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Therapeutic window for cyclooxygenase-2 related anti-inflammatory therapy after status epilepticus

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ABSTRACT

As a prominent inflammatory effector of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE_2) mediates brain inflammation and injury in many chronic central nervous system (CNS) conditions including seizures and epilepsy, largely through its receptor subtype EP2. However, EP2 receptor activation might also be neuroprotective in models of excitotoxicity and ischemia. These seemingly incongruent observations expose the delicacy of immune and inflammatory signaling in the brain; thus the therapeutic window for quelling neuroinflammation might vary with injury type and target molecule. Here, we identify a therapeutic window for EP2 antagonism to reduce delayed mortality and functional morbidity after status epilepticus (SE) in mice. Importantly, treatment must be delayed relative to SE onset to be effective, a finding that could be explained by the time-course of COX-2 induction after SE and compound pharmacokinetics. A large number of inflammatory mediators were upregulated in hippocampus after SE with COX-2 and IL-1β temporally leading many others. Thus, EP2 antagonism represents a novel anti-inflammatory strategy to treat SE with a tightly-regulated therapeutic window.

Introduction

Though the central nervous system (CNS) was historically considered immune-privileged, inflammation within the brain, also known as neuroinflammation, is now emerging as a salient feature of many chronic neurological conditions that might contribute to the pathogenesis of these diseases (Aronica and Crino, 2011; Lucas et al., 2006; Varvel et al., 2015; Wee Yong, 2010). Resembling its extraneural counterpart, neuroinflammation involves a wide range of inflammatory molecules that mediate both pro- and anti-inflammatory events. Among these, cyclooxygenase (COX) is widely recognized for its pivotal role in initiation then prolongation of inflammatory responses via its prostanoid products, consisting of prostaglandin E_2 (PGE_2), PGD_2, $PGF_{2\alpha}$, prostacyclin I_2, and thromboxane A_2. COX-2 is the isozyme primarily responsible for prostanoid production under acute or chronic inflammatory conditions, whereas COX-1 is constitutively expressed in various tissues maintaining normal homeostatic functions of prostanoids (Hla and Neilson, 1992; Jiang and Dingledine, 2013a). COX-2 is rapidly induced in principal brain neurons in response to injury or excessive neuronal activity, and its overexpression is often associated with neurotoxicity and tissue injury in acute conditions including seizures (Kelley et al., 1999; Serrano et al., 2011; Takemiya et al., 2006) and ischemia (Iadecola et al., 2001; Kawano et al., 2006), as well as in chronic inflammatory diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Creutzfeldt–Jakob disease (Minghetti, 2004).

Although the COX-2 downstream signaling pathways involved in inflammation and tissue injury have not been fully identified, PGE_2 signaling through its EP2 receptor appears to play a major role in peripheral inflammation and pain observed in chronic inflammatory diseases such as rheumatoid arthritis, skin and vascular inflammation, and inflammatory hyperalgesia (Jiang and Dingledine, 2013a). In the brain, PGE_2 is a predominant COX-2 product and the EP2 receptor is also widely expressed. Extensive studies in the past decade reveal that PGE_2 signaling via EP2 mediates pro-inflammatory effects in models of innate immunity (Ganesh et al., 2013; Johansson et al., 2013; Montine et al., 2002), AD (Johansson et al., 2015; Liang et al., 2005), ALS (Liang et al., 2008) and more recently status epilepticus (SE) (Jiang et al., 2012, 2013), and slows the clearance of toxic substances such as amyloid beta (Aβ) peptides (Keene et al., 2010; Shie et al., 2005a, 2005b) and

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α-synuclein (Jin et al., 2007). Although advances have been made in our knowledge of degenerative diseases, there remains controversy whether upregulated COX-2 and PGE_2/EP2 signaling in the brain are net beneficial or detrimental (Andreasson, 2010; Jiang et al., 2010; Jiang and Dingledine, 2013a; Mohan et al., 2012), highlighting the delicate balance of pro- and anti-inflammatory responses following CNS injuries. Therefore, the therapeutic window for quelling COX-2-engaged neuroinflammation might vary with the injury types. In the present study, we identify a therapeutic window for anti-inflammatory treatment via inhibition of the EP2 receptor that opens approximately 2 h after onset of pilocarpine-induced SE in mice. This tight therapeutic window inspired us to investigate the expression of a number of inflammatory mediators in hippocampus after SE and we found that inhibition of the EP2 receptor blunts the delayed induction of pro-inflammatory genes after SE. Thus the rapid and robust induction of COX-2 might trigger or perpetuate EP2-mediated inflammatory reactions in the brain following SE.

