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Suppressing pro-inflammatory prostaglandin signaling attenuates excitotoxicity-associated neuronal inflammation and injury

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Abstract

Glutamate receptor-mediated excitotoxicity is a common pathogenic process in many neurological conditions including epilepsy. Prolonged seizures induce elevations in extracellular glutamate that contribute to excitotoxic damage, which in turn can trigger chronic neuroinflammatory reactions, leading to secondary damage to the brain. Blocking key inflammatory pathways could prevent such secondary brain injury following the initial excitotoxic insults. Prostaglandin E₂ (PGE₂) has emerged as an important mediator of neuroinflammation-associated injury, in large part via activating its EP2 receptor subtype. Herein, we investigated the effects of EP2 receptor inhibition on excitotoxicity-associated neuronal inflammation and injury *in vivo*. Utilizing a bioavailable and brain-permeant compound, TG6–10–1, we found that pharmacological inhibition of EP2 receptor after a one-hour episode of kainate-induced status epilepticus (SE) in mice reduced seizure-promoted functional deficits, cytokine induction, reactive gliosis, blood-brain barrier impairment, and hippocampal damage. Our preclinical findings endorse the feasibility of blocking PGE₂/EP2 signaling as an adjunctive strategy to treat prolonged seizures. The promising benefits from EP2 receptor inhibition should also be relevant to other neurological conditions in which excitotoxicity-associated secondary damage to the brain represents a pathogenic event.

Keywords

Blood-brain barrier; EP2 receptor; Epilepsy; Excitotoxicity; Kainate; Neuroprotection; PGE₂; Prostaglandin; Seizure

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2019.02.011>.

1. Introduction

Neuronal excitotoxicity is a pathogenic process by which brain cells and tissues are irreversibly damaged by excessive excitatory neurotransmission that is predominantly mediated by glutamate and its ion channel receptors (Traynelis et al., 2010). Exogenous excitotoxins such as kainate can over-stimulate these ionotropic receptors that allow Ca^{2+} entry into neurons, as high levels of Ca^{2+} in turn activate intracellular enzymes including endonucleases, proteases, and phospholipases, leading to cellular injury and death. Excitotoxicity caused by over stimulation of glutamate receptors has been thought a common pathophysiological mechanism underlying the aggravation of a wide range of acute and chronic neurological conditions including epilepsy, ischemia, intracranial hemorrhage, spinal cord injury, head trauma and neurodegenerative diseases (Wang and Qin, 2010; Mayor and Tymianski, 2018). As such, prolonged seizures such as status epilepticus (SE) cause initial brain injuries mainly through the overactivation of glutamate receptors (Fujikawa, 2005; Barker-Haliski and White, 2015). The excitotoxicity-associated necrotic and apoptotic neuronal death in turn directly or indirectly triggers a succession of inflammatory processes that can escalate to secondary neuronal death and the development of acquired epilepsy (Dingledine et al., 2014).

As a key pro-inflammatory mediator and immediate early gene, cyclooxygenase-2 (COX-2) is highly regulated by neuronal activities (Kaufmann et al., 1996; Hartings et al., 2017; Qiu et al., 2017), and is often rapidly and vigorously induced within the brain during excitotoxic genesis triggered by seizures or cerebral ischemia (Marcheselli and Bazan, 1996; Nakayama et al., 1998; Du et al., 2016), leading to the biosynthesis of prostaglandin E₂ (PGE₂) by brain cells. PGE₂ in turn, via acting on four membrane receptors EP1-EP4, mediates a handful of G protein-dependent and independent downstream signaling events (Dey et al., 2016; Jiang et al., 2017). Among these four PGE₂ receptors, Gas-coupled EP2 is widely expressed in the brain and is essential for some physiological functions of the central nervous system (CNS), such as neuronal plasticity, learning and memory (Savonenko et al., 2009; Yang et al., 2009). Moreover, PGE₂ signaling via neuronal EP2 receptor can defend cerebral neurons against ischemic and other excitotoxic insults in a cAMP-dependent manner (McCullough et al., 2004). However, EP2 signaling might also be associated with secondary neuronal toxicity in models of chronic inflammation and neurodegeneration (Andreasson, 2010; Jiang and Dingledine, 2013a), which has been suggested to link to brain innate immunity as EP2 receptor activation suppresses the beneficial functions of microglia in these conditions (Johansson et al., 2013, 2015). Moreover, EP2 receptor activation that initially causes microglial activation could later promote delayed death of active microglia, thereby potentially contributing to the resolution phase of brain inflammation (Quan et al., 2013; Fu et al., 2015). It appears that whether EP2 activation plays a beneficial or destructive role within the brain is very likely defined by the cellular milieu as well as the insult types, highlighting the cellular and molecular complexities present in neuroinflammatory pathways. The role of EP2 receptor in neuronal inflammation and degeneration triggered by exogenous excitotoxins such as kainate has not been studied *in vivo* to date.

