Parecoxib, A Selective Cyclooxygenase Inhibitor, Attenuates C-Jun N-Terminal Kinase Activation in Experimental Subarachnoid Hemorrhage Induced Early Brain Injury

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Abstract

Background: Increased evidence has disclosed early brain injury (EBI) might determine the outcome of patients suffering from aneurismal subarachnoid hemorrhage (SAH). This study is of interest to examine the efficacy of parecoxib, a cyclooxygenase-2 (COX-2) inhibitor, on SAH-induced EBI.

Methods: The rodent SAH model was employed. 500/1000/2000ug/kg/day parecoxib was administered via osmotic mini-pump. CSF samples were collected to examine IL-1β, IL-6, IL-8 and TNF-α. Cerebral cortex was harvested for C-Jun N-terminal kinase (cJNK), NMDARs (western blot), B-cell lymphoma 2 (Bcl-2), caspases (rt-PCR) measurement.

Results: Parecoxib dose-dependently reduced the bio-expression of cJNK when compared with the SAH groups. No significant differences were found in the levels of IL-8 among the experimental groups. The levels of IL-1β, IL-6 and TNF-α were increased in animals subjected to SAH, compared with healthy controls, but not in the medium- and high-dose parecoxib treatment group. Moreover, the levels of TNF-α in the SAH-only and SAH-plus vehicle groups were significantly elevated, and treatment with parecoxib (1000ug/kg) reduced the level of healthy controls. Cleaved caspase-3 and -9a were both significantly reduced in 2000ug/kg parecoxib treatment groups. Likewise, NMDAR-2a was significantly suppressed in parecoxib treatment SAH groups (p <0.01).

Conclusion: Treatment with parecoxib exerts its neuroprotective effect through the dual effect of inhibiting cJNK-activated neuro-inflammation and reduced mitochondrion-related caspase-9a expression. Besides, parecoxib decreased CSF levels of TNF-α and IL-1β, which contributes to the anti-delayed vasoconstriction effect. This study lends credence to support the COX-2 inhibitor could attenuate SAH-induced EBI.

Keywords: Early brain injury; Parecoxib; C-Jun N-terminal kinase; Cyclooxygenase 2; N-methyl-D-aspartate receptor; Subarachnoid hemorrhage

Abbreviations

BA: Basilar Artery; BAX: BCL2-Associated X Protein; Bcl-2: B-Cell Lymphoma 2; Caspases: Cysteine Requiring Aspartate Proteases; cJNK: C-Jun N-Terminal Kinase; COX: Cyclooxygenase; CSF: Cerebrospinal Fluid; EBI: Early Brain Injury; ET: Endothelin; GFAP: Glial Fibrillary Acidic Protein; HRP: Horseradish Peroxidase; IEL: Internal Elastic Lamina; IL-1&-6: Interleukin 1 and 6; MAPK: Mitogen-Activated Protein Kinase; MCP-1: Monocyte Chemoattractant Protein-1; NMDA: N-Methyl-D-Aspartate; Nr2: Nuclear Factor-Erythroid Related Factor 2; PBS: Phosphate-Buffered Saline; PGEs: Prostaglandins; RANTES: Regulated-upon-Activation Normal T Cell Expression and Presumably Secreted; ROS: Reactive Oxygen Species; SAH: Subarachnoid Hemorrhage; TNF-α: Tumor Necrotic Factor-α.

Introduction

Delayed ischemic neurological deficit, and acute cerebral ischemia subordinate to subarachnoid hemorrhage (SAH) has become a major disability in patients following a ruptured aneurysm [1-5]. Despite more than fifty years of studies focusing on the correction and prevention of delayed vasospasm, the efforts, in a multifaceted way, trying to reverse this condition, have proven to illustrate poor outcome. Increased evidence shows early brain injury (EBI) [6-10] enrolled cortical spreading depression, early cortical depolarization waves, and impaired neurovascular coupling, play key roles in neurological deterioration after SAH. Connolly stated the mortality rate in SAH patients remained 27% to 44%, and more than half of all SAH patients survive with serious cognitive and functional impairment [11]. These results stimulate efforts to dissect the cellular and molecular basis of EBI accompanying SAH to establish rational therapeutic targets.

