Hippocampal Degeneration after Traumatic Brain Injury: Role of the PGE$_2$ EP1 Receptor

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Abstract

Over the past decade, prostaglandin E$_2$ (PGE$_2$) EP1 receptor blockers have been studied as a promising strategy for the treatment of neurological disorders and as a potential safer alternative to the cyclooxygenase-2 inhibitors. Preclinical data have demonstrated their efficacy in the treatment of ischemic and excitotoxic conditions by improving behavioral and anatomical outcomes and by promoting cell survival. However, according to recent reports, EP1 receptor roles are complex and the neuroprotective effects of its inhibition might be compensated or overpowered by adverse effects or toxicity in models of brain trauma and intracerebral hemorrhage. Consequently, the goal of this study was to investigate the effect of a selective EP1 receptor antagonist, SC-51089, on delayed neurodegeneration induced by traumatic brain injury (TBI) using a controlled cortical impact (CCI) model with two different injury magnitudes in mice. The data demonstrate that neurological deficit scores at 24 and 48 h after CCI rose with increasing injury magnitude. Repeated post-treatment with 20 µg/kg of SC-51089 has no significant effects on neurological deficit scores compared to vehicle groups with either magnitude. Of interest, 10 days after the severe CCI in the SC-51089 treatment group, the delayed hippocampal tissue loss was greater compared to controls. The data, in combination with published reports, suggest that the EP1 inhibition worsened delayed degenerative processes in the hippocampus at subacute time points after TBI, and that this effect is more profound with increased trauma severity, likely due to the increased contribution of hemorrhagic injury.

ABBREVIATIONS

CCI: Controlled Cortical Impact; COX: Cyclooxygenase; GPCR: G-Protein-Coupled Receptors; DG: Dentate Gyrus; NDS: Neurological Deficit Score; PGE$_2$: Prostaglandin E$_2$; PGF$_{2\alpha}$: Prostaglandin F$_{2\alpha}$; TBI: Traumatic Brain Injury

INTRODUCTION

Traumatic brain injury (TBI) is one of the major causes of death and disability and it currently has no effective treatment. TBI has a hemorrhagic and ischemic stroke component and is often associated with chronic neurological conditions such as chronic post-traumatic stress disorder, epilepsy, and cognitive dysfunction; the prevalence of these often comorbid complications is associated with the type and severity of injury resulting in brain lesions and hippocampal degeneration [1]. Brain damage following TBI results from several coexisting mechanisms, including excitotoxicity and intracerebral or subarachnoid hemorrhage and is associated with hemoglobin breakdown product toxicity, edema, and upregulation of proinflammatory mediators [2-4]. Moreover, different anti-inflammatory strategies have been currently proposed for treatment of TBI [5]. The role of mobilization of intracellular calcium ([Ca$^{2+}$]) has long been suggested as a key mechanism involved in brain pathologies of TBI [6]. Experimental and clinical evidence suggests that neuroinflammation involving the upregulation of cyclooxygenase (COX) enzymes in TBI is associated with so-called proinflammatory prostaglandins [7-11], notably prostaglandin E$_2$ (PGE$_2$) and PGF$_{2\alpha}$ [12], leading to overactivation of a class of G-protein-coupled receptors (GPCRs), particularly, EP1 and FP receptors. Interestingly, the amino acid sequences of the EP1 and FP receptors have high homology, these receptors share the same branch of the phylogenetic tree with the EP1 [13], and both GPCRs exert their effects via pathways involving [Ca$^{2+}$]$_{i}$ [13-17]. Further, it has been shown that EP1 and FP GPCRs augment brain damage in mouse preclinical models of excitotoxicity and focal cerebral ischemia [17-21]. Interestingly, the blockade of the
EP1 receptor was tested as a strategy in the treatment of epilepsy [22,23] but to reach a desired effect, it required a higher dose of one of the commonly used antagonists, SC-51089 [23], than those used in models of stroke and N-methyl-D-aspartate-induced excitotoxicity [16,17]. The preclinical data also suggest the utility of the possible therapeutic application of the EP1 receptor antagonist in neurodegenerative conditions, including Alzheimer and Huntington diseases [21,24].

