Cyclooxygenase-2 Inhibition Provides Lasting Protection Following Germinal Matrix Hemorrhage in Premature Infant Rats

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Introduction

Germinal matrix hemorrhage (GMH) is the most common neurological disease of premature infants, partly because this germinal region is most vulnerable to spontaneous bleeding within the first 3 days of preterm life [1]. Intracerebroventricular expansion partly contributes to long-term brain injury through mechanical compression of surrounding tissues [2–4]. Devastating outcomes include hydrocephalus, mental retardation, and cerebral palsy [1, 5, 6]. Current treatment modalities are largely ineffective, and GMH has been thus far not preventable [7].

Importantly, the blood constituent thrombin is an established factor in hydrocephalus formation [8–10], which binds and trans-activates a subfamily of G protein-coupled receptors named proteinase-activated receptors (specifically PAR-1 and PAR-4) [11], theoretically leading to increased COX-2 expression [12]. Therefore, we hypothesized that modulation of brain injury through thrombin, PAR-1,-4, and COX-2 could be an eventual strategy to help improve outcomes after GMH.

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Methods

All studies, protocols, and procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University. Postnatal day 7 (P7) neonatal rats were subjected to stereotactic ganglionic eminence collagenase infusion. Groups were as follows: animals were euthanized at either of two time points 72 h (short-term) or 4 weeks (long-term). Short-term COX-2 expression was evaluated in the context of PAR-1 (SCH-79797) and PAR-4 (P4pal10) inhibition; pups in the long-term group were administered the selective COX-2 inhibitor (NS-398) as routinely performed [13].

Animal Surgeries

P7 Sprague-Dawley rat pups (14–19 g) were randomly allocated to either GMH or sham operation. A stereotactically guided, 0.3 U bacterial collagenase infusion model was used to model preterm right-sided ganglionic eminence bleeds [14–16]. Timed pregnant rats were purchased from Harlan Laboratories (Indianapolis, IN, USA), and pups of equally both genders were subjected to collagenase infusion [15]. Briefly, general anesthesia was obtained by using isoflurane (3 % in 30/70 % oxygen/medical air). Anesthetized pups were positioned prone, with heads secured onto the neonatal stereotactic frame (Kopf Instruments, Tujunga, CA, USA). The scalp was then sterilized (using betadine solution), and a small midline incision made to expose the bregma. Using a standard dental drill, a 1-mm cranial burr hole was made (bregma coordinates: 1.8 mm anterior, 1.5 mm lateral, 2.8 mm deep), through which a 26-gauge needle was lowered, and at this position, clostridial collagenase VII-S (0.3 U: Sigma, St. Louis, MO, USA) was infused at 0.25 μl/min into the right basal ganglion. Needles were left in place for 10 min after infusion to prevent backflow. Thereafter, the needle was slowly withdrawn at rate of 1 mm/min; burr holes

R.L. Applegate et al. (eds.), *Brain Edema XVI: Translate Basic Science into Clinical Practice*, Acta Neurochirurgica Supplement, Vol. 121,
DOI 10.1007/978-3-319-18497-5_36,

were 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 all the above without collagenase infusion, as routinely performed [13].

Animal Perfusion and Tissue Extraction

The animals were fatally anesthetized with isoflurane (≥5 %) followed by cardiovascular perfusion with ice-cold PBS for Western blot analyses. Forebrains were dissected and snap-frozen with liquid nitrogen and then stored in –80 °C freezer, awaiting quantification as routinely performed [13].

Western Blotting

For the protein immunoblot [13], the concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA, USA). The samples were then subjected to SDS-PAGE on 4–20 % gels, and then transferred to nitrocellulose membrane X 100 min at 100 V (Bio-Rad). Blotting membranes were incubated for 1 h with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween 20, and these were then incubated overnight with the primary antibody, anti-COX2 (1:200; Cayman Chemical, Ann Arbor, MI, USA). Membranes were then incubated using secondary antibodies (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and processed with an ECL Plus kit (GE Healthcare and Life Science, Piscataway, NJ, USA). For an internal control, the same membrane was probed using an antibody against β-actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) after being stripped. Relative densities of resultant protein immunoblot images were semiquantitatively analyzed by Image J software (4.0, Media Cybernetics, Silver Spring, MD, USA) as described elsewhere [17].