Blood clots, existing in the subarachnoid space, are able to induce acute arteries and arteriolar constriction, passive venous obliteration and delayed arterial spasm [1, 11-12]. Previous studies have focused on
the vasoconstriction of arteries; however, the precise mechanism and pathogenesis of arterial constriction and venous obliteration associated EBI remain unclear. A growing body of both direct and indirect evidence shows possible mechanisms to produce EBI include oxidative stress,[13] nitric oxide(NO)/nitric oxide synthase(NOS) uncoupling [14], matrix metalloproteinase -9 (MMP-9) induced BBB breakdown [15], nuclear factor erythroid related factor 2 (Nrf2) pathway related oxidation [16], activation of c-Jun N-terminal kinase pathway, [14,17,18] increased proinflammatory cytokines [19] vascular endothelial growth factor, and mitogen-activation protein kinase [20]. Energy over-loaded after hemoglobin degrading has been shown to induce EBI. Owing to lack of effective therapies to achieve a better outcome in SAH patients, studies have become focused on the pathogenesis of EBI.

Parecoxib, a intravenous administration form of a cyclooxygenase-2 (COX-2) selective nonsteroidal anti-inflammatory drug (named celecoxib), is used to treat osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute pain, and juvenile rheumatoid arthritis [7]. COX (a prostaglandin H (PGH) synthase) is a key regulatory enzyme that catalyzes the conversion of arachidonic acid and associated prostaglandin (PG) G2 and PGH2 to a variety of eicosanoids, such as PGE2, PGD2, PGF2α, PGI2, and thromboxane (TX) A2 [21]. Recently, COX-2 inhibitor has been reported to have a neuroprotective effect in the experimental intracerebral hemorrhage model as well as induce apoptosis in Huh7 and HepG2 cell line cultures [22]. In the study of SAH rabbits, intramuscular administration of parecoxib was shown to attenuate SAH induced vasospasm and basal artery wall thickness [23]. Owing to the concerning side effects of major cardiovascular events, increased risk could be avoided by shortening the duration of administered COX-2 inhibitor and reducing the therapeutic dosage in patients who have pre-existing cardiovascular risk factors or disease. In this study, parecoxib was administered via an osmotic mini-pump at a minimum dosage and within a short duration, devoid the mentioned side effect of cardiovascular and renal problems. 0.9% saline was used as a vehicle.

Taking these findings together, we propose that COX-2 inhibitors, with its unique property in PGs expression, may be effective in SAH-induced EBI. Given the importance of arterial lesion formation, associated pro-inflammatory cytokine stimulation on leukocyte and endothelial dysfunction in the rat SAH model was used to test the hypothesis that COX-2 inhibitor significantly attenuates caspase-related apoptosis and C-Jun N-terminal kinase (cJNK)-associated inflammation following experimental SAH.

Methods

Materials

Parecoxib has been previously characterized as a potent inhibitor of COX-2 and was bought from Sigma Laboratory, Taipei, Taiwan. Monoclonal anti-rat IL-1β, IL-6, IL-8, NeuN, MCP-1 and GFAP antibody were obtained from Abcam (Cambridge, MA 02139-1517, USA), BD Transduction Lab (BD Biosciences, San Jose, CA, 95060, USA), Upstate Biotech (Lake placid NY, USA), and Santa Cruz Biotech (Santa Cruz Biotechnology, Inc. Santa Cruz, CA 95060, USA), respectively. Rabbit anti-rat cJNK (p55 and p46) antibody was purchased from R&D Systems, Inc (Minneapolis, MN 55413 USA). CMN protein extraction kits were from Biochain (Hayward, CA 94545, USA). Parecoxib in a mini-pump was prepared by Ms. Wu SC (Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, ROC), and 0.9% saline was used as a vehicle.

Induction of experimental SAH

Fifty-four male Sprague-Dawley rats, weighing between 320–450 g (BioLasco Taiwan Co., Ltd., authorized by Charles River Lab), were used in this study. All the experimental protocols were authorized and superintended by the University of Kaohsiung Medical Animal Research Committee. The rats received anesthesia by an intraperitoneal injection of a mixture of 0.9 mg/gm xylocine and 5.0 mg/gm ketave (0.1 ml fresh arterial blood was withdrawn and injected into the cisterna magna using a stereotactic apparatus). After the induction, animals were placed in ventral recumbent position for 30 minutes to let ventral blood clot formation. After monitoring for respiratory distress and giving mechanical ventilation if necessary, the animals were returned to the vivarium until becoming fully awake. A habitat with a 12 hr light-dark cycle and access to food and water ad lib was offered.

