled receptors, named proteinase-activated receptors (PARs; specifically PAR-1 and PAR-4),6 leading to phosphorylation and activation of mammalian target of rapamycin (mTOR)7 and increased cyclooxygenase (COX)-2 expression.8 Therefore, we hypothesized that modulation of the thrombin-(PAR)-1, -4–(COX)−2/mTOR pathway could be a promising strategy to improve outcomes after GMH.

Materials and Methods

Animal Surgeries

All studies, protocols, and procedures were approved by the Loma Linda University IACUC. One hundred fifty-seven P7 rat pups (comparable with human 30–32 gestational weeks; 14–19 g; Harlan Laboratories, Indianapolis, IN) were randomly subjected to either GMH or sham operations. GMH was induced by a stereotactically guided infusion (using bacterial collagenase, denatured collagenase, blood, or thrombin) to mimic preterm right-sided ganglionic eminence bleeds.9 Details are in the online-only Data Supplement.

Tissue Processing and Analysis

Rats were euthanized at 72 hours (for Western blot), at various time points between 6 hours and 21 days (for thrombin assay), and after 28 days (for neuropathological analysis) post GMH, and analyzed by blinded experts.9–11 Details are in the online-only Data Supplement.

Animal Treatments and Experimental Groups

For the 72-hour (short term; n=49) Western blot study, GMH animals received intraperitoneal coinjections of PAR-1 (SCH79797) and PAR-4 (P4pal-10) antagonists (1, 3, 7, 10, or 15 mg/kg given 1, 24, and 48 hours post GMH). For thrombin assay time-course study (n=49, n=7 per time point), GMH animals were euthanized at 0 hour, 6 hours, 1 day, 5 days, 7 days, 10 days, and 21 days after GMH. For the 28-day (long term; n=26) study, COX-2 (NS398) or mTOR (rapamycin) treatment consisted of 6 intraperitoneal injections (1, 6, 24, 36, 48, and 60 hours after GMH). Inhibitors were prepared using distilled water containing 5% dimethyl sulfoxide (DMSO) solvent. Controls received the vehicle (5% DMSO). All drugs were purchased from Sigma-Aldrich (St. Louis, MO).10,11

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Assessment of Neurological Deficits
Cognitive (T-maze, Water-maze) and sensorimotor (Rotarod, Foot Fault) testing from 21 to 28 days post GMH was performed by experienced blinded investigators as described.9–11 Details are in the online-only Data Supplement.

Statistical Analysis
P values of <0.05 were considered statistically significant. Neurobehavioral data were analyzed using 1-way ANOVA on ranks with Student–Newman–Keuls post hoc test. All other data were analyzed by 1-way ANOVA with Tukey post hoc test. Data are expressed as mean±SEM.

Results
Hemorrhage progression and hydrocephalus formation after GMH are shown in Figure I in the online-only Data Supplement, and changes in body weight after GMH are shown in Figure II in the online-only Data Supplement.

GMH-Activated Thrombin
Thrombin activity increased at 6 and 24 hours after collagenase infusion compared with sham (P<0.05), and then normalized over 5 to 21 days (Figure 1B).

Molecular Mediators of Posthemorrhagic Hydrocephalus
Posthemorrhagic hydrocephalus at 28 days was greatest in the group receiving direct intraparenchymal infusion of collagenase into the ganglionic eminence (P<0.05; Figure 1C and 1D) compared with intracerebroventricular injections of collagenase, heat-deactivated collagenase, donor blood, or thrombin.

Western Blots
Early PAR-1 and PAR-4 signal inhibition reduced mTOR phosphorylation and COX-2 expression (P<0.05; Figure 2A and 2B) in a dose responsive fashion at 72 hours post GMH induction.

Early Signal Inhibition Improved Long-Term Outcome
Treatments using either COX-2 or mTOR inhibitors significantly ameliorated long-term cortical thickness, ventricular volume, and neurodeficits (P<0.05; Figures 2C and 2D and 3A–3D) compared with vehicle-treated animals at 28 days post GMH.

Discussion
This study investigated the effectiveness of modulating thrombin-PAR-1 and PAR-4 in reversing COX-2 and phospho-mTOR (p-mTOR) upregulation, as well as the effect of direct COX-2 and p-mTOR inhibition on posthemorrhagic hydrocephalus, and neurological deficits. Previous studies hypothesized the mechanism of hydrocephalus involved increased production of infiltrating extracellular matrix proteins throughout the cerebroventricular system, leading to the disruption of cerebrospinal fluid outflow.1,2,9,11–13 Our results suggest that thrombin-induced PAR-1, PAR-4 stimulation upregulates detrimental signaling: exacerbating inflammatory (ie, COX-2 mediated) and proliferative responses (ie, p-mTOR mediated) that are potentially upstream of extracellular matrix protein dysregulation.1,5,7–9,11,14 Multiple parallels (especially thrombin)1,2,7–11 exist between our study and the pathophysiology of adult intracerebral hemorrhage.4,5,12–14
Thus, in extension, our findings may have a much broader therapeutic implication in terms of further adult stroke mechanistic study (these fall outside the scope of this article15).