Taking advantage of our recently developed small-molecule antagonists for the EP2 receptor (Jiang et al., 2012; Jiang and Dingledine, 2013b), our previous studies suggest that systemic pharmacologic inhibition of EP2 receptor overall reduced brain inflammation and injury following pilocarpine-induced seizures in mice (Jiang et al., 2013, 2015). The present study was aimed to elucidate the role of EP2 receptor in the secondary neuronal death following excitotoxic challenge *in vivo*. We examined the effects of a bioavailable and brain-permeable EP2 antagonist on functional recovery, cytokine induction, blood-brain barrier integrity, reactive gliosis, and neuronal injury in a mouse model of SE induced by systemic administration of a classical excitotoxin, kainic acid.

2. Materials and methods

2.1. Chemicals and drugs

Kainic acid was purchased from Tocris Bioscience, and injectable diazepam was from Besse Medical. Compound TG6–10–1 was initially synthesized as previously described (Ganesh et al., 2014a, 2014b, 2018), and later obtained from MedChem Express. The selectivity and potency of TG6–10–1 from different sources and batches were evaluated blindly and compared for consistency in potency and selectivity (Kang et al., 2017; Qiu et al., 2019).

2.2. Seizure induction and drug treatment

All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (the *Guide*) from the NIH. Adult C57BL/6 mice (8–10 weeks old, male) were housed under a 12-hr light/dark cycle with food and water *ad libitum*. A total of 72 mice were used in this study.

Forty-eight mice were used in the post-kainate treatment experiment: 8 mice for control and 40 for seizure induction. Kainate (30 mg/kg, i.p.) was injected into 40 mice to induce seizures that were classified as previously reported (Racine, 1972; Schauwecker and Steward, 1997) (Table 1). Saline injection was used for the 8 control mice. Twenty-one out of 40 kainate-treated mice experienced status epilepticus (SE), the onset of which was defined as the occurrence of three stage-4 seizures (Table 1). To reduce the variation that might be introduced by repeated doses of kainate, no additional kainate was administered to those 19 mice that did not enter SE initially; they thus were removed from the study. One hour after SE began, mice were treated with diazepam (10 mg/kg, i.p.) to interrupt seizures (Khan et al., 2018). After recovery for another hour, all 21 SE mice were randomized and administered either vehicle (10% DMSO, 50% PEG 400, 40% ddH₂O) or TG6–10–1 (5 mg/kg, i.p.) twice daily for three consecutive days. This treatment paradigm was designed to counteract the COX-2 induction and PGE₂ surge within the brain following SE (Jiang et al., 2015). Thus, compound TG6–10–1 should be able to suppress majority of the pathological function of PGE₂/EP2 signaling; whereas its impact on the normal physiological role of the receptor should be limited (Du et al., 2016). During recovery, animals were fed moistened rodent chow, monitored daily and injected with 5% dextrose in lactated Ringer's solution (Baxter) (0.5 ml, s.c. or i.p.) when necessary. All 10 antagonist-treated SE mice and 9 out of 11 vehicle-treated SE mice survived. The 8 control mice were

also randomly treated with vehicle or TG6–10–1, as they all survived. After three days, all 27 surviving animals were euthanized under deep anesthesia with isoflurane and perfused with ice-cold phosphate buffered saline (PBS) to wash blood out of the brain. All 27 mouse brains were collected and cut into two hemispheres: one hemisphere was immersed in 4% paraformaldehyde fixative overnight for further histological analysis; the other hemisphere was dissected for hippocampus for biochemical study.

Twenty-four mice were used for the pre-kainate treatment experiment and divided into 2 groups: 12 in vehicle group; 12 in antagonist group. For pre-kainate treatment, mice were treated with either vehicle or compound TG6–10–1 (5 mg/kg, i.p.), and 30 min later by kainate (30 mg/kg, i.p.). Animal behavioral seizures were observed and classified for up to 3 h using the Racine scale (Table 1).

2.3. Animal behavioral tests

Animal functional recovery from SE was monitored daily by using two simple behavioral tests – nest building and Irwin test, as they can be done quickly without posing extra stress during the post-SE recovery. Nesting is a sensitive indicator of brain lesion especially in the hippocampus (Deacon, 2006), thus normal nesting activity might suggest reduced hippocampal damage. The ability of an animal to construct its nest overnight from a supplied nestlet square (2 " × 2 ") was assessed on a rating scale of 1–5.1: the nestlet was more than 90% intact; 2: nestlet was partially torn with 50–90% untouched; 3: more than 50% nestlet was shredded without a nest site; 4: an obvious but flat nest was built; 5: a perfect nest with walls higher than mouse body was built. A modified Irwin test was used as another measurement for animal functional recovery after SE (Roux et al., 2005). Animals were observed for ptosis, exophthalmia, lacrimation, body posture, running and walking, hypoactivity, hypothermia, and vocalization when handled. Each observation item was scored as: 0 = normal; 1 = mild to moderate impairment; 2 = severe impairment.