Neurological Deficits

All neurobehavior assessments were conducted in a blinded manner by experienced investigators [13–16]. Animals were assessed using a series of tests. Neurological deficit was quantified using a series of six tests measuring functional deficits (100 = severe, 50 = moderate, 0 = none): (1) proprioceptive limb placing, (2) lateral limb placement, (3) forelimb placement, (4) postural reflex, (5) back pressure toward edge, and (6) lateral pressure toward edge. These are routinely performed in brain-injured juvenile rats [18]. A T-maze was used to assess short-term (working) memory ability [19]; for each trial, rat were placed into the stem (40 cm × 10 cm) of the T-maze and allowed to explore until either the left or right path was chosen. Following a sequence of 10 trials, the rate of spontaneous alternation (0 % = none and 100 % = complete; alternations/trial) was recorded [18, 20].

Histological Slides

Animals were terminally anesthetized with isoflurane (≥5 %), followed by cardiovascular perfusion with ice-cold PBS and 10 % paraformaldehyde. Brains were removed and separated from surrounding tissues and post-fixed in 10 % paraformaldehyde and then 30 % sucrose (weight/volume) for total of 3 days. Histopathological pictographs used 10-μm thick coronal sections, caudally cut every 600 μm on a cryostat (Leica Microsystems LM3050S), then mounted and stained on poly-L-lysine-coated slides.

Statistical Analysis

Significance was based on <0.05. Data were statistically analyzed using one-way ANOVA, followed by Tukey post hoc test for significant analyses. Statistical analyses were performed using SigmaPlot version 10.0 for Windows.

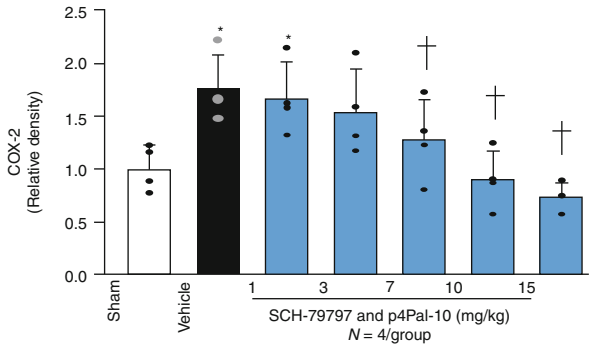
Results

Early combined PAR-1 and PAR-4 signal inhibition reduced COX-2 expression ($p<0.05$; Fig. 1) in a dose-responsive manner measured 72 h after collagenase infusion. Thereafter, in a separate cohort of animals, direct inhibition of COX-2 by NS-398 further reduced hydrocephalus (Fig. 2) and also improved long-term neurobehavioral outcome ($p<0.05$; Fig. 3).

Conclusion

Translational stroke studies, in particular those involving animal modeling, are greatly needed to safely integrate basic preclinical investigations ahead of eventual clinical applications [21–25]. This study therefore investigated the value of modulating thrombin–PAR-1 and PAR-4 with reversing COX-2 upregulation, as well as the effect of direct COX-2 inhibition on post-hemorrhagic hydrocephalus and

on neurological deficits. In prior studies, others hypothesized that hydrocephalus mechanisms involved increased production of infiltrating extracellular matrix (ECM) proteins throughout the cerebroventricular system and that these would lead to the obstruction of CSF outflow [1, 2, 10, 14, 15, 26–30]. Our data suggest that thrombin-induced PAR-1, -4 stimulation could upregulate harmful signaling, exacerbating inflammatory signaling (i.e., COX-2 mediated) upstream of ECM dysregulation [1, 8, 12, 14, 15, 31–34]. Thus, we hypothesized that thrombin binding to PAR-1, -4 receptors could consequently upregulate COX-2 protein. Furthermore, we investigated inhibition of PAR-1, -4 using a combined treatment with SCH79797 (PAR-1 antagonist) and p4pal10 (PAR-4 antagonist), which also significantly improved COX-2 after 72 h. Next, we asked whether directly inhibiting COX-2 following GMH could circumvent long-term negative outcomes. Our findings demonstrated that vehicle-treated animals had significantly worsened outcomes compared with shams, and treatment with NS398 (COX-2 inhibitor) significantly improved not only neuropathology but also and neurological ability. Therefore, by decreasing the early inflammatory COX-2 signaling pathway, we improved long-term outcome in juvenile animals. In summary, this study is the first to show that normalization of thrombin–PAR-1, -4 signals positively affect early COX-2 expression levels and improve long-term outcomes following collagenase infusion-mediated GMH.