Materials and methods

Chemicals and drugs

Methylscopolamine bromide, terbutaline, and pilocarpine were purchased from Sigma-Aldrich. Pentobarbital was purchased from Akorn Pharmaceuticals. Compound TG6-10-1 was synthesized and characterized as previously reported (Jiang et al., 2012, 2013).

Animals

The wildtype C57BL/6Cr mice were purchased from Charles River. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and conducted in accordance with its guidelines. Every effort was made to minimize animal suffering.

Seizure model

C57BL/6Cr mice (8–10 weeks old) were housed under a 12-h light/dark cycle with food and water ad libitum. To minimize peripheral side effects of pilocarpine, mice were injected with methylscopolamine and terbutaline (2 mg/kg each in saline, i.p.). After 30 min, pilocarpine (280 mg/kg in saline, freshly prepared, i.p.) was injected to induce status epilepticus (SE). Control mice received methylscopolamine and terbutaline, followed by saline injection instead of pilocarpine. Seizures were classified as previously described (Borges et al., 2003; Jiang et al., 2012, 2013). 0: normal behavior—walking, exploring, sniffing, and grooming; 1: immobile, staring, jumpy, and curled-up posture; 2: automatisms—repetitive blinking, chewing, head bobbing, vibrissae twitching, scratching, face-washing, and "star-gazing"; 3: partial body clonus, occasional myoclonic jerks, and shivering; 4: whole body clonus, "corkscrew" turning & flipping, loss of posture, rearing and falling; 5: (SE onset): non-intermittent seizure activity; 6: wild running, bouncing, and tonic seizures; and 7: death.

Administration of EP2 antagonist

Mice underwent SE for 1 h and were administered pentobarbital (30 mg/kg in saline, i.p.) to terminate SE. Mice were then randomized and administered either vehicle (10% DMSO, 50% PEG 400, 40% ddH_2O) or TG6-10-1 (5 mg/kg, i.p.) at different time points after SE onset. DMSO was used to enhance the solubility of TG6-10-1, although DMSO has been reported to exert anti-oxidant or anti-inflammatory effects of its own (Rosenberg et al., 2007). However, a synergistic effect between DMSO and TG6-10-1 is limited in our studies in that the naive control mice that received vehicle or vehicle plus EP2 antagonist did not show difference in weight, nesting behavior, neuroinflammation, or blood–brain barrier (BBB) integrity. Moreover, mice pretreated with vehicle and vehicle plus EP2 antagonist showed very similar seizure progression after pilocarpine injection (Jiang et al., 2013). After SE was terminated by pentobarbital, mice 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. One to four days after SE groups of mice were euthanized under deep isoflurane anesthesia and perfused with ice-cold phosphate buffered saline (PBS) to wash blood out of the brain. The brains were collected and then immersed in 4% paraformaldehyde (PFA) fixative for at least 10 h for histology study, or the hippocampi were dissected and stored at −80 °C for further studies.

Nesting behavior

The ability of a mouse to construct its nest overnight from a supplied nestlet square was assessed on a rating scale of 1–5 (Deacon, 2006). 1: the nestlet is more than 90% intact; 2: nestlet is partially torn with 50–90% untouched; 3: more than 50% nestlet is shredded without a nest site; 4: an obvious but flat nest is built; and 5: a perfect nest with walls higher than mouse body is built.