2.4. Quantitative real-time PCR (qPCR)

The total RNA from mouse hippocampus was isolated using TRIzol (Invitrogen) with the PureLink RNA Mini Kit (Invitrogen). RNA purity and concentration were measured by A260/A280 ratio and A260 value, respectively. The first-strand complementary DNA (cDNA) synthesis was performed with 1 µg RNA, 0.25 µg random primers, and 200 units of SuperScript II Reverse Transcriptase (Invitrogen) in a reaction volume of 20 µl at 42 °C for 50 min. The reaction was ended by heating at 70 °C for 15 min. The qPCR was performed using 8 µl of 50 × diluted cDNA, 0.4 µM of primers, and 2 × B-R SYBR® Green SuperMix (Quanta BioSciences) with a final volume of 20 µl in the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories) (Jiang et al., 2015). Cycling conditions were as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and then 60 °C for 1 min. Melting curve analysis was used to verify single-species PCR product. Fluorescent data were acquired at the 60 °C step. The cycle threshold for GAPDH was subtracted from the cycle threshold measured for each gene of interest to yield CT. Samples without cDNA template served as negative controls. Primers used for qPCR are listed in Table 2.

2.5. Western blot analysis

Hippocampal tissues were homogenized on ice in 0.5 ml RIPA buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS) plus a mixture of protease and phosphatase inhibitors (Roche Applied Science). The homogenates were centrifuged (13,000×g, 15 min, 4 °C) and protein concentration in the supernate was measured by Bradford assay (Thermo Fisher Scientific). The supernates (10 µg protein each) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk at room temperature for 2 h, then incubated overnight at 4 °C with primary antibodies: mouse anti-GFAP (1:2,000, Santa Cruz Biotechnology), rabbit anti-Iba1 (1:2,000, Wako Chemicals), rabbit anti-albumin (1:5,000, GenWay Biotech), or mouse anti-GAPDH (1:1,000, Abcam). This procedure was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3,000, Santa Cruz Biotechnology) at room temperature for 2 h. The blots were developed by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific) and scanned. The band intensity was quantified using ImageJ (NIH) (Jiang et al., 2015). The protein expression level of a specific gene was first normalized to the loading control GAPDH and then to the mean of control group, which was set as 100%.

2.6. Histopathology

Fixed mouse brains were embedded in paraffin and 8-µm coronal sections were prepared (Jiang et al., 2012, 2013). The paraffin wax was removed by xylene. For immunostaining, the sections first were permeabilized with 0.25% Triton X-100 in PBS for 15 min and blocked in 10% goat serum in PBS for 60 min. This procedure was followed by incubation with primary antibodies: rabbit anti-GFAP (1:750, Abcam) or rabbit anti-Iba1 (1:750, Wako Chemicals) at 4 °C overnight. Then the sections were incubated with fluorescent secondary antibody – goat anti-rabbit Alexa Fluor 488 (Invitrogen) at room temperature for 2 h. Images were obtained using EVOS FL Auto Cell Imaging System (Invitrogen).

Neuronal injury in the hippocampus was assessed by Fluoro-Jade staining. In brief, sections were immersed in 0.06% potassium permanganate for 15 min with gentle agitation, rinsed for 1 min in distilled water, and then transferred to the Fluoro-Jade B reagent solution (0.001%, w/v, in distilled water with 0.1% acetic acid) for 30 min with gentle agitation in the dark. Sections were rinsed with three 1 min changes of distilled water and rapidly air dried. The slides were immersed in xylene and then coverslipped with D.P.X. mountant (Sigma-Aldrich). Sections between bregma –1.31 and –2.69 (one for every 10) were examined with a fluorescence microscope. The neuronal injury of each animal was indicated by the average count of Fluoro-Jade positive cells (~15 sections/mouse). The evaluation of cell injury was done without knowledge of treatment.

2.7. Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software) by one- or two-way ANOVA with post-hoc Bonferroni or Dunnett's test, *t*-test, Fisher's exact test, or Mann-Whitney *U* test as appropriate. Outliers were searched for by the Grubbs' test, and none was

found in this study. $P < 0.05$ was considered statistically significant. All data are presented as mean $\pm$ or $\pm$ SEM.

