Inhibition of the prostaglandin receptor EP2 following status epilepticus reduces delayed mortality and brain inflammation

Jianxiong Jiang1, Yi Quan2, Thota Ganesh3, Wendy A. Pouliti3, F. Edward Dudek4, and Raymond Dingledine3

1Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322; and 2Department of Physiology, University of Utah School of Medicine, Salt Lake City, UT 84108

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Prostaglandin E2 is now widely recognized to play critical roles in brain inflammation and injury, although the responsible prostaglandin receptors have not been fully identified. We developed a potent and selective antagonist for the prostaglandin E2 receptor subtype EP2, TG6-10-1, with a sufficient pharmacokinetic profile to be used in vivo. We found that in the mouse pilocarpine model of status epilepticus (SE), systemic administration of TG6-10-1 completely recapitulates the effects of conditional ablation of cyclooxygenase-2 from principal forebrain neurons, namely reduced delayed mortality, accelerated recovery from weight loss, reduced brain inflammation, prevention of blood-brain barrier opening, and neuroprotection in the hippocampus, without modifying seizures acutely. Prolonged SE in humans causes high mortality and morbidity that are associated with brain inflammation and injury, but currently the only effective treatment is to stop the seizures quickly enough with anticonvulsants to prevent brain damage. Our results suggest that the prostaglandin receptor EP2 is critically involved in neuroinflammation and neurodegeneration, and point to EP2 receptor antagonism as an adjunctive therapeutic strategy to treat SE.

inflammatory cytokine | electroencephalography | epileptogenesis | gliosis | neuronal injury

As a dominant product of cyclooxygenase-2 (COX-2 or PTGS2) in the brain, prostaglandin E2 (PGE2) is emerging as a crucial mediator of many COX-2-driven pathological events in the central nervous system (CNS) (1). PGE2 acts on 4 G protein-coupled receptors named EP1, EP2, EP3, and EP4. Among these, the EP2 receptor is widely expressed in the brain and plays important physiologic functions, such as in neuronal plasticity (2, 3). However, recent studies have identified a possible link between EP2 signaling and secondary neurotoxicity in models of chronic inflammation and neurodegeneration (1, 4-6). In a rodent model of amyotrophic lateral sclerosis, for example, EP2 receptor knock-out mice exhibit improved survival, down-regulation of proinflammatory enzymes, and reduced oxidative stress (6).

Prolonged status epilepticus (SE) in humans is associated with brain injury and substantial morbidity. Mortality is high during refractory SE that requires general anesthesia (7, 8), and the 30-d mortality is about 35-37% for adults who experience at least 60 min of SE (9). Outcome in humans is dependent upon age, etiology, and SE duration (8-10), and currently the only effective treatment is to stop the seizures quickly enough to prevent brain damage (10). Most deaths from nonrefractory SE occur in the 2-wk period after successful treatment rather than during the seizure episode itself (9), pointing to a delayed but cascading set of responsible events. In mice, prolonged SE induced by pilocarpine causes >25% delayed mortality (11), and is associated with a series of molecular and cellular events in the brain, including neurodegeneration, and selective inflammatory reactions involving reactive microglia and astrocytes (12). Although the underlying cellular and molecular mechanisms are incompletely understood, it is known that the rapidly up-regulated COX-2 after seizures promotes brain inflammation and secondary neurodegeneration (13-19). We recently showed that COX-2 of neuronal origin was responsible for these effects (19). However, which of the nine prostanoid receptors (1) mediates SE-induced inflammation and neurodegeneration is not fully understood.

We hypothesize that EP2 receptor activation contributes to neuronal injury and associated morbidities following SE. We reported previously that brief exposure of mice to a modestly brain-permeable EP2 antagonist soon after pilocarpine-induced SE reduced early neurodegeneration in the hippocampus as assessed 24 h later (20). Here we report that administration of an EP2 antagonist with improved pharmacokinetic properties in mice beginning 4 h after pilocarpine-induced SE produced a broad range of beneficial effects, including a sharp reduction in delayed mortality and neuroinflammation through a mechanism of action that does not involve acute anticonvulsant effects. Recognizing the untoward consequences of COX-2 inhibitors (21), EP2 receptor inhibition could be an appealing therapeutic strategy to reduce brain inflammation and injury following SE.