Quantitative real-time PCR (qRT-PCR)

Total RNA from mouse hippocampus was isolated using TRIzol (Invitrogen) with the PureLink RNA Mini Kit (Invitrogen). RNA concentration and purity were measured by A260 value and A260/A280 ratio, respectively. First-strand complementary DNA (cDNA) synthesis was performed with 1 μg of total RNA, 200 units of SuperScript II Reverse Transcriptase (Invitrogen), and 0.25 μg random primers in a reaction volume of 20 μl at 42 °C for 50 min. The reaction was terminated by heating at 70 °C for 15 min. qRT-PCR was performed by 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). 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 geometric mean of the cycle thresholds for β-actin, GAPDH and HPRT1 was subtracted from the cycle threshold measured for each gene of interest to yield ΔCT. Samples without cDNA template served as the negative controls. Primers used for qRT-PCR were as follows: β-actin, forward 5′-AAGGCCAACCGTGAAAAGAT-3′ and reverse 5′-GTGGTACGACCAGAGGCATAC-3′; GAPDH, forward 5′-TGTCCGTCGTGGATCTGAC-3′ and reverse 5′-CCTGCTTCACCACCTTCTTG-3′; HPRT1, forward 5′-GGAGCGGTAGCACCTCCT-3′ and reverse 5′-CTGGTTCATCATCGCTAATCAC-3′; COX-2, forward 5′-CTCCACCGCCACCACTAC-3′ and reverse 5′-TGGATTGGAACAGCAAGGAT-3′; mPGES-1, forward 5′-ATCAAGATGTACGCGGTGGC-3′ and reverse 5′-GAGGAAATGTATCCAGGCGA-3′; iNOS, forward 5′-CCTGGAGACCCACACACTG-3′ and reverse 5′-CCATGATGGTCACATTCTGC-3′; NOX-2, forward 5′-TGCCACCAGTCTGAAACTCA-3′ and reverse 5′-GCATCTGGGTCTCCAGCA-3′; IL-1β, forward 5′-TGAGCACCTTCTTTTCCTTCA-3′ and reverse 5′-TTGTCTAATGGGAACGTCACAC-3′; IL-6, forward 5′-TCTAATTCATATCTTCAACCAAGAGG-3′ and reverse 5′-TGGTCCTTAGCCACTCCTTC-3′; TNF-α, forward 5′-TCTTCTGTCTACTGAACTTCGG-3′ and reverse 5′-AAGATGATCTGAGTGTGAGGG-3′; TGF-β1, forward 5′-TCAGACATTCGGGAAGCAGT-3′ and reverse 5′-ACGCCAGGAATTGTTGCTAT-3′; CCL2, forward 5′-CATCCACGTGTTGGCTCA-3′ and reverse 5′-GCTGCTGGTGATCCTCTTGTA-3′; CCL3, forward 5′-TGCCCTTGCTGTTCTTCTCT-3′ and reverse 5′-GTGGAATCTTCCGGCTGTAG-3′; CCL4, forward 5′-CATGAAGCTCTGCGTGTCTG-3′ and reverse 5′-GGAGGGTCAGAGCCCATT-3′; EP2, forward 5′-TCTTTAGTCTGGCCACGATGCTCA-3′ and reverse 5′-GCAGGGAACAGAAGAGCAAGGAGG-3′; GFAP, forward 5′-GACAACTTTGCACAGGACCTC-3′ and reverse 5′-ATACGCAGCCAGGTTGTTCT-3′; S100B, forward 5′-TCGGACACTGAAGCCAGAG-3′ and reverse 5′-AGACATCAATGAGGGCAACC-3′; Iba1, forward

5′-GGATTTGCAGGGAGGAAAAG-3′ and reverse 5′-TGGGATCATCGAGGAATTG-3′; CD11b, forward 5′-CCAGTAAGGTCATACAGCATCAGT-3′ and reverse 5′-TTGATCTGAACAGGGATCCAG-3′.

Western blot analysis

Hippocampi from PBS-perfused mice were homogenized on ice in 0.5 ml RIPA buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a mixture of protease and phosphatase inhibitors (Roche Applied Science). The homogenates were centrifuged (12,000 ×*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: rabbit anti-COX-2 (1:1000, Abcam), rabbit anti-EP2 (1:1000, Santa Cruz Biotechnology), rabbit anti-IL-1β (1:1000, Santa Cruz Biotechnology), mouse anti-GFAP (1:2000, Santa Cruz Biotechnology), or mouse anti-GAPDH (1:1000, Abcam). This procedure was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology) at room temperature for 2 h. The blots were developed by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific) and scanned. The band intensities were quantified by ImageJ (NIH).