3. Results

3.1. Post-treatment with EP2 antagonist facilitates functional recovery after systemic administration of kainate

We previously reported that pharmacological inhibition of EP2 receptor reduced hippocampal inflammation and neurodegeneration following pilocarpine-induced SE in mice (Jiang et al., 2013, 2015). However, it is important to confirm these benefits in another classical seizure model because model-specific findings are unlikely relevant to human conditions and could mislead future translational efforts. The experimental design is shown in Fig. 1A. We chose to use EP2 antagonist TG6–10–1 because it has improved pharmacokinetics properties, such as plasma half-life and brain-plasma ratio among the first-generation EP2 antagonists (Ganesh et al., 2013, 2014a, 2014b). A single dose of kainate (30 mg/kg, i.p.) was used to induce seizures in mice; no additional kainate was administered to animals that did not enter SE after the initial kainate injection and thus were excluded from the experiment. SE was allowed to proceed for about 1 h and then interrupted by treatment with diazepam, which was followed by intraperitoneal administration of TG6–10–1 to animals twice daily. All mice that survived SE were sacrificed three days after SE for biochemical and histological analyses.

There was no difference in either seizure progression [$F(1, 399) = 0.1667$, $P = 0.6833$] (Fig. 1B) or latency to SE [$t(19) = 1.576$, $P = 0.1316$] (Fig. 1C) between vehicle and EP2 antagonist groups after kainate injection, confirming that mice from these two experimental groups had been effectively randomized. There was no statistical difference in 3-day mortality after kainate SE between these two groups ($P = 0.4762$) (Fig. 1D). However, treatment with the EP2 antagonist accelerated the regain of animal weight when compared to vehicle-treated mice [$F(1, 72) = 9.808$, $P = 0.0025$; $P = 0.0295$ at day 3] (Fig. 1E). In addition, mice that received TG6–10–1 treatment showed improved nesting activity following SE [$F(1, 72) = 26.94$, $P < 0.0001$; $P < 0.001$ at days 2 and 3] (Fig. 1F), and reduced Irwin scores [$F(1, 72) = 29.46$, $P < 0.0001$; $P = 0.0124$ at day 1; $P < 0.001$ at day 2; $P = 0.0050$ at day 3] (Fig. 1G). These behavioral observations suggest that the post-administration of EP2 antagonist TG6–10–1 improved functional recovery of mice from kainate-induced SE.

3.2. Effect of post-SE inhibition of EP2 receptor on inflammation-associated genes

Prolonged seizures can trigger a series of inflammatory reactions and processes within the brain that can precede the development of epilepsy, i.e., epileptogenesis (Loscher et al., 2013; Vezzani et al., 2013; Varvel et al., 2015). We next examined the effect of the EP2 antagonist on seizure-induced brain inflammation by measuring the mRNA expression of a number of inflammation-associated genes within the hippocampus three days after kainate-induced SE. We found that post-SE treatment with TG6–10–1 decreased the SE-promoted hippocampal expression of oxidative stress-related enzymes including phagocyte NADPH oxidase (NOX-2 or gp91^{phox}) [$F(3, 23) = 13.5$, $P < 0.0001$] and inducible nitric oxide

synthase (iNOS) [$F(3, 23) = 5.784, P = 0.0042$], as well as a number of key pro-inflammatory cytokines and chemokines, such as interleukin 1 β (IL-1 β) [$F(3, 23) = 10.5, P = 0.0002$], IL-6 [$F(3, 23) = 3.035, P = 0.0497$], chemokine (C-C motif) ligand 2 (CCL2) [$F(3, 23) = 6.184, P = 0.0031$], CCL3 [$F(3, 23) = 19.43, P < 0.0001$], CCL4 [$F(3, 23) = 13.3, P < 0.0001$], transforming growth factor β 1 (TGF- β 1) [$F(3, 23) = 18.39, P < 0.0001$], and tumor necrosis factor α (TNF- α) [$F(3, 23) = 4.353, P = 0.0144$] (Fig. 2). Interestingly, hippocampal COX-2 [$F(3, 23) = 3.115, P = 0.0459$], microsomal prostaglandin E synthase-1 (mPGES-1, i.e., the inducible form of PGE₂ synthase) [$F(3, 23) = 2.038, P = 0.1365$], and EP2 [$F(3, 23) = 4.76, P = 0.01$] were also significantly induced by kainate SE, suggesting an elevated COX-2/mPGES-1/PGE₂/EP2 signaling pathway as a whole within the hippocampus following SE. Post-SE treatment by the EP2 antagonist prevented the significant increase of mRNA levels of all three inflammatory prostaglandin signaling-associated genes (Fig. 2). These qPCR results together suggest that EP2 receptor activation might play an important role in the induction of these inflammation-associated genes within the hippocampus following prolonged seizures.