General design of experiments and treatment groups

The animals were subdivided into the following groups (9 rats/group): 1) sham operated (no SAH); 2) SAH only; 3) SAH plus vehicle; 4) SAH plus parecoxib (500 ug/kg/day); 5) SAH plus treatment with parecoxib (1000 ug/kg/day); and 6) treatment with 2000 ug/kg parecoxib in SAH rats. The dosage was adjusted according to our pilot study, devoid of hepato-renal toxicity and G-I bleeding. The first injection was given at 1hr after induction of SAH by using an osmotic mini-pump (Alzet corp, Palo Alto, CA 94301, USA). After re-anesthesia, CSF sampling was obtained through a 30-gauge needle into the cranial-cervical junction by using stereotactic apparatus. The animals were sacrificed by perfusion–fixation 72hr after SAH. Cortical tissue were sampled by means of placing a 22-gauge needle inserted 3 mm in depth into the skull bone (N=5) through a Burr hole craniectomy (2 mm apart from the bregma) at a 24 hr interval.

Perfusion–Fixation

By the end of the study, the animals were re-anesthetized by administration of 7 mg/kg Zoletil 50 (a combination of tiletamine hypochloride and zolazepam hypochloride. VIRBAC, L.I.D., Carros 06516, France). The femoral artery was catheterized to monitor blood pressure and obtain blood samples to determine arterial blood gas, BUN, Cr, GOT and GPT levels. Perfusion–fixation was performed as opening the thorax, and the left ventricle was canalled with a NO18 catheter with clamping the descending aorta, and opening the right atrium. 120 ml of 70 mm Hg of 0.01M phosphate buffer (pH 7.4) was used for perfusion, followed by fixation with 120 ml 2% paraformaldehyde in the PBS solution at 36°C under a perfusion pressure of 80 mm Hg. The harvested brain was immersed in a fixative at 4°C overnight. Visual inspection made sure that formed blood clots covered the basilar artery (BA) in all SAH animals.

Hemodynamic measurements

Heart rate, blood pressure, and rectal temperature were monitored before and after parecoxib administration at intervals of 12hr after the induction of SAH by a tail-cuff method (SC1000 Single Channel System, Hatteras Instruments, NC, 27518, USA) and rectal thermometer (BIO-BRET-2-ISO. FL 33780, USA).
Neurological assessment

A modified limb-placing tests (MLPT), [26] and a modified Voetsch neuro-score test [27] were performed before and at 24hr after the induction of SAH by an investigator blinded to the experiment set. The behavioral examination comprised two parts: ambulation and placing/stepping reflex examinations were performed before and after animals were subjected to SAH. The modified limb-placing tests (MLPT) was composed of two limb-placing tasks to examine the sensorimotor integration of the forelimb and the hindlimb through tactile and proprioceptive stimulation. The ambulation task was performed by suspending the rat 10 cm over a table and evaluated the stretch of the forelimbs towards the table: normal stretch, 0 points; abnormal flexion, 1 point and then, by positioning the animal along the edge of the table, and let its forelimbs placed out of the edge and allowed to move freely. The same performance of hindlimb was checked as normal performance, 0 point; performance with a delay (2 s) and/or incomplete, 1 point. A motor deficit index (MDI) was calculated for each rat at a set time interval. The final index was the sum of the scores of walking with lower extremities and placing/stepping reflex. Animals with MDI score more than three were defined as paraplegic, whereas animals with MDI score less than three were regarded as neurological deficit. The modified Voetsch neuro-score was to evaluate the head movement, confrontation, reflex stimulated by ear pinch, proprioception and four limbs movement. The modified Voetsch neuroscore is a vertebrobasilar scale score of sensorimotor ability. To examine the spontaneous movement of head: moves in all dimensions, 3 points; prefers one side, 2 points; only movement to 1 side, 1 point; flexed to unilateral side, 0 point. Confrontation task was defined as: fully responsive, 3 points; moderately responsive, 2 points; minimally responsive, 1 point and coma, 0 point. The hearing test by auditory startling: finger rubbing, 3 points; snap of fingers, 2 points; loud clapping, 1 point and no startling, 0 point. Pain reflex after ear pinch: brisk and symmetrical reaction, 3 points; slightly diminished or asymmetrical reaction, 2 points; severely diminished and asymmetrical reaction, 1 point; no reaction, 0 point. To check the sensation of the neck in a cage: reached 2 walls, 3 points; touched 1 wall, 2 points; moved along the base, 1 point and no movement, 0 point. To check cranialcaudal movement, turned bilaterally, 3 points; preferred circling 1 side, 2 points; only turned circling to 1 side, 1 point; fallen to 1 side, 0 point. To test the axial sensation, brisk and symmetrical reaction to stimuli, 3 points; slightly diminished or asymmetrical reaction, 2 points; greatly diminished and asymmetrical movement, 1 point and no reaction, 0 point. To evaluate forelimb and hindlimb movement: equal and bilateral movement, 3 points; slight asymmetry, 2 points; prominent asymmetry, 1 point; paresis, 0 point. To check the ability to climbing, the rat is able to climb to top, 3 points; impaired climbing, 2 points; stationary gripping, 1 point and fell immediately, 0 point. The final stage is to examine movement to the stimuli of beam, explored both ends, 3 points and some movements, 2 points. The sum of neuroscores ranged from 0 to 30, which stood for normal performance to comatose status and paraplegia.