Results

Development and Characterization of EP2 Receptor Antagonist. We previously reported a prostaglandin receptor EP2 antagonist, TG4-155, with a relatively short plasma half-life (~0.6 h) and low brain:plasma ratio (~0.3) after systemic administration in mice (20, 22). We recently created an analog compound, TG6-10-1 (Fig. S1A), which has a superior pharmacokinetic profile making it suitable for more extensive testing. EP2 receptor activation by PGE2 stimulates adenylate cyclase to elevate cytoplasmic cAMP level. We used a time-resolved fluorescence resonance energy transfer assay to monitor PGE2-induced cAMP accumulation in C6 glioma (C6G) cells overexpressing human EP2 receptor (23). The potency of compound TG6-10-1 was evaluated by its effects on the concentration–response curves of PGE2 in C6G-EP2 cells. Cells were incubated first with vehicle, 0.01, 0.1, 1, or 10 μM TG6-10-1 for 10 min, and then with increasing concentrations of PGE2 for 40 min to activate the EP2 receptor. TG6-10-1 produced concentration-dependent, parallel rightward shifts in the PGE2 concentration–response curve without affecting the maximal response (Fig. S1B). Schild regression analysis indicated that TG6-10-1 has a competitive mechanism of antagonism of the EP2 receptor with an equilibrium dissociation constant for the antagonist-receptor complex (Kd) of 17.8 nM (Fig. S1C).


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To whom correspondence should be addressed. E-mail: jjiang3@emory.edu.

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The selectivity of TG6-10-1 was evaluated against other prostanoid receptors in cell-based functional assays. In a comparison of Schild Kᵢ values, TG6-10-1 displayed at least 300-fold selectivity for the EP2 receptor over human EP3, EP4, and IP receptors, 100-fold selectivity against human EP1, 25-fold selectivity against human FP and TP, and 10-fold selectivity against human DP1 receptors (Fig. S1D). These results indicate that of the eight canonical prostanoid receptors, TG6-10-1 shows low-nanomolar antagonist activity against only EP2 and DP1, the receptor activated by prostaglandin D2 (PGD₂). In addition, other off-target activity assays showed that TG6-10-1 had negligible effect on a panel of 40 enzymes, ion channels, receptors, and neurotransmitter transporters (IC₅₀ > 10 μM), except that TG6-10-1 weakly inhibited the serotonin 5-hydroxytryptamine 2B (5-HT2B) receptor with IC₅₀ = 7.5 μM (Table S1). At a high concentration (10 μM), TG6-10-1 had little or no effect on the enzymatic activity of COX-1 (7% inhibition) and COX-2 (14% inhibition), and inhibited the leukotriene B₄ (LTB₄) receptor BLT1 by 1% (Table S1). TG6-10-1 displayed a plasma half-life of ~1.6 h and a brain:plasma ratio of 1.6 after systemic administration (5 mg/kg, i.p.) in mice (Fig. S1E). Thus, compared with the original compound (20, 22), the redesigned compound TG6-10-1 has much improved pharmacokinetic properties, which justifies its use for in vivo study.

**EP2 Receptor Antagonist Reduces Delayed Mortality, Accelerates Recovery from Weight Loss, and Improves Functional Recovery of Mice After Status Epilepticus.** COX-2 is rapidly up-regulated in selected forebrain neurons after a seizure (13, 24), and ablation of the COX-2 gene restricted to these neurons brings a broad range of beneficial effects in mice that had experienced SE (11, 19). We investigated the effect of EP2 receptor inhibition by TG6-10-1 after pilocarpine-induced SE in C57BL/6 mice. SE was allowed to proceed for 1 h and then terminated by pentobarbital. Three hours later (i.e., 4 h after SE onset), vehicle or TG6-10-1 was administered (5 mg/kg, i.p.). Two additional doses of TG6-10-1 were administered at 21 and 30 h after SE onset (Fig. L4) to approximately match the temporal pattern of COX-2 induction after pilocarpine. All in vivo studies used this treatment protocol. Given TG6-10-1 pharmacokinetics (Fig. S1E), its brain concentration should be more than twice its EP2 receptor Kᵢ for at least 5 h following each injection, but TG6-10-1 should inhibit DP1 for less than 1 h following injection. None of the other prostanoid receptors is sensitive enough to TG6-10-1 to be appreciably inhibited by this dose. Thus, most of the effects of TG6-10-1 on prostanoid receptors can be attributed to inhibition of EP2, although DP1 can potentially contribute.