3.3. EP2 receptor inhibition decreases seizure-promoted reactive gliosis

Featured by the proliferation and hypertrophy of glial cells including astrocytes and microglia, reactive gliosis is not just considered a common neuropathologic characteristic but also a potential contributor to many CNS disease mechanisms including acquired epileptogenesis (Varvel et al., 2015; Aronica et al., 2017; Wyatt-Johnson et al., 2017). We found that a one-hour episode of SE was sufficient to promote marked gliosis within the hippocampus, assessed three days after SE by measuring the expression of GFAP for astrogliosis and Iba1 for microgliosis at both mRNA level [GFAP: $F(3, 23) = 21.54, P < 0.0001$; Iba1: $F(3, 23) = 15.57, P < 0.0001$] (Fig. 3A) and protein level [GFAP: $F(3, 23) = 26.8, P < 0.0001$; Iba1: $F(3, 23) = 6.328, P = 0.0027$] (Fig. 3B). However, the post-SE treatment with EP2 antagonist TG6–10–1 substantially decreased SE-induced expression of these two reactive gliosis biomarkers within the hippocampus also at both mRNA level ($P < 0.05$ for GFAP; $P < 0.001$ for Iba1) (Fig. 3A) and protein level ($P < 0.01$ for GFAP; $P < 0.05$ for Iba1) (Fig. 3B). Immunohistochemistry results further revealed that the seizure-induced glial proliferation occurred throughout the hippocampal subregions CA1, CA3 and dentate hilus (Fig. 3C). These results together demonstrate that EP2 receptor activation is involved in the hippocampal gliosis following kainate-induced SE.

3.4. Effect of EP2 receptor inhibition on blood-brain barrier integrity after seizures

With the neuroinflammatory reactions and processes increasing in response to insults to the brain, the neurovascular units can become more permeable (Hawkins and Davis, 2005). As such, blood-brain barrier damage was found in both human patients with epilepsy and rodents after prolonged seizures (Marchi et al., 2007). The integrity of blood-brain barrier can be assessed by measuring the degree of extravasation of serum proteins such as albumin into the brain parenchyma (van Vliet et al., 2007). Here we found that a one-hour episode of SE induced by kainate was able to increase the permeability of the blood-brain barrier, demonstrated by the augmented presence of serum albumin in the hippocampus three days after SE detected by western blot analysis [$F(3, 23) = 4.49, P = 0.0127$]; while the SE-induced albumin extravasation was largely prevented by the post-SE treatment with EP2

antagonist TG6–10–1 ($P < 0.05$) (Fig. 4). These results together revealed a powerful anti-inflammatory action by post-SE inhibition of EP2 receptor that can restore the blood-brain barrier integrity in this mouse kainate seizure model.

3.5. EP2 antagonist reduces SE-promoted hippocampal injury

Repetitive severe seizures can kill brain cells and also lead to chronic epilepsy, thus engagement of neuronal death pathways has been proposed to be an essential step of acquired epileptogenesis, particularly in the adult brain (Dingledine et al., 2014). We thus next evaluated neuronal injury in the hippocampus three days after kainate SE. Coronal brain sections were prepared and stained with Fluoro-Jade B reagent to label degenerating neurons, and the positively stained cells in each section were counted to indicate the injury level. Kainate-induced SE caused substantial hippocampal neuronal damage in vehicle-treated mice (Fig. 5A); whereas the post-SE treatment with EP2 antagonist TG6–10–1 reduced the Fluoro-Jade B positive cells by 90% ($P < 0.001$) in CA1, 80% in CA3 ($P < 0.05$), and 70% ($P < 0.01$) in the dentate hilus (Fig. 5B).

Furthermore, in these SE mice ($N = 19$) we found a positive correlation between neuronal injury indicated by Fluoro-Jade B staining and neuroinflammation implied by the mRNA levels of IL-1 β , IL-6 and TNF- α in all three hippocampal subregions CA1 ($R = 0.62042$, $P = 0.00459$), CA3 ($R = 0.57553$, $P = 0.00993$) and dentate hilus ($R = 0.59341$, $P = 0.0074$) (Fig. 6). We chose the expression of IL-1 β , IL-6 and TNF- α as a surrogate for neuroinflammation because these three prototypical inflammatory cytokines are the most widely studied cytokines in animal seizure models and human epilepsy patients, owing to their contributions to inflammation within the brain and epileptogenesis (Wilcox and Vezzani, 2014; Vezzani and Viviani, 2015; Patel et al., 2017). Though an increase in the mRNA levels of cytokines does not necessarily always lead to an increase in their protein levels, these positive correlations insinuate that the neuroprotection from inhibiting EP2 receptor activation is strongly associated with the anti-inflammatory action of the antagonist.

3.6. Pre-treatment of EP2 antagonist has no effect on kainate-induced seizures

So far, we demonstrated that post-kainate treatment with EP2 antagonist TG6–10–1 showed evident anti-inflammatory and neuroprotective effects. However, these benefits from EP2 inhibition after SE might be the direct result of a potential anticonvulsant effect. To explore this possibility, we pre-treated mice with TG6–10–1 about 30 min before kainate administration (Fig. 7A), and the behavioral seizures were scored using the Racine scale (Table 1). Pre-treatment with TG6–10–1 did not alter either the temporal evolution of convulsive seizures after kainate administration [$F(1, 721) = 0.03001$, $P = 0.8625$] (Fig. 7B) or the latency of mice to generalized seizures [stage-3: $t(20) = 0.824$, $P = 0.4196$; stage-4: $t(17) = 1.19$, $P = 0.2503$; stage-5: $t(13) = 1.075$, $P = 0.3019$] (Fig. 7C). Thus, pre-treatment with TG6–10–1 did not modify the progression of or animal susceptibility to kainate-induced convulsive seizures. The neuroprotection from inhibiting EP2 receptor can likely be attributed to the anti-inflammatory action, instead of a direct anticonvulsant effect, of the antagonist (Fig. 7D).