Preclinical data suggest that both selective and non-selective COX-2 inhibitors used in the clinic might be also used for treatment of neurological disorders, notably in acute conditions such as stroke and TBI [7-11]. However, because prostaglandins are involved in many vital physiological functions, the clinical application of COX-2 inhibitors affecting total prostaglandin production is limited due to serious cerebrovascular and gastrointestinal adverse effects [25], and their chronic administration often requires adjuvant prostaglandin therapy to compensate for their deficits. Thus, drugs selectively targeting certain cognate prostaglandin receptors involving the downstream COX-2 cascades have been suggested as a safe and more efficient alternative in the treatment of neurological disease [13,26]. Inhibition of the EP1 receptor following acute brain injuries has long been considered a promising strategy for the treatment of neurological disorders [13,26]. However, we and others have documented that the EP1 receptor role is complex and its blockade differentially affects outcomes in different neurological conditions such as ischemia [17-21], hemorrhagic stroke [27,28], excitotoxicity [17,19,20], epilepsy [22,23], surgical brain injury [29], and TBI [30].

TBIs comprise several features of brain ischemia and intracerebral hemorrhage, and the excitotoxicity resulting from these conditions is believed to be a major triggering mechanism of secondary injuries and delayed neuronal cell death in the traumatic brain. Our recent study using a selective EP1 receptor antagonist and EP1 receptor knockout mice revealed no significant effects on the neurological deficits, anatomical pathologies, or activation of reactive astrocytes and microglia at acute time points in a model of mild-to-moderate TBI [30]; in addition, how the EP1 receptor is involved in the development of chronic degenerative processes and whether their outcomes is affected by the severity of TBI remains unclear. In human TBI, hippocampal degeneration is among well recognized pathologies associated with development of sub-acute or chronic cognitive deficits [31,32]. Neuroprotective agents affecting delayed cell death have long been considered as putative therapeutic strategies for TBI but despite some success in preclinical research they translational potential has remained elusive and discovery of new pathways as targets of novel therapeutics remain critical [33]. On the other hand, recent studies from our group demonstrated that pharmacological inhibition or genetic deletion of EP1 receptors might be a promising strategy for prevention of neuronal death in a preclinical models of cerebral ischemia involving excitotoxicity by increasing a number of viable cells in the hippocampus at sub-acute time points after stroke [21] that have not been tested in TBI. To our knowledge, only two previous studies focusing on EP1 receptor in brain trauma have been published to date. Both these studies focused on acute time points. Our study used genetic deletion and post-treatment blocking the EP1 receptor in the mild-to-moderate TBI model [30]. The second study from Dr. Zhang and colleagues in which they gave the EP1 antagonist before resecting the front lobe [29]. Thus, exploring different unexplored relevant outcomes (e.g. hippocampal degeneration and neuronal viability) and sub-acute time-points are important.

Thus, the goal of this study was to explore potential neuroprotective features of EP1 receptor inhibition on the characteristic features of human TBI-delayed hippocampal lesions and neuronal death after TBI of different severity using a preclinical animal model. Controlled cortical impact (CCI) is a common preclinical TBI model that allows us to reproduce anatomical and neurological deficits characteristic of human TBI in an experimental animal in controlled fashion including progressive hippocampal degeneration after acute impact [34-39]. In this study, we applied two different CCI parameters to produce experimental TBI with different magnitudes affecting a pattern of excitotoxic, ischemic, and hemorrhagic injury components that can be differently affected by pharmacological treatments targeting the EP1 receptor and can be accessed through neurobehavioral testing to evaluate severity of neurological deficits at acute time points, and overall brain pathology using Cresyl violet stain and hippocampal cell viability using hematoxylin and eosin stain at a sub-acute time points following experimental injury,

**MATERIALS AND METHODS**

**Animals**

Age-matched young adult (1.5–3.1 months old) wildtype C57BL/6 male mice weighing 21.4 ± 3.6 g (mean ± SD, n = 18) were used. The study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures used in this study were approved by the University of Florida Institutional Animal Care and Use Committee. All mice were housed under controlled conditions (23 ± 2°C; 12-h reversed light/dark cycle), with access to food and water ad libitum. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize the pain and distress of the experimental animals.

**Experimental animals and CCI procedures**

All surgical procedures in this study were performed accordingly to previously described protocol [30,40]. Briefly, anesthesia was induced and maintained using 4% and 2% isoflurane in a 25% oxygen/air mixture, respectively. CCI was stereotactically induced using a PCI3000 PinPoint Precision Cortical Impactor (Hatteras Instruments, Cary, NC, USA) using 3-mm diameter impact tip and a constant velocity of 3 m/sec. Two different magnitudes of injury were achieved by applying compression for distances of 1 and 1.5 mm but a constant time of 100 msec, allowing for the experimental production of mild-to-moderate and severe contusive TBI, respectively. The level of severity was assumed based on considerations of behavioral and anatomical outcomes [41]. Following the surgery, the mice were injected intraperitoneal with warm saline to prevent dehydration and placed in a temperature-controlled chamber for at least 1 h for a full recovery and were then transferred to the housing room. One SC-51089-treated animal was euthanized at a humane time point due to a complication after surgery.
Drug treatment regimen