Immuno-staining with MCP-1, NeuN polyclonal and GFAP monoclonal antibody and brain edema studies

The basilar arteries (BA) were frozen and cut into 25-µm-thick sections with the assistance of ultra-microtome E (Leica EM UC7, Union Optical & Instruments and Nanotechnology. New Taipei City 22101, Taiwan.) Five BA cross-sections of each animal (the middle third) were analyzed by two investigators blinded to the experiment set. A MCP-1 recruited monocytes, memory T cells, and dendritic cells were detected using a video-assisted microscope (x400). Briefly, coronal sections of the isolated rat basilar arteries were stored overnight on slides at -80°C. Rabbit monoclonal anti-rat MCP-1 antibody, polyclonal anti-rat NeuN antibody, glial fibrillary acidic protein (GFAP) monoclonal antibody were used at a dilution of 1:40, 1: 20 and 1:40, respectively, and immunostaining was performed for 40 min at 25°C followed by drying overnight as described in the mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [28]. A modified wet-dry weight method was used for determining the water content of the brain at 48 hr, when the weight loss was robust and reached a peak for 2 days after the induction of SAH [28]. The brain cortex was harvested from the skull bone, pondered on a precision microbalance (Precision Weighing Balances, Bradford, MA 01835, USA), and then let dried in an oven at 60°C for 48 hr. The dry specimen was re-weighed, and brain edema index was calculated as (wet cortex weight / dry cortex weight).

Quantification of mRNA expression of IL-1β, IL-6, IL-8, and MCP-1

ABI PRISM 7900 System (Applied Biosysytems, Foster City, CA, 94404, USA) was used to examined the expression of cytokines mRNA. As indicated by the manufacturer’s instructions, the mRNAs for IL-1β, IL-6, IL-8, and MCP-1 expression were estimated, while 18S was assigned as a housekeeping gene owing to its stable expression in the condition of SAH stimuli. Each sample was launched into a TaqMan® Human Cytokine Card that enclosed probes and primers for specific targets as well as the 18S ribosomal RNA as a standard control. This procedure was performed through an affixed filling reservoir and a vacuum loading process via the ABI PRISM® Card Filling Station.

Target mRNAs were standardized according to the reference gene (18S), and final data were expressed as a relative fold from baseline values. Comparative mRNA expression was set by the Livak and Schmittgen method [30]. The results were analyzed once a 5-fold increase was observed in the mRNA levels compared with baseline to let data consistent.

Immuno-blotting for cJNK (p55 and p46)

To evaluate glia-mediating neuro-inflammation, the protein expression of JNK in brain tissue homogenates was evaluated via western blot analysis. Samples containing 30µg of protein stirred with LDS sample buffer (contains 40% glycerol, 4% lidoche dodecyl sulfate (LDS), 0.8 M triethanolamine–CI pH 7.6, 4% Ficoll™-400, 0.025% phenol red, 0.025% Crossmas G250, 2 mM EDTA disodium, NuPAGE® LDS Sample Buffer (x4) NP0007; Invitrogen, Carlsbad, CA 92008, USA). Samples were then obtained after being loaded for 8% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then separated after centrifuging at 15,000 rpm for 10 min. The sample was mounted onto a polyvinylidene difluoride membrane and then incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline with 0.2% Tween 20) at room temperature. Rabbit anti-rat cJNK polyclonal antibody (1:200; Chemicon International, Temecula, CA 92590, USA) was used by β-Actin (monoclonal anti-β- actin, dilution 1:40,000; Sigma–Aldrich, Taipei, 100, Taiwan) was used as a control. A secondary antibody was conjugated with horseradish peroxidase (HRP) in TBS-t at room temperature for 1hr. Optical densities were measured by an enhanced Pierce chemiluminescent image analyzer (a GS-700 digital densitometer, GMI, Ramsey, MN 55303, USA).
The analysis of NMDAR -1, -2a, -2b by western blotting