4. Discussion

Our study was designated to evaluate the effects of pharmacological inhibition of EP2 receptor on excitotoxicity-associated neuronal injury and inflammation *in vivo*. We found that systemic treatment with a brain-permeable EP2 antagonist TG6–10–1 after kainate-induced SE in mice accelerated functional recovery, decreased cytokine induction and reactive gliosis, prevented blood-brain barrier deterioration, and reduced neuronal injury in the hippocampus. Importantly, these beneficial effects from EP2 inhibition were unlikely the direct result of reduced seizure activities, as pre-treatment with the EP2 antagonist had no effect on the progression of convulsive SE after kainate administration regarding the seizure duration or intensity.

The EP2 receptor is expressed in brain neurons and glia to mediate a wide range of physiological and pathological functions. Intriguingly, accumulating evidence from recent studies on cell type specific ablation in animal models of CNS conditions such as Parkinson's disease and Alzheimer's disease suggests that EP2 receptor activation in innate immune cells – particularly microglia – is detrimental (Andreasson, 2010; Johansson et al., 2013, 2015). In contrast, neuronal EP2 receptor activation by PGE₂ or butaprost has been demonstrated to be neuroprotective against glutamate receptor-mediated excitotoxicity in cortical/hippocampal primary neuronal cultures in the absence of microglia (McCullough et al., 2004). We also reported neuroprotection by several positive allosteric modulators that selectively potentiate EP2 receptor activation (Jiang et al., 2010, 2018). These seemingly conflicting results supports our hypothesis that EP2 receptor activation exerts both beneficial and deleterious effects, which might be determined by the injury types and the responding cellular and molecular components (Jiang and Dingledine, 2013a). Whether the EP2 receptor activation in microglia is detrimental and neuronal EP2 signaling exerts beneficial effects remain to be determined in animal seizure models. Nonetheless, results from the present study suggest that pharmacological inhibition of EP2 receptor after kainate SE is overall anti-inflammatory and neuroprotective (Fig. 7D).

Pilocarpine and kainate are the two most widely used chemoconvulsants for seizure induction in rodents. These two models share many features, e.g., SE can last for several hours, and then is followed by a tranquil phase of a few days or weeks without noticeable seismic activity. Later on, the animals gradually display unprovoked, spontaneous recurrent seizures (SRSs) with increasing frequency and no remission (Covolan and Mello, 2000; Reddy and Kuruba, 2013). In addition, SE induced by pilocarpine or kainate promotes long-lasting brain inflammation and injury in rodents (Pont et al., 1995; Maj et al., 1998; Borges et al., 2003; Rizzi et al., 2003; Varvel et al., 2015; Umpierre et al., 2016), which also simulates human SE conditions. However, pilocarpine triggers seizures via activating muscarinic acetylcholine receptor M1 subtype (Bymaster et al., 2003), whereas kainate is pro-convulsive owing to its acting on the glutamate receptor subtype GluR6 in principal neurons and GluR5 in interneurons (Mulle et al., 1998; Ben-Ari, 2010). Thus, these two chemoconvulsants will have distinct peripheral and central effects. Indeed, less than 5% of gene expression changes in dentate granule cells observed after pilocarpine, kainate or electrically-triggered SE were in common (Dingledine et al., 2017), attesting to fundamental differences among these animal models. We previously reported that the pharmacological

inhibition of EP2 receptor commencing 4 h after the onset of pilocarpine-induced SE recapitulated most benefits of the conditional ablation of COX-2 restricted to forebrain neurons (Serrano et al., 2011; Levin et al., 2012), such as increased post-SE survival, reduced functional deficit and blood-brain barrier impairment, and decreased brain inflammation and injury (Jiang et al., 2013, 2015). In the present study, these beneficial effects of EP2 inhibition or COX-2 ablation were also demonstrated in the mouse kainate SE model, suggesting they are not model-specific findings. Importantly, these results together exclude any direct effect of the tested compound on either acetylcholine system or glutamate system; rather, the beneficial effects of TG6–10–1 likely derive from its anti-inflammatory actions.