To study the effect of the EP1 antagonist, mice were treated with three subcutaneous injections of SC-51089 at doses of 20 μg/kg immediately, and 24 and 48 h after CCI, an optimal regimen for related ischemia and excitotoxic models [16,17]. Control groups received injections of the same volume of saline. Before the second injections, mice were briefly anesthetized with isoflurane to avoid potential injury to the surgery site due to handling. Solutions of the SC-51089 were prepared in sterile saline immediately before use from stock solution aliquots (10 mg/mL in dimethyl sulfoxide) that were prepared previously and stored at -20°C [30]. A concentration of dimethyl sulfoxide in the formulation for injection was 0.05%.

Neurological Deficit Scores (NDS)

Neurobehavioral deficit scores were assessed in all mice used in the study at 24 and 48 h after CCI using a 24-point scale, as we previously described [40]. The NDS test used in the study was originally developed for testing our neurobehavioral outcomes following experimental intracerebral hemorrhage [42] and further adopted with minor modifications for TBI [40] taking into account several common features of these disorders. Briefly, NDS test comprises individual tests for assessment of animal behavior performed with minimal interaction with investigator consecutively performed in the same animal. A total NDS was calculated as a sum score obtained from the assessment of six individual tests for body symmetry, gait, circling behavior, climbing, front limb symmetry, and compulsory circling scored between 0 and 4 points for no deficit and gradually increase based on criteria of severity. The NDS testing in the treatment groups was performed side-by-side by an investigator who was blinded to the treatment and all tests were video recorded. NDS scoring was done directly during the test and the scores were confirmed off-line with video recordings. The NDS assessments were performed before consecutive SC-51089 injections performed on the first and second days after CCI.

Histopathology

Mice were survived for 10 days after injury and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in the same solution. Brains were harvested and embedded in paraffin using standard histological processing in the Cell & Tissue Analysis Core at the University of Florida McKnight Brain Institute (Gainesville, FL, USA). Coronal sections (10 μm thick) were obtained from throughout the entire brain and were processed for histological analysis using Cresyl violet and hematoxylin and eosin staining as described previously [40]. All slides were scanned using ScanScope (Aperio Technologies, Vista, CA, USA) and analyzed using ImageScope (Aperio Technologies) and ImageJ (NIH, Bethesda, MD, USA) software in a blinded manner.

Statistical analysis

Sample size was calculated by power analysis to obtain statistical significance of anticipated differences with means of at least 2 standard deviations with a significance level of α = 0.05 and a power of 1 – β = 0.80 using a build-in function of SigmaStat 3.1 software. Statistical comparisons between outcomes in drug- and vehicle-treated groups were done using Student’s t-test for quantitative histopathological data and the Mann-Whitney non-parametric rank sum test for NDS. Data are presented as the mean ± standard error, and P values <0.05 were considered significant [43]. Non-parametric data were presented using box and whisker plot showing median, range, and 25th and 75th percentile. Statistical analyses were performed using GraphPad software.