To address the question of molecular mediators of GMH, our first aim demonstrated that intraparenchymal infusion of collagenase generated the majority of hydrocephalus. This is likely the sum contribution of blood products14 (eg, red blood cell lysis and inflammation) and thrombin. In fact, thrombin demonstrated greatest activity in the acute phase, between 6 and 24 hours post ictus, with tendency to remain elevated ≤10 days and normalized by 21 days.

Figure 1. A, Proposed mechanism (B) Time-course of thrombin activity (n=7 per group; * P<0.05 compared with sham). C, Representative 2-mm coronal brain section pictographs (28 days after infusion). D, Quantification of ventricular volume (n=6 per group; * P<0.05 compared with vehicle). GMH indicates germinal matrix hemorrhage; ICV, intracerebroventricular; and RAPA, rapamycin.
We next hypothesized that thrombin binds to PAR-1, PAR-4 and consequently upregulates COX-2 and p-mTOR. Because thrombin is most active in the acute phase, we examined levels at 72 hours post ictus, and determined COX-2 and p-mTOR were significantly greater in vehicle-treated animals than in sham. Furthermore, inhibiting PAR-1, PAR-4 using SCH79797 (PAR-1 antagonist) and p4pal10 (PAR-4 antagonist) significantly normalized COX-2 and p-mTOR levels at 72 hours.

Then, we asked whether directly inhibiting COX-2 or p-mTOR after GMH could circumvent long-term posthemorrhagic ventricular dilation, cortical cell loss, and improve sensorimotor and neurocognitive outcomes. Our findings demonstrated that vehicle-treated animals had significantly worsened outcomes compared with shams, and treating with either NS398 (COX-2 inhibitor) or rapamycin (mTOR inhibitor) significantly improved brain neuropathology and neurological ability. Thus, by attenuating early inflammatory...
(ie, COX-2) and proliferative (ie, p-mTOR) signaling pathways, we improved long-term outcome in the juvenile animals.

In summary, this study is the first to show thrombin-PAR-1, thrombin-PAR-4 signal inhibition normalizing early COX-2 and p-mTOR expression levels, and this in turn, improving long-term neurological outcomes after GMH.

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Disclosures
None.

References
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Supplemental Material

**Animal Surgeries**
All studies, protocols and procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University. One hundred and fifty-seven P7 Sprague Dawley rat pups (14–19 g; Harlan Laboratories; Indianapolis, IN) were randomly allocated to either GMH or sham operation. The number of pups needed for the experiments and study mechanisms was accounted for in each test group. The sample size estimates for all groups assumed type 1 error rate = 0.05 and power = 0.8 on a two-sided test. Sample size estimates were then made using data from our previous experiments (per previous assumptions, mean values, standard deviation, and up to a 20% change in means). A stereotactically guided, 0.3 U bacterial collagenase infusion model was used to mimic preterm right-sided ganglionic eminence bleeds\(^1-3\) (except for the cohort using either heat-deactivated collagenase, \(30\mu l\) of donor blood, or corresponding amount of thrombin [5U], directly injected into the ventricles to study hydrocephalus). Time pregnant rats were purchased from Harlan Laboratories (Indianapolis, IN) and pups of both genders were subjected to spontaneous germinal matrix hemorrhage (GMH) using collagenase infusion.\(^2\) Briefly, general anesthesia was achieved by isoflurane (3% in 30/70% Oxygen/Medical Air). The anesthetized rat pup was positioned prone, with its head secured onto a neonatal stereotactic frame (Kopf Instruments, Tujunga, CA). Next, the scalp was sterilized (using betadine solution), and a small midline incision was made to expose the bregma. Then, a 1 mm cranial burr hole was made using a standard dental drill (coordinates from bregma: 1.8mm anterior, 1.5mm lateral, 2.8mm in depth) through which a 26-gauge needle was stereotaxically lowered into the rodent’s brain. At this position, clostridial collagenase VII-S (0.3 U, Sigma; St. Louis, MO) was infused at a rate of 0.25\(\mu l/min\) into the right striatum. The needle was left in place for 10 minutes following infusion completion to prevent backflow of collagenase. Thereafter, the needle was slowly withdrawn at a rate of 1mm/min; the burr hole was sealed with bone wax; and the scalp was sutured closed. All animals were allowed to recover under observation on a 37°C warm heating blanket before being returned to their dams. Shams received needle insertion only without collagenase infusion.