5. Conclusion

In summary, pharmacological inhibition of EP2 receptor affords a broad spectrum of anti-inflammatory and neuroprotective effects after kainate-induced SE in mice. The benefits from post-SE treatment with TG6–10–1 are unlikely model specific (Jiang et al., 2013), thereby holding promise in translational possibility. Our data present reinforcing evidence for the feasibility that pharmacologically targeting a specific downstream prostanoid receptor can provide an alternative strategy to the blockade of the entire COX cascade, in the wake of controversial and untoward consequences of COX inhibition in epilepsy studies (Rojas et al., 2014, 2019; Dey et al., 2016; Dhir, 2019; Ravizza and Vezzani, 2018). It should be noted that diazepam alone cannot completely terminate SE in animal models. Instead, the anticonvulsant gradually mitigates both electrographic activities and convulsive seizures, which usually can last for several hours after the administration of diazepam and then subside (Gualtieri et al., 2012; Rojas et al., 2018). Whether TG6–10–1 provides similar anti-inflammatory and neuroprotective effects in the absence of anticonvulsant treatment would be an interesting topic for the future studies. Nonetheless, our findings support the notion of EP2 antagonism as an adjunctive strategy, together with anticonvulsants and glutamate receptor blockers, to treat SE (Fig. 7D). Future studies should also be directed to determine whether EP2 receptor inhibition prevents and/or modifies the development of SRSs following SE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Prostaglandin E2 (PGE₂) is an essential pro-inflammatory mediator largely via activating the EP2 receptor.
- The effects of an EP2-selective antagonist on excitotoxicity in kainate-treated mice were evaluated.
- Inhibition of the EP2 receptor mitigated functional deficits associated with kainate-induced status epileptic (SE).
- EP2 inhibition after SE reduced cytokine induction, reactive gliosis, blood-brain barrier breakdown, and neuronal injury.
- Blocking PGE₂/EP2 signaling did not alter the intensity or duration of convulsive seizures induced by kainic acid.

Fig. 1. Post-SE treatment with EP2 antagonist facilitates functional recovery after SE.

(A) Experiment procedure for post-treatment of EP2 antagonist. Mice were injected with kainate (30 mg/kg, i.p.) to induce seizures. The SE was allowed to persist for 60 min and interrupted by diazepam (10 mg/kg, i.p.). Then mice were randomly treated with vehicle (10% DMSO, 50% PEG 400, 40% ddH₂O) or EP2 antagonist TG6-10-1 (5 mg/kg, i.p.) twice daily for three consecutive days. The animals were checked daily for body weight, mortality and behavior. (B) After kainate injection, mouse behavioral seizure score was tabulated every 5 min until the seizure was interrupted by diazepam 1 h after SE onset. (C)

The latency to reach behavioral SE after kainate injection (*N.S.* = not significant, *t*-test). **(D)** 3-day survival rates of animals that received vehicle or TG6–10–1 after kainate SE (*N.S.* = not significant, Fisher's exact test). **(E)** Animal body weight change after SE (**P* < 0.05 compared with vehicle group, two-way ANOVA with *post-hoc* Bonferroni test). **(F)** Animal nesting behavior after SE (****P* < 0.001 compared with vehicle group, two-way ANOVA with *post-hoc* Bonferroni test). Data are shown as mean ± SEM. **(G)** Modified Irwin test after SE (**P* < 0.05; ***P* < 0.01; ****P* < 0.001 compared with vehicle group, two-way ANOVA with *post-hoc* Bonferroni test). Data are shown as mean ± SEM (*N* = 11 for vehicle group and 10 for TG6–10–1 group).

Fig. 2. EP2 inhibition decreases the induction of pro-inflammatory mediators after SE.

The expression of tested inflammatory genes in hippocampus was measured by qPCR for their mRNA levels in mice 3 days after kainate SE (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA and post-hoc Bonferroni test). Data are shown as mean $\pm$ SEM ($N = 9$ and 10 for vehicle group and TG6-10-1 group, respectively).

Fig. 3. Post-SE treatment with EP2 antagonist mitigates seizure-induced glial activation. Three days after kainate-induced SE, the expression of biomarkers GFAP for astrocyte activation and Iba1 for microglia in the mouse hippocampus were measured by qPCR for their mRNA levels (**A**) and western blot for protein levels (**B**) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA and *post-hoc* Bonferroni test). Data are shown as mean $\pm$ SEM ($N = 9$ and 10 for vehicle group and TG6–10–1 group, respectively). (**C**) Hippocampal immunostaining for GFAP and Iba1 to visualize reactive astrocytes and microglia, respectively, in mice 3 days after kainate-induced SE. Scale bar, 500 μ m.

Fig. 4. EP2 antagonist preserves blood-brain barrier integrity following SE.

(A) Three days after kainate-induced SE, serum albumin leak into the hippocampal parenchyma of saline-perfused brains was used to evaluate the integrity of the blood-brain barrier and was assessed by western blot analysis with GAPDH as loading control. (B) The Western blot film was scanned, and the band intensity was quantified by ImageJ to indicate the relative level of albumin protein within the hippocampus (* $P < 0.05$, one-way ANOVA and *post-hoc* Dunnett's test). Data are shown as mean $\pm$ SEM ($N = 9$ and 10 for vehicle and TG6-10-1 treatment, respectively).