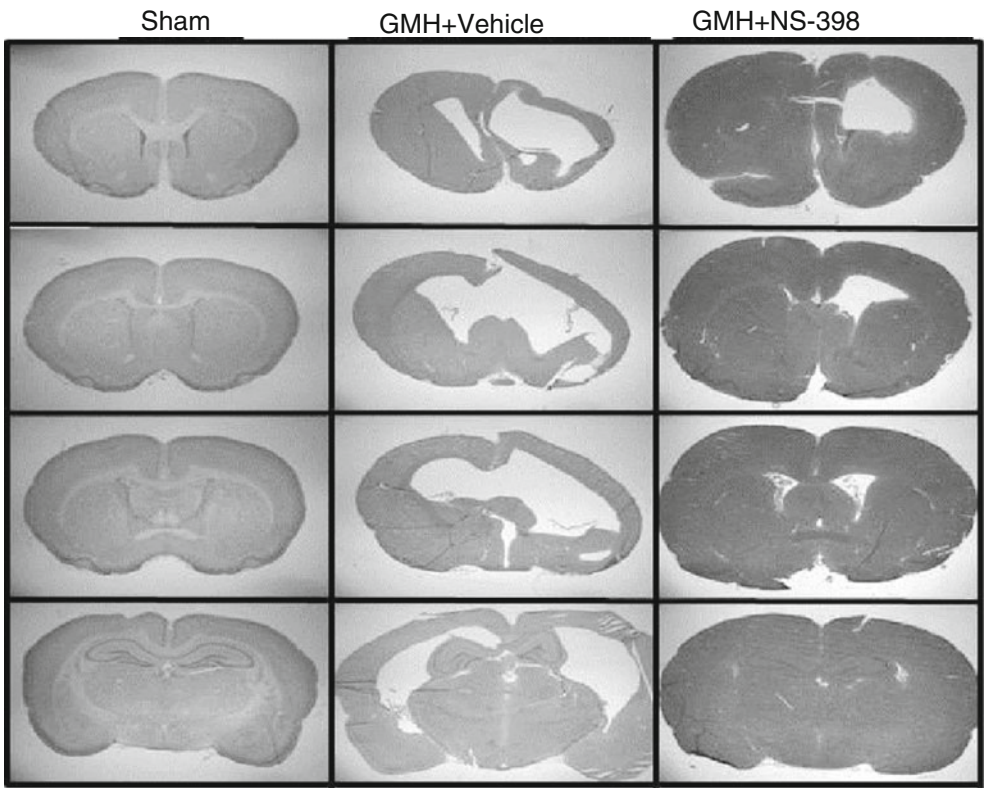


Brain COX-2 epression 3 days post GMH following SCH-79797 and p4Pal-10 co-administration.

Fig. 1 COX-2 expression post-GMH; dose response following PAR-1 and PAR-4 co- administration; 72 h after collagenase infusion; (*asterisk*) <0.05 compared with sham; (*cross*) <0.05 compared with GMH (vehicle); *SEM* standard error of the mean; $n = 4$/group

Acknowledgment This study was partially supported by National Institutes of Health grant RO1 NS078755 (Dr. Zhang).

Disclosures None



NS-398 reduced hydrocephalus
28 days after GMH

Fig. 2 Pictographs showing relative cortical thickness, ventricular and overall brain size between groups

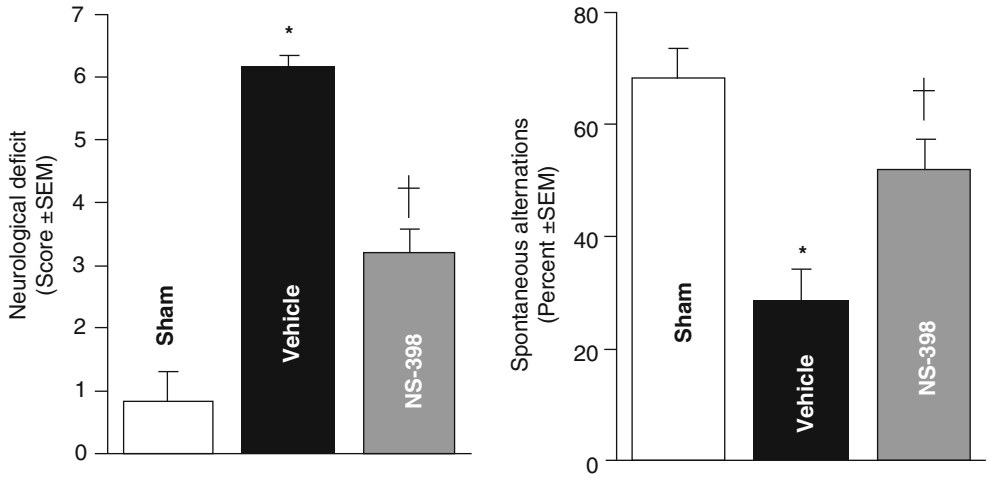


Fig. 3 *Left panel*, Neurological deficits (sensorimotor skill); *Right panel*, T-maze (spontaneous alternations) measured 1 month following collagenase infusion; (*asterisk*) <0.05 compared with sham; *SEM* standard error of the mean; $n=4$/group

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