**Tissue Processing and Analysis**
Standard protocols\(^1-3\) were used for Western blot analysis at 72h post-GMH. Briefly, deeply anesthetized pups were transcardially perfused using 50ml PBS, and the whole brain tissue samples were collected. Both hemispheres (i.e. bilateral forebrain) were placed into a single test tube for further analysis. Following tissue preparation and protein extractions of the bilateral hemispheres, western blotting was performed using the following primary antibodies: anti-COX-2 (1:200; Cayman Chemical, Ann Arbor, MI), or anti-phospho-MTOR (1:1000; Cell Signaling Technology, Danvers, MA). The appropriate secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were visualized using ECL Plus chemiluminescence kit (Amersham Bioscience, Arlington Heights, IL) and further semi-quantitatively analyzed using Image J (4.0, Media Cybernetics, Silver Springs, MD). Results are expressed as a relative density ratio, adjusted to sham.
Standard protocols\textsuperscript{1-3} were used for Thrombin assay. Briefly, animals were transcardially perfused with phosphate-buffered saline at 6hr, 1d, 5d, 7d, 10d and 21d after GMH. Brain samples were then homogenized and thrombin amidolytic activity was measured using the thrombin-specific chromogenic substrate, S2238 (Anaspec, Fremont, CA) as previously described.\textsuperscript{4} The final concentration of the s2238 solution was 20mM in phosphate-buffered saline. Thrombin standards were made using rat thrombin (Sigma Aldrich, St. Louis, MO) at concentrations of 0, 1.5625, 3.125, 6.25, 12.5, 25, and 50 mU/ml. Reaction mixtures consisted of 10ul of brain sample supernatant and 1.5ul of the s2238 chromogenic substrate mixture, which were then added to 90ul of phosphate-buffered saline. Reaction mixtures were allowed to incubate for 1hr at room temperature, after which the sample absorbance were spectrophotometrically measured at 405nm.

Standard protocols\textsuperscript{1-3} were used for Histopathological analysis at 28d post-GMH. Briefly, deeply anesthetized pups were transcardially perfused using 50ml ice-cold PBS and 10\% paraformaldehyde. Whole brain tissue samples were post-fixed in 10\% paraformaldehyde solution (at 4°C for 3 days), then dehydrated in 30\% sucrose for the same amount of time. 10 μm frozen coronal brain sections were cut every 600 μm using the cryostat (CM3050S, Leica Microsystems), then mounted, and cresyl violet stained on poly-L-lysine-coated slides. Morphometric analysis,\textsuperscript{2} was conducted using computer-assisted (ImageJ 4.0, Media Cybernetics, Silver Spring, MD) hand delineation of the ventricle system (lateral, third, cerebral aqueduct, and fourth), hemisphere (cortex, subcortex), corpus callosum (white matter), caudate, thalamus, and hippocampus. Borderlines of these structures are based on criteria derived from stereologic studies using optical dissector principles.\textsuperscript{1-3, 5-6} Volumes were calculated: \textit{(Average \textit{[(Area of coronal section) \times Interval \times Number of sections]})}.\textsuperscript{1-3}

\textbf{Assessment of Neurological Deficits}

Delayed juvenile cognitive (T-maze, Water-maze) and sensorimotor (Rotarod, Foot Fault) testing was performed at 21-28 days post-GMH. Briefly, the T-Maze\textsuperscript{1-2} assessed short-term (working) memory ability. For each trial, the pup was placed into the stem (40cm×10cm) and allowed to explore until either the left or right arm was chosen. From the sequence of 10 trials, the rate of spontaneous alternation (0\% = none and 100\% = complete; alternations/trial) was calculated. The Morris Water-maze,\textsuperscript{1-3} measured spatial learning and memory on three daily blocks. The apparatus consists of a metal pool (110cm diameter) filled with water to within 15cm of the upper edge and containing a small platform (11cm diameter) for the animal to climb onto. The swim path length was digitally analyzed by Noldus Ethovision tracking software. Cued trials (maximum of 60 sec/trial) measured general associative learning, sensorimotor ability, and motivation to escape the water with the platform visible above the water surface. The platform’s location changed every other trial. Spatial trials (maximum of 60 sec/trial) measured spatial learning with the platform submerged, but discoverable. Probe trials measured spatial memory by recording the amount of time in the target quadrant after the escape platform was removed. For the rotarod,\textsuperscript{2-3} this test assessed motor and coordination ability. The apparatus (Columbus Instruments, Columbus, OH) consists of a horizontally rotating cylinder requiring continuous walking in order to avoid falling (7cm diameter, 9.5cm width) and programmed to either maintain a constant velocity, or to accelerate 2rpm every 5sec.
The time to fall was recorded by photobeam circuit. For Foot Fault testing,1-2 this documented the number of completed limb missteps through the openings while the animal explored an elevated wire (3mm) grid (20cmx100cm) over 120sec. All neurobehavior assessments were conducted in a blinded fashion by experienced investigators.1-3

**Supplemental References**
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Supplemental Figure legends

**Supplemental Figure I.** Early hemorrhage progression and hydrocephalus formation following GMH. A. Timeline showing the progression of hemorrhage at 6, 24 and 72 hours after collagenase infusion (0.3U). B. Histological brain sections, stained with cresyl violet, confirm presence of post-hemorrhagic hydrocephalus at 28 days after GMH-induction. Photographs of the bleed show coronal view along the needle tract. 28 day histological sections were taken between 1 mm rostral and caudal of the needle tract.

**Supplemental Figure II.** Change in body weight 1, 9, 14, 21, and 28 days after collagenase infusion (GMH). *P*<0.05 compared with sham; †*P*<0.05 compared with vehicle.