Fig. 5. EP2 inhibition decreases kainate-induced neuronal injury in the hippocampus. (A) Neuronal injury in the hippocampus was visualized by Fluoro-Jade B staining 3 days after kainate SE. Scale bar, 100 μ m. (B) Quantification of degenerating neurons in hippocampal subregions CA1, CA3, and dentate hilus (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, Mann-Whitney U test). Data are shown as mean +SEM ($N = 9$ and 10 for vehicle and TG6-10-1 treatment).

Fig. 6. Correlation between neuroinflammation and neuronal injury after SE.

Pearson's correlation coefficient analysis was performed to examine the relationship between neuronal damage shown by Fluoro-Jade B positive cells and neuroinflammation indicated by the mRNA expression levels of three prototypical inflammatory cytokines (IL-1 β , IL-6 and TNF- α) separately (**3 top rows**) or jointly (**bottom row**) in the hippocampus of mice that experienced kainate SE ($N = 19$).

Fig. 7. Pre-treatment with EP2 antagonist has no effect on kainate-induced seizures.

(A) Experiment procedure for pretreatment with EP2 antagonist (5 mg/kg, i.p.) before seizure induction by kainate (30 mg/kg, i.p.) in mice. (B) The behavioral seizure score was tabulated every 5 min for up to 3 h ($N = 12$ mice per group). (C) Latency of mice to generalized seizures (stages 3, 4 and 5) after kainate administration in mice (*N.S.* = not significant, *t*-test). Data are shown as mean $\pm$ SEM. (D). A proposed model depicting the mode of action of TG6-10-1 for SE treatment. Prolonged seizures cause excitotoxicity-associated neuronal death, which in turn directly or indirectly triggers a succession of inflammatory processes such as COX cascade, cytokine storm and reactive gliosis within the

brain that can escalate to secondary neuronal death. EP2 antagonism is emerging as an adjunctive anti-inflammatory strategy, along with anticonvulsants and glutamate blockers, to treat SE.

Table 1

Modified Racine scale for convulsive seizures after kainate administration.

Seizure Score	Observed Motor Behavior
0	Normal behavior – walking, exploring, sniffing, grooming
1	Arrest and rigid posture
2	Head bobbing
3	Partial body clonus (unilateral forelimb clonus), myoclonic jerk, lordotic posture
4	Rearing with bilateral forelimb clonus
5	Rearing and falling (loss of postural control)
6	Tonic-clonic seizure with running and jumping
7	Death

Table 2

Primer sequences for qPCR.

Gene	Forward primer	Reverse primer
NOX-2	5'-TGCCACCAGTCTGAAACTCA-3'	5'-GCATCTGGGTCTCCAGCA-3'
iNOS	5'-CCTGGAGAGCCACACACTG-3'	5'-CCATGATGGTGCACATTCTGC-3'
IL-1 β	5'-TGAGCACCTTCTTTTCTTCA-3'	5'-TTGTCTAATGGGAACGTCACAC-3'
IL-6	5'-TCTAAATTCATATCTTCAACCAAGAGG-3'	5'-TGGTCCCTTAGCCACTCCTTC-3'
CCL2	5'-CATCCACGTGTGGCTCA-3'	5'-GCTGCTGGTGATCCTCTTGTA-3'
CCL3	5'-TGCCCTTGCTGTCTCTCT-3'	5'-GTGGAATCTTCGGGTGTAG-3'
CCL4	5'-CATGAAGCTCTGCGTGTG-3'	5'-GGAGGGTCAAGCCCAATT-3'
TGF- β 1	5'-TCAGACATTCGGGAAGCAGT-3'	5'-ACGCCAGGAATTGTTGCTAT-3'
TNF- α	5'-TTCCTCCCAAGGCTATAAAGG-3'	5'-CTGTCTCCTCCTGGGCTAGT-3'
COX-2	5'-CTCCACCGCCACCACTAC-3'	5'-TGGATTGGAACAGCAAGGAT-3'
mPGES-1	5'-ATCAAGATGTACGGGTGGC-3'	5'-GAGGAATGTATCCAGGCGA-3'
EP2	5'-TCTTTAGTCTGGCCACGATGCTCA-3'	5'-GCAGGGAACAGAGCAAGGAGG-3'
GFAP	5'-GACAACTTTGCACAGGACCTC-3'	5'-ATACGCAGCCAGGTTGTTCT-3'
Iba1	5'-GGATTTCAGGGAGGAAAAG-3'	5'-TGGGATCATCGAGGAATTG-3'
GAPDH	5'-TGTCCTGCTGTGGATCTGAC-3'	5'-CCTGCTTCAACCCTTCTTG-3'