The cortical homogenates were centrifuged at 15,000 rpm for 10 min twice. The protein extracts (30µg) were obtained via 10% sodium-decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) and then transferred onto a nitrocellulose membrane. These membranes were further nurtured in a blocking buffer, composed of 5% skim milk in Tris-buffered saline containing Tween 20 (TBS-t) (50 mmol/L Tris, pH 7.5, 0.15 mmol/L NaCl, 0.05% Tween 20) and incubated with anti-NMDAR-2a (1:1000), anti-NMDAR-2b (1:1000), anti-NMDAR-1 (1:1000), and anti-HO-1 (1:1000) in TBS-t at room temperature for 1hr. Anti-β-actin antibody was used as a standard control. The immunoblots were developed via a GS-700 digital scan and Molecular Analyst®. Relative optical densities were obtained by comparison between the measured values and the mean value. The pro-caspase-9 was catalyzed and cleaved into caspase-9a (majority) and -9b. We chose cleaved caspase-9a to represent the activated caspase-9.

Results

General observation

Through all the course of this experiment, all animals showed no significant differences in the physiological parameters recorded, including glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), blood urea nitrogen (BUN), creatinine, pH, blood pressure and arterial blood gas analysis among all groups. It proved that continued parecoxib pumping in the selected dosage has a number of pleiotropic effects, but devoid of hepatic and renal toxicity and bowel bleeding (Table 1). According to the brain edema after the induction of SAH, there is no significant difference among all the experimental groups (Figure 1, lower panel). Increased MCP-1(+) cells in the adventitia of vascular wall, NeuN (+) neurons with vacuolar formation in the cortex and GFAP(+) astrocytes were observed in the SAH groups, when compared with the parecoxib treatment SAH group, which correspond to the observation of MCP-1 mRNA (Figure 1 upper and middle panels).

Estimation of Bcl-2, cleaved caspase-3 and -9a mRNA by rt-PCR

The levels of activated caspase-3 and caspase-9a mRNA in the cortical homogenates were determined by TriPure RT-PCR Reagent (Roche Diagnostics Corp. IN, 46256, USA). According to the supplier’s instructions, the PCR primer sequences were designed according to the cleaved caspase-3, and -9a and glyceraldehyde-3-phosphate dehydrogenase (GAPDH as a standard control) gene sequences in GenBank. Bcl-2 (231bp; forward: 5’-CTGTACGGCCCAAGCATGCG-3’; reverse: 5’-GGATTAGACAGACAGTGG-3’); Caspase-3 (393bp; forward: 5’-GCTTTGTTTCTGATCGATC-3’; reverse: 5’-CATGGGATCTGTTTCTTC-3’); caspase-9a (889bp; forward: 5’-GCTCTTCCATTGTCTTCCTCC-3’; reverse: 5’-CCATGGCTTTCCGGTTACTGC-3’); GAPDH (347bp; forward: 5’-CCAGTGAGTTTCCCGTTC-3’. After incubation with Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (Promega, WI 53711, USA), the cleaved caspase -3, -9a and GAPDH cDNA were amplified. The amplified cDNA fragments were detected via agarose gel electrophoresis swirled with 1 µL of ethidium bromide. The intensity of the bands was measured via a comet assay method. Owing to its stable expression under subarachnoid hemorrhage stimuli, GAPDH mRNA was used as a housekeeping gene for multiplexing. The tissue of every five animals was used for rt-PCR and three measurements for each animal specimen performed to obtain a mean value. The pro-caspase-9 was catalyzed and cleaved into caspase -9a (majority) and -9b. We chose cleaved caspase-9a to represent the activated caspase-9.

Statistical analysis

Data are expressed as the means ± standard deviation. For group comparisons, one-way analysis of variance was used for analyses (ANOVA) followed by the Newman-Keuls post-hoc test and the Student t test statistically. Difference, at a probability value less than 0.01, was considered significant.
was considered as motor deficit index (MDI). The values of MDI in the SAH and SAH+vehicle groups were 2.92 ± 0.46 and 2.87 ± 0.40 respectively, compared with a score of 0 and 1.20 ± 0.46 in the healthy controls and 2000ug/kg parecoxib respectively. Treatment with parecoxib significantly improved the MDI in the SAH groups (Table 2). The modified Voetsch neuro-scores were dose-dependently improved in the parecoxib + SAH group and the healthy controls when compared with the SAH groups (Table 3).

Figure 1: The monocyte chemoattractant protein-1(MCP-1), NeuN and glial fibrillary acidic protein (GFAP) immunostaining and brain edema index (BEI) among the groups. Upper panel represents MCP-1(+) monocytes ( Red arrow head), NeuN (+) neurons (white arrow head) with vacuolated neucli and GFAP(+) astrocytes (yellow head) immunostaining of the sham-operated group (A), the SAH only group (B), the SAH rats treated with vehicle (C), 500ug/kg/day parecoxib treatment SAH group (D), 1000ug/kg/day parecoxib treatment in SAH rats (E) and SAH animals received 2000ug/kg/day parecoxib treatment (F). Standard bar= 400um. Vacuolar formation (white arrow head) was observed in neurons in SAH groups, which was absent in the sham-operated and parecoxib treatment SAH groups. Standard bar= 50um. Lower panel revealed the brain edema index. Data in the figure are presented as mean ± SD. (#, ##, ###:P>0.01, indicates comparison among the 500/1000/2000ug/kg/day parecoxib treatment and SAH groups respectively).

Figure 3: The expression of cJNK (p55/p46) in SAH rats. (p<0.01 compared to the SAH-only group).

mRNA expression of IL-1β, IL-6, IL-8, and MCP-1

After SAH, the CSF levels of IL-1β, IL-6, IL-8, and MCP-1 were found to increase 1000- and 3000-fold at 72hr, when compared with the sham operated group. Administration of 2000 ug/kg parecoxib reduced cytokine levels by 7%, 25%, 42% and 21% for IL-1β, IL-6, IL-8, and MCP-1 respectively, 24hr after SAH (Figure 2). Level of IL-1β was reduced in the parecoxib treatment groups in a dose-dependent mechanism in this study. 2000 ug/kg/day parecoxib significantly reduced the levels of IL-1β, IL-6 and MCP-1 in animals at the time points of 24hr and 72hr after the induction of SAH. Meanwhile, treatment with parecoxib tended to reduce the level of pro-inflammatory cytokines; the IL-8 level (at 72hr) failed to achieve statistical difference from the SAH group (Figure 2).

The expression of NMDAR-1, NMDAR-2a, and NMDAR-2b protein

To examine the glutamate-related neuronal death in animals subjected to SAH, NMDAR-1, NMDAR-2a and NMDAR-2b was used via rt-PCR (Figure 4). The level of NMDAR-1 protein was significantly reduced in the parecoxib treatment groups in a dose-dependent mechanism in this study.
induced in the SAH groups (SAH only, treatment with vehicle) when compared with the sham-operated groups (p<0.01). Parecoxib (at 1000 and 2000 ug/kg/day) decreased NMDAR-2a expression when compared with the SAH groups (P<0.01). Parecoxib dose-dependently reduced the expression of NMDAR-1 (P<0.01). Increased NMDAR-2b expression was noted in all the SAH groups (Figure 4, P >0.01).

The expression of activated Caspase-3 and -9a mRNA

Cleaved caspase-3 and -9a (the major functional variant) were used to examine the neuronal apoptosis. The activated caspase-9a was observed to increase in the SAH groups when compared with the sham-operated groups (p<0.01). 1000 ug/kg and 2000 ug/kg parecoxib decrease cleaved caspase-3 level when compared with the SAH groups respectively (Figure 5, p<0.01). Only high-dose parecoxib could attenuate apoptosis-related caspase-9a and caspase-3 when compared with the SAH groups.

Figure 2: Bar graph depicting parecoxib on the time-course (24hr and 72hr) change of pro-inflammatory cytokines after induction of SAH. Data are depicted for IL-1 β, IL-6, IL-8, MCP-1. Although trends to decrease in IL-8 and IL-6, the effect of parecoxib is relevant to IL-1β and MCP-1. Data in the figure are presented as mean ± SD(n = 9). *, **, ***: P<0.01, and #, ##, ###: P>0.01 when compared with the SAH group.

Figure 3: The immunohistochemical study of cJNK( P55/P46) expression (western blot). Upper panel represents the western blot of the SAH only group (A), the SAH + vehicle group (B), 500ug/kg/day parecoxib treatment SAH group (C), 1000ug/kg/day parecoxib treatment in SAH rats (D) and SAH animals received 2000ug/kg/day parecoxib treatment (E). Bar graphs revealed the assay showed induced cJNK immunoreactivity in the SAH groups when compared with the control group. Parecoxib dose-dependently reduced the expression of cJNK when compared with the SAH groups. Data in the figure are presented as mean ± SD. (*,**: P<0.01, #: P>0.01, indicates comparison among the 500, 1000, 2000ug/kg parecoxib and SAH groups respectively.)

Figure 4: Bar graph demonstrating parecoxib on the expression of NMDAR-1, NMDAR-2a, and NMDAR-2b protein (western blot). Top panel: all groups are identified to those stated in the legend of Figure 3. Bottom panel shows parecoxib is able to decrease the expression of NMDAR-2b (at high dose), and NMDAR-2a (dose-dependently), but does not decrease SAH-induced NMDAR-1 expression. All values are mean ± SD. *, **: P<0.01, and #: P>0.01, compared with the SAH group.
cytochrome c release, Bcl-2 mRNA was evaluated. The Bcl-2 mRNA was induced in the treatment of 2000 ug/kg/day parecoxib in SAH rats, when compared with the SAH group (Figure 6, p<0.01).

ApoPrimer for Bcl-2 mRNA

To assess the regulation of apoptosis through mitochondrial cytochrome c release, Bcl-2 mRNA was evaluated. The Bcl-2 mRNA was induced in the treatment of 2000 ug/kg/day parecoxib in SAH rats, when compared with the SAH group (Figure 6, p<0.01).

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Discussion

In this study, we have shown that parecoxib, a selected cyclooxygenase-2 inhibitor, ameliorates neurologic deficits in the behavior studies and brain damage, including neuronal death, and brain edema in the MCP-1 and NeuN immunostaining in rats subjected to SAH. EBI may promote delayed neuronal death by SAH-induced inflammation, associated increased BBB permeability, cerebral edema and intracranial pressure, and resulting neuronal dysfunction [8]. The family of cyclooxygenases (COX) includes two isoforms: COX-1, which functions in cellular homeostasis, and COX-2, which is responsible for up-regulating inflammatory cytokines and mitogens [21,30]. Induced COX-2 has profound effects on the brain via the complex effects of prostanoids that are abundant in the central nerve system, which modulate glutamate release, cerebral vasoconstriction, induction of reactive oxygen species (ROS) release, and make alterations on neuroendocrine function [7]. Besides, it is possible that COX-2 up-regulation in injured neurons could contribute to neuronal death following brain injury. Inhibition of COX-2 activity has been demonstrated to protect against ischemic damage, kainate-induced seizures, and neuroinflammation-induced neurotoxicity. [21] In this study, parecoxib was observed to be able to reduce early COX2 expression via inhibiting cJNK (p55) expression (dose-dependently), reduce IL-1β, IL-6 and MCP-1 (at 2000 ug/kg/day at the time point of 24hr and 72hr after the induction of SAH), IL-8 (of the dosage of 2000 ug/kg/day at 24hr after the induction of SAH), and also reduce cleaved caspase-1 (at 1000 and 2000 ug/kg/day) and Caspase-9a (at 2000 ug/kg/day).

Disrupted energy metabolism within the central nerve system was believed to cause deadly damage to neurons in both acute and chronic neurological disorders [32]. Overstimulation of glutamate receptors, especially NMDARs related to intracellular glutamate release, results in acute and delayed neuron injury, which included chronic diseases: Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, HIV associated dementia, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and acute disorders: stroke, central nervous system (CNS) trauma and seizures [33]. Both might be initiated by distinctive mechanisms but might share an ultimate similar pathway to neuronal injury. Excitotoxicity, owing to over-activation of NMDA-sensitive glutamate receptors, allows excessive Ca2+ influx through the NMDAR-associated ionotropic channel. Increased intracellular Ca2+ resulted in free peroxide radicals production and excessive proteolytic activity, which finally led to cellular damage and death [34]. Unwarranted glutamate released from the necrotic cells leads to a cascade of auto-destructive events and progressive cellular death, which can last for hours or even days after the primary injury. In the present study, parecoxib was found to reduce the NMDAR-1, and NMDAR-2a (at 1000 and 2000 ug/kg) hours after the induction of SAH. Unlike pro-inflammatory cytokines observed in CSF and released from microglia or immigrating from the breakout of BBB in cerebral ischemia, activated NMDARs were short-lived and believed primary to the activation of other neuro-toxic events as early cyclooxygenase-2 activation.

There are three major pathways observed in the caspase-related apoptosis of mitochondria existing in mammals: the cell-surface (extrinsic) receptor-related pathway, the apoptosome (intrinsic) pathway and the cytotoxic lymphocyte-initiated granzyme B pathway [35-41]. Shi stated there are 14 caspases identified in mammals, subdivided into three groups: one is the initiator caspases, such as caspase-8 and -9, one is the downstream effector caspases, like...
capase-3, -6, and -7, and the other include pro-inflammatory enzyme-enrolled capasases-1, -4, -5, -11, -12, and -13. Capaspe-3 is believed to be the major effector caspase in neuronal apoptosis [42]. It has been observed that caspases act on upstream intrinsic apoptosis, and on downstream of cytochrome c release [43]. Caspase-9 behaves as an initiator and has been shown able to uncouple the mitochondria and increase ROS production [13,40]. In this study, parecoxib was observed able to reduce the caspase-9a (2000 ug/kg/day) and decrease caspase-3 (of a dosage of 1000 and 2000 ug/kg/day). It is believed parecoxib, at a selected dosage, has a neuroprotective effect by blocking both initiator caspase-9a and effector caspase-3 activation.

Four conserved Bcl-2 homology (BH) domains were found in Bcl-2 family members, designated BH1, BH2, BH3, and BH4 [43,44]. An anti-apoptotic characteristic was conserved in a carboxy-terminal hydrophobic domain, through which BCL-2 is essential for its targeting to membranes such as the mitochondrial outer membrane [44]. The anti-apoptotic molecules, such as Bcl-2 or Bcl-XL, can inhibit the activation of BAX following a death signal. In contrast to inactive Bax, [43] Bcl-2 is an integral membrane protein heavily localized to mitochondria, exerts an anti-apoptotic effect. 2000 ug/kg/day parecoxib enhanced the cytoplasm Bcl-2 in the SAH study. The mechanism that parecoxib exerts anti-apoptotic effect via inducing cytoplasmic Bcl-2 expression merits a further study lateral.

Increased adhesion molecules and pro-inflammatory cytokines were observed increased in cerebrospinal fluid (CSF) after aneurismal SAH [5,24]. However, the relationships among the development of inflammatory response, early brain injury, and delayed cerebral ischemia in the brain after SAH need to be clarified. The cumulative findings indicate that the up-regulation of pro-inflammatory cytokines is antecedent to radiographic vasospasm (peak at 4th to 14th days after SAH) in human, and attenuation of cytokines tends to minimize vascular constriction and reduced early brain ischemia in animals. [6,44] Monocyte chemoattractant protein-1 (MCP-1) is able to predict outcome and vasospasm following aneurismal SAH and is highly expressed to reduce the tight junctions of vascular endothelium [19,46]. Elevation in the mRNA levels for IL-1β, IL-6, IL-8, TNF-α and adhesion molecules has been observed in the pilot study. In the study, parecoxib was observed to be able to reduce IL-1β and MCP-1 (at the dose of 1000 and 2000 ug/kg/day) and tended to reduce the IL-8 level. This result indicated parecoxib selectively reduced the inflammatory cytokines.

cJNK, belonging to the family of mitogen-activated protein kinase (MAPK), plays a role in T cell differentiation and cellular apoptosis pathway [14,39]. Though mediated by regulated-upon-activation normal T cell expression and presumably secreted (RANTES) and colony stimulating factor 2 (CSF2), cJNK is believed to be able to modulate cellular apoptosis, neuro-degeneration, proliferation and differentiation, cytokine production and subsequent inflammatory reactions [17]. In Jadhav et al’s study, through modulating cJNK/ MAPK-related molecules, microRNA was able to suppressed microglia elicited neuro-inflammation [18]. In the study of Hela cell culture, cleaved caspase-3 was demonstrated to disassemble the JIP1/JNK complex and set off the initiation of apoptosis, which corresponds to our findings [17]. Through suppression of cleaved caspase-3 (at a dosage of 1000 and 2000 ug/kg/day), parecoxib was able to reduce cJNK, and attenuated associated cytokine activation.

In summary, the results of this study show that continued administration of parecoxib, at a selected dosage, is safe and efficacious in the prevention of EBI in this experimental model and is meritorious of further investigation. Decreased levels of NMDAR-1 as well as caspase-3 may contribute to the anti-apoptotic effect of this compound. Besides, parecoxib, by reducing cJNK activation, exerts a dual effect on the MAEK-related apoptotic pathway and IL-1β related inflammatory cascade.

Conclusion

The outcome of SAH patients is persistently devastating, and has changed little after decades of research and treatment focusing on cerebral vasospasm. These accumulated results arouse interest to consider the pathogenesis of SAH-induced EBI and its effects dictating the patient’s outcome. The ultra-short activated cyclooxygenase-2 following SAH leads a cascade of neuro-inflammatory reaction. The breakout of BBB accompanying SAH may be another complicated pathway underlying the development and maintenance of EBI. This study shows that administration of parecoxib diminishes SAH-induced cerebral edema and subsequent glutamate-related excitotoxicity in a rodent model of SAH. This study suggests that parecoxib, an early cyclooxygenase-2 inhibitor, could prove clinically useful in treating SAH-induced early brain injury.

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