Substantial delayed mortality occurs in the week following SE in mice and humans (9, 11). A significant increase in survival was observed in post-SE mice that received TG6-10-1 compared with those in the vehicle group (Fig. 1B). Administration of TG6-10-1 improved 1-wk survival from 60 to 90% after SE (P = 0.029; Fig. 1B). SE also caused substantial weight loss of the animals, which is gradually regained over a week in vehicle-treated mice. TG6-10-1 accelerated the recovery of lost weight (P < 0.01 at day 4; Fig. 1C). About half of the mice (9 of 17) that received vehicle continued weight loss between days 1 and 4 after SE, whereas only 1 of 17 TG6-10-1-treated mice lost weight during the same period (P < 0.01; Fig. 1D). Weight gain by mice experiencing SE followed by TG6-10-1 treatment approximated that of control mice that had not experienced SE (Fig. 1D). Four days after SE, all 17 animals that received TG6-10-1 showed normal behavior, such as locomotion, drinking, eating, and nest building, whereas 5 of 17 (29%) of the animals receiving vehicle were not able to build nests by day 4 (P < 0.05; Fig. 1E). Nesting is a sensitive indicator of brain lesions, especially in the hippocampal area (25). Taken together, these results demonstrate that administration of the EP2 antagonist with the schedule shown in Fig. 1A improved survival, accelerated recovery of lost weight, and improved functional recovery following SE.
EP2 Antagonist Reduces Neurodegeneration in Hippocampus. We next evaluated neurodegeneration in hippocampi from mice that received TG6-10-1 or vehicle at 4, 21, and 30 h after SE and were sacrificed on day 4. Coronal brain sections were stained with Fluoro-Jade (0.001%, wt/vol), and the number of Fluoro-Jade-positive cells in hippocampal subregions cormu ammonis area 1 (CA1), CA3, and dentate hilus was determined. Pilocarpine-induced SE caused substantial hippocampal neurodegeneration in vehicle-treated mice 4 d after SE (Fig. 4A), whereas no positive staining was detected in mice treated with vehicle or TG6-10-1 alone rather than pilocarpine (Fig. S2). Systemic administration of TG6-10-1 reduced the SE-induced neurodegeneration score by 66% (P < 0.05) in CA1 and by 52% in CA3, and reduced cell loss in hilus by 55% (P < 0.01) (Fig. 4B). These results support the conclusion that EP2 receptor activation promotes neuronal death following SE.

Discussion
We show that an EP2-selective competitive antagonist with 10-fold weaker potency against DP1, when administered systemically beginning 4 h after onset of pilocarpine-induced SE, mitigates many of the deleterious consequences of SE including delayed mortality, weight loss, functional deficit, opening of the blood–brain barrier, formation of a cytokine storm, gliosis, and neurodegeneration in the hippocampus. Because COX-2 produces products that act on nine different receptors (1), it was unexpected...
Brain inflammation is now recognized as a common feature of chronic neurodegenerative disorders, with the COX-2 cascade being widely believed to play a central role (36, 37). The role of the EP2 receptor in inflammation appears to be tissue- and context-dependent. In the periphery, genetic ablation of EP2 inhibits phorbol ester-induced expression of IL-1α and macrophage infiltration into the skin (38). In the CNS, however, selective EP2 activation reduces microglial migration toward injured tissue (39), whereas ablation of the EP2 receptor limits lipopolysaccharide-induced, microglia-mediated inducible nitric oxide synthase (iNOS) production and neurotoxicity in mixed cortical cultures (40). As the resident forms of macrophages in the brain, microglia appear to play a pivotal role in seizure-induced immune responses because activated microglia are a major source of a host of proinflammatory and neurotoxic factors including cytokines, chemokines, free radicals, and prostanooids in injured neuronal tissues (41, 42); activated astrocytes might also contribute to brain inflammation by producing iNOS in response to EP2 activation (43). Our work shows that an EP2 receptor antagonist significantly reduced the seizure-mediated induction of seven cytokines and chemokines, among them IL-1β and IL-6, and also blunted the induction of activated glial markers (Fig. 2).

IL-1β plays critical roles in a variety of cellular events such as cell differentiation, proliferation, and apoptosis, and inhibition of either IL-1β synthesis or its receptor attenuates epileptiform activity in a chronic model of epilepsy (44). Interestingly, IL-1β can induce COX-2 in the brain (45). IL-6 is one of the major mediators of both the acute phase and chronic responses during inflammation (46, 47). IL-6 can also induce COX-2 (48). Elevated COX-2 in turn synthesizes more PGE2 to maintain EP2 receptor activation. This self-reinforcing cycle of EP2 receptor activation could contribute to long-term inflammation and sustained neuronal injury, which might underlie a molecular mechanism of chronic neuronal inflammation and injury in a range of neurodegenerative diseases. Inhibition of the EP2 receptor could break this cycle and, therefore, reduce chronic inflammatory reactions (Fig. 6). The anti-inflammatory effects of the EP2 receptor antagonist suggest that the EP2 receptor plays a significant role in immune responses in the brain, as it does in the periphery.

Disruption of the blood–brain barrier is another common event in numerous neurological disorders including seizures and stroke (26, 27, 49), and contributes to the development of brain injury (39). Whether blood–brain barrier disruption is only a consequence of seizures or can also affect progression of the disease has been questioned for decades (50). However, recent evidence indicates that leukocyte infiltration together with signals via a leaky blood–brain barrier and astrocyte-derived cytokines enhance inflammation in the brain and contribute to epileptogenesis (26, 28, 51), although the immune responses mediated by those infiltrated leukocytes might afford some counteracting benefits (52).

Importantly, extravasation of albumin into the brain after SE appears to promote the progression of epilepsy, because injection of albumin directly into the brain promotes gliosis and intensifies subsequent development of spontaneous seizures (29). COX-2 and prostanooid signaling pathways have long been known to be involved in regulation of blood–brain barrier permeability (53). Recently, the prostaglandin receptor EP1 has been reported to promote blood–brain barrier leakage by tyrosine phosphorylation of occludin at tight junctions after cerebral ischemia (49). In addition, during seizures, EP1 receptor activation up-regulates blood–brain barrier efflux transporter P-glycoprotein, which reduces brain access and efficacy of therapeutic agents such as phenytoin (54). In our study, leakage of serum albumin into the brain via a damaged blood–brain barrier after SE was abolished by an EP2 receptor antagonist (Fig. 3). Our results indicate a role for the prostaglandin receptor EP2 in neuroinflammation and blood–brain barrier disruption after SE. Blood–brain barrier opening and brain inflammation interact that systemic inhibition of a single prostanoid receptor, EP2, would completely recapitulate the multiple beneficial effects of conditionally ablating COX-2 from a restricted population of forebrain neurons (11, 19). By contrast, the EP1 receptor appears to be responsible for much of the neuronal injury that follows cerebral ischemia (31). These results point to an important role for the EP2 receptor and perhaps DP1 in the neuropathogenesis of SE. More importantly, our finding raises the possibility that an EP2 antagonist could be used as adjunctive treatment of prolonged SE to reduce delayed mortality. Delayed mortality in patients after SE is often associated with acute or gradual cardiac decompensation (32). Pilocarpine-treated rats also develop chronic changes in autonomic control of cardiac function characterized by decreased parasympathetic activity leading to sympathetic dominance (33–35) and increased risk for ventricular arrhythmias. The location of the EP2 receptor responsible for SE-associated delayed mortality is not known, although a similar reduction in delayed mortality was observed in a conditional knockout of COX-2 limited to principal forebrain neurons (11), suggesting the relevant EP2 receptor is central rather than peripheral. Additional work is needed to test the hypothesis that EP2 receptor activation modulates cortical networks that influence brainstem circuits responsible for sympathovagal balance.

**Fig. 4.** EP2 receptor antagonist reduces neurodegeneration in hippocampus after SE. (A) Neurodegeneration in hippocampi from animals treated with vehicle or EP2 antagonist T66-10-1 was assessed by Fluoro-Jade staining 4 d after pilocarpine-induced SE. Arrows point to damaged neurons. No positive staining was detected in control mice treated with vehicle or T66-10-1 only (Fig. S2). (Scale bar, 50 μm.) (B) Quantification of neurodegenerating neurons in hippocampal subregions CA1, CA3, and dentate hilus. Coronal brain sections were examined for neurodegeneration with Fluoro-Jade (F-J) staining. One of every five sections was counted throughout the hippocampus. Neuronal injury in CA1 and CA3 was quantified by averaging the injury scores of sections from the same animal (in each section: 0, <3 Fluoro-Jade–positive cells; 1, 3–30 cells; 2, 31–100 cells; 3, extensive Fluoro-Jade staining, frequently in patches). Neuronal injury in the hilus was evaluated by counts of Fluoro-Jade–positive cells per section (n = 8–9 mice per group. *P < 0.05, **P < 0.01, one-way ANOVA and post hoc Bonferroni test with selected pairs). Data are shown as mean ± SEM.
with and enhance each other, with the blood–brain barrier usually becoming more permeable during brain inflammation. Taken together, activation of both prostaglandin receptors EP1 and EP2 are involved in blood–brain barrier breakdown. EP1 receptor activation directly modifies blood–brain barrier components, whereas the mechanism by which the EP2 receptor mediates blood–brain barrier breakdown, and the cellular targets, is not yet known.