Immunohistochemistry

Immunohistochemical staining of fixed coronal brain sections was carried out by either fluorescence or 3,3′-diaminobenzidine (DAB) staining. For fluorescence immunostaining, floating sections (25 μm) were permeabilized with 0.25% Triton X-100 in PBS at room temperature for 15 min. The sections were then blocked in 10% goat serum in PBS at room temperature for 60 min. This procedure was followed by incubation with primary antibody (rabbit anti-COX-2, 1:1000, Abcam) at 4 °C overnight. Then the sections were washed with PBS and incubated with fluorescent secondary antibody: goat anti-rabbit Alexa Fluor® 488 at room temperature for 2 h, followed by washing. Images were obtained with a fluorescent microscope (Carl Zeiss). For DAB immunostaining, paraffin brain sections (8 μm) were permeabilized with 0.25% Triton X-100 at room temperature for 15 min, then blocked with serum, followed by incubation in primary antibody (rabbit anti-COX-2, 1:1000, Abcam) at 4 °C overnight. The sections were incubated in species-specific biotinylated secondary antibody, and then followed by incubation in avidin–biotin–peroxidase complex, and color development with 0.6 mg/ml DAB/0.03% hydrogen peroxide (Vector Labs).

Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software) by one- or two-way ANOVA with post-hoc Bonferroni or Dunnett's test, Fisher's exact test, or paired *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 ± or + SEM.

Results

Therapeutic window for targeting prostaglandin receptor EP2 to treat SE

Pharmacological inhibition of PGE_2 receptor subtype EP2 beginning hours after SE onset brought a broad range of beneficial effects in mice (Jiang et al., 2012, 2013). However, intraventricular administration of the EP2 agonist butaprost immediately after SE showed moderate neuroprotection in a rat SE model through an unidentified mechanism (Serrano et al., 2011). These seemingly incongruent observations reveal the complexity of immune reactions in the brain after SE and suggest that the therapeutic window for quelling neuroinflammation following SE might be delayed. To investigate the possibility of such a therapeutic window, we tested a brain-permeant EP2 antagonist, TG6-10-1, in a mouse pilocarpine SE model. Compound TG6-10-1 was created by introducing a trifluoromethyl group in the methylindol ring, aiming to improve its pharmacokinetic properties (Fig. 1A) (Jiang et al., 2012). Mice were pretreated with methylscopolamine and terbutaline to minimize the peripheral adverse effects of pilocarpine, then 30 min later seizures were induced by systemic administration of pilocarpine (280 mg/kg, i.p.), a non-selective muscarinic receptor agonist. Mice typically developed SE within 40 to 60 min. The SE was allowed to proceed for 1 h, and then terminated by pentobarbital injection. Surviving mice were then randomized and treated with vehicle or TG6-10-1 (5 mg/kg, i.p.) at several sets of time points after SE onset (Fig. 1B). Multiple doses of TG6-10-1 were used due to its moderately short plasma half-life (1.6–1.8 h), although it has a favorable brain/plasma ratio (1.2–1.6) in mice (Ganesh et al., 2014a, 2014b; Jiang et al., 2013). Delayed mortality, body weight change and nesting behavior were monitored daily in the mice. Following pilocarpine injection, mice in different treatment groups experienced similar behavioral seizures (Fig. 1C), and latencies to SE (Fig. 1D), before they were treated with pentobarbital. Cortical electroencephalography (EEG) recording demonstrated that TG6-10-1 administered in the delayed treatment 1 treatment protocol (Fig. 1B) had no effect on the timing or severity of SE in mice treated with pilocarpine (Jiang et al., 2013).

We previously reported that administration of TG6-10-1 beginning 4 h after SE onset (delayed treatment 1 in Fig. 2A) improved the 7-day survival from 60% to 90% (Jiang et al., 2013). Here, injection of TG6-10-1 twice daily for 2 d beginning 2 h after