RESULTS AND DISCUSSION

Neurological deficits

NDS at 24 and 48 h were increased in all animals that underwent experimental TBI. The NDS was essentially higher in the severe TBI group than in the mild-to-moderate group, although the differences were not statistically significant between groups with different CCI magnitudes (P = 0.0796 and P = 0.1367 at 24 and 48 h, respectively, n = 4-6) (Figure 1). The level of neurological deficits after a mild-to-moderate level of experimental injury was consistent with our previous data using the same CCI parameters showing significantly increased NDS compared to the craniotomy-only sham group. Repeated treatment with SC-51089 (20 μg/kg) had no significant effect on the NDS impaired after CCI (Figure 1). The NDS scores obtained in this study were in the same range of those obtained in our previous TBI studies with respective injury severity [30,40]. In addition, the reliability of NDS scoring approach used in this study was supported by our previous studies demonstrating robustness of this test and its sensitivity to obtain statistical significance between CCI and sham groups, and treatments with small-molecules (e.g. GPCR) following TBI [40] and between wildtype and EP1 receptor knockout mice in a preclinical model of intracerebral hemorrhage [27]. The effects reported here are consistent with our previous mouse acute TBI study where we used SC-51089 at considerably higher repeated doses (100 μg/kg), which was reported to be effective in different animal models of stroke and excitotoxicity [16,17] and models of neurodegenerative diseases, including Huntington [24] and Alzheimer [21]. The lack of significant effects from SC-51089 in the TBI model could also be explained by potential caveats such as poor drug bioavailability at the target site and the specificity commonly observed in many commercially available prostaglandin receptor antagonists [44]. To avoid undesired unspecific effects, in this study, we used the lower doses of SC-51089. Furthermore, the selection of the repeated SC-51089 dose (20 μg/kg) and treatment regimen in this study was based on efficacy reports of improvement in behavioral and anatomical outcomes in two different preclinical models of acute brain injury: focal ischemia and chemically induced excitotoxicity, suggesting a direct neuroprotective effect of this EP1 receptor antagonist [16,17]. In addition, SC-51089 at a lower dose improved behavioral outcomes in a model of surgical brain injury [29]. However, repeated treatment with SC-51089, with the dose range used in this and our previous study (i.e., 20 and 100 μg/kg), did not improve NDS after CCI [30]. All aforementioned effects have also been confirmed using mice lacking the EP1 receptor [30]. In contrast, treatment with an antagonist of a functionally and structurally related FP receptor [24] improved NDS after CCI at 24- and 48-h time points [40].
**Figure 1** The effect of EP1 receptor blockade on behavioral outcomes after experimental TBI with different magnitudes. (A and B) Neurological deficits scores in saline- and EP1 antagonist SC-51089-treated animals at 24 and 48 h following CCI with a compression distance of 1.0 mm (mild-to-moderate) and 1.5 mm (severe), respectively. P values were obtained using the Mann–Whitney non-parametric rank sum test (n = 4-5 per group).

**Figure 2** The effect of EP1 receptor blockade on hippocampal degeneration outcomes after severe experimental TBI. (A and B) Cresyl violet-stained brain sections collected 10 d after CCI with the 1.5 mm compression distance. Contingency graph demonstrates fractions of animals with different levels of hippocampal degeneration (Lesion Type I and II) following CCI with a compression distance of 1.0 mm (mild-to-moderate) and 1.5 mm (severe), (n = 4-5 per group).

**Anatomical pathology**

CCI resulted in significant cortical and hippocampal lesions evident 10 d after experimental injury. The brain lesions from CCI with a compression distance of 1.0 mm (mild-to-moderate TBI) 10 d post-injury were characterized by loss of cortical tissue (i.e., cavitation) and hippocampal distortion with continued structural tissue integrity, although altered in comparison to contralateral hippocampi. These structural brain changes are consistent with our previous data using the same CCI parameters [30,40]. With an increase in magnitude using a compression distance of 1.5 mm (severe TBI), the severity of lesions was also increased. Based on evident morphopathological changes, the lesions were categorized into two groups: type I and II. The qualitative characteristics of type I lesions were similar to those observed with mild-to-moderate injury. Type II lesions were characterized as a complete loss of the ipsilateral hippocampus. Cortical cavitation was presented in both of these lesion types. It should be mentioned that CCI induces injury by compression of the cortex and other underlying brain tissues and does not directly cause hippocampal damage. The loss of cortical and hippocampal tissue occurs primarily due to secondary brain injury-induced...
necrosis involving calpain proteolysis [45,46]. Figure 2 demonstrates examples of type I and II lesions. As shown in this figure, most of the saline-treated animals have type I lesions (three of four animals tested). However, all animals in the SC-51089-treated group have type II lesions (n = 5), suggesting that repetitive post-treatment with SC-51089 worsened outcomes in severe experimental TBI. In contrast, post-treatment with a selective antagonist, SC-51089, had no significant effects on cortical and hippocampal lesions in mice with mild-to-moderate experimental TBI, which is consistent with our previous report [30]. Overall, these data are consistent with our previous data in an acute TBI model [30] and with data obtained in a related model of surgical brain injury [29].