Although downstream COX-2 signaling pathways promoting neurodegeneration are not completely understood, recent evidence suggests that EP2 receptor activation by PGE2 might underlie neuronal injury in some models of chronic inflammation and neurodegeneration (1, 4–6). Global ablation of the EP2 receptor reduced oxidative stress and improved survival in animal models of Alzheimer’s disease and amyotrophic lateral sclerosis (5, 6). We demonstrate here that an EP2 receptor antagonist significantly decreases delayed neurodegeneration in mice when administered after SE (Fig. 4 and Fig. S2). By contrast, EP2 ablation can increase infarct volume in models of focal ischemia (55), and we have shown that intraventricular administration of an EP2 agonist immediately after SE can be neuroprotective in a rat pilocarpine model (19). These seemingly incongruent observations are probably due to the complexity of inflammatory signaling in the brain, and could reflect dual consequences of EP2 activation—early neuroprotection followed by later neurodegeneration.

Continuous electrographic recordings for 48 h showed that TG6-10-1 is not a frank anticonvulsant in the pilocarpine model; furthermore, TG6-10-1 had no detectable effect on pilocarpine-induced electrographic activity, thus confirming a different mode of action from benzoiazepines, phenytoin, propofol, or pentobarbital. Neuroprotection by an EP2 receptor antagonist supports the involvement of this key prostaglandin receptor in delayed neurodegeneration after SE. The electrographic data support the hypothesis that neuroprotection by EP2 inhibition after SE is not the consequence of a direct anticonvulsant effect (Fig. 5 C and D and Fig. S3); rather, the neuroprotective effect of TG6-10-1 likely derives from the anti-inflammatory actions of the compound (Fig. 6). This conclusion is supported by extensive evidence concerning the neuroprotective mechanisms of IL-1β inhibitors (56), TNF-α inhibitors (57), and COX-2 inhibitors (14–18), because the targets of these inhibitors are down-regulated by the EP2 antagonist (Fig. 24). The cellular targets of the EP2 antagonist, the mechanism by which blood–brain barrier breakdown is prevented by the EP2 antagonist, and the role—if any—of the DP1 receptor in these phenomena are all important topics for future study. Nonetheless, our findings reinforce the notion that the prostaglandin receptor EP2 should be explored as a therapeutic target to oppose neuroinflammation and neurodegeneration, recognizing the cardio- and cerebrovascular adverse effects during long-term use of selective COX-2 inhibitors (21). Our results also support the notion of EP2 antagonism as an adjunctive strategy, along with benzoiazepines and general anesthetics, to treat status epilepticus.

Materials and Methods

Please see SI Materials and Methods for details about cell culture, chemicals and drugs, cell-based cAMP assay, potency of Tg6-10-1 on prostanoid receptors, off-target activity, pharmacokinetics, animals and seizure model, quantitative real-time PCR, Western blot, histopathology, and EEG analysis.

Drug Administration After Pilocarpine Treatment. Mice underwent SE for 1 h, and SE was then terminated by pentobarbital (30 mg/kg in saline, i.p.). After 3 h, mice were randomized and received three doses of vehicle (10% DMSO,
50% PEG 400, 40% ddH(2)O or TG6-10-1 (5 mg/kg, i.p.) at 4, 21, and 30 h after SE onset. Mice were fed moistened rodent chow, monitored daily, and injected with 5% by the Institutional Animal Care and Use Committee of Emory University and conducted in accordance with its guidelines. Every effort was made to minimize animal suffering.


Statistical Analysis. Statistical analyses were performed using Prism (GraphPad Software) by one- or two-way ANOVA with post hoc Bonferroni, Dunnett’s, or Student’s t-test, or t-test as appropriate. Survival was assessed using Kaplan–Meier analysis. P < 0.05 was considered to be statistically significant. All data are presented as mean ± SEM.

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