Hippocampal cell viability

To further investigate the cellular pathology of the hippocampus, viable and dead cell numbers were assessed in all hippocampal regions 10 d after injury. Dead and dying cells in the hippocampal regions CA1, CA2, and CA3 were scattered along the pyramidal cell layer, whereas in the contralateral dentate gyrus (DG) region in both the saline and treatment groups, dead or dying cells were preferably located closer to the edge stratum oriens and/or of the polymorph layer of the CA3 region, depending on the distance from the bregma. In its ipsilateral counterpart, the dead or dying cells were more evenly spread within the granule cell layer, which was especially evident in the animals with a type II lesion, where most parts of the hippocampus were degraded. However, we did not observe differences in dead cell numbers between control and SC-51089 treatment groups. Because the ipsilateral hippocampi were completely degraded in all SC-51089-treated mice from the severe TBI group at the time points used in this study, the analysis was performed only in the mild-
to-moderate TBI group (Figure 3). Quantitative analyses on hippocampal cell viability were performed separately in the two brain segments of the rostral and caudal diencephalon located within the CCI impact area (Figure 3A). Figure 3B demonstrates typical examples of the of the alterations in the pyramidal cell layer of the ipsilateral hippocampus following CCI, with increased numbers of the degenerating or "dead" neurons that were characterized by various degrees of cell body shrinkage, intensely stained cytoplasm, and condensed or fragmented nuclei. The significant change in viable cell density was observed in the DG region of the contralateral hippocampi, whereas in its ipsilateral counterparts and other hippocampal regions, no statistically significant differences were observed (Figure 3C). No significant changes in areas covering the pyramidal or granule cell layers in CA1-3 and DG regions, respectively, between corresponding control and SC-51089 treatment groups, were observed, suggesting that the changes in density are not due to hippocampal edema. Published data obtained in a related model of surgical brain injury also demonstrated a lack of effects of the EP1 receptor antagonist SC-51089 applied prior to surgery on edema or cell death [29]. Similarly, our previous study in mild-to-moderate TBI using the same model demonstrated a lack of effect on anatomical outcomes, including cortical lesions and hippocampal edema at acute time points [29]. In contrast, post-treatment with an antagonist of the FP receptor, which is functionally and structurally related to the EP1 receptor [13], in the same TBI model and under similar treatment conditions, improved hippocampal edema but not cortical lesions [40].

Limitations of the study

One potential limitation of this study is that the drug treatment regimen was based on preclinical data obtained in a related but different preclinical model of stroke and excitotoxicity. Although acute ischemic stroke and TBI share some common features involving inflammatory pathways, and excitotoxicity is also a major mechanism triggering pathology in TBI, the temporal and spatial profiles of secondary injury, as well as involvement of EP1 receptors, might be different. However, secondary brain injury following TBI shares common features with neuropathological consequences of stroke, including oxidative stress and neuroinflammation. The usability of such an approach of justification of treatment with a prostaglandin receptor antagonist in a TBI model based on the results obtained in preclinical models of ischemic stroke was suggested from our previous study focusing on the effect of the antagonist of a functionally and structurally related FP receptor in a mild TBI model that demonstrated the similarity of the effects on some behavioral and anatomical outcomes in these models. Our previous study using SC-51089 at higher doses in two different treatment regimens and EP1 receptor knockout mice also showed no statistically significant effects compared with vehicle or wildtype controls in a mild-to-moderate TBI model [30]. On the other hand, recent studies in models of intracerebral hemorrhage performed using EP1 receptor knockout mice and its selective antagonist suggested that activation of the EP1 receptor in this condition is beneficial to overall behavioral and anatomical outcomes [27,28]. Thus, taken together, these data, in combination with previously published reports, suggest that a lack of the effects of the EP1 receptor blockade in TBI is associated with limited therapeutic properties of the compound or contribution of a specific pathway.

CONCLUSION

Although EP1 receptor blockade has been investigated as a treatment strategy for chronic and acute neurological disorders, including traumatic and surgical injuries to the brain, this preclinical study is the first report focusing on the roles of this receptor in delayed degenerative processes following TBI and linking the outcomes with severity of injury. The data obtained here suggest that treatment with the EP1 receptor antagonist may have a negative effect on TBI-induced cell death in selected hippocampal regions and global hippocampal degeneration affecting both ipsilateral and contralateral hippocampi at a subacute time points, worsening overall anatomical outcomes that are not evident at acute time points tested in our previous study [30]. The data suggest that treatment with the EP1 receptor antagonist following TBI could potentially worsen cognitive and other behavioral outcomes associated with hippocampal function. Importantly, the data suggest that the deleterious effect of EP1 blockade is more profound with increasing severity of the experimental TBI, presumably due to augmentation of the hemorrhagic component characteristic of severe brain trauma. These data, in combination with previous data obtained in preclinical models of injury associated with intracerebral hemorrhage, suggest that hemorrhagic features associated with many neurological disorders, such as hemorrhagic transformation of ischemic stroke and microbleeds associated with Alzheimer disease [47], which are also reported in various animal models [48], could be limiting factors for clinical applications of the EP1 receptor antagonists proposed as a promising strategy for treatment of certain neurological conditions, and care should be taken in further translational studies involving EP1 receptor ligands in brain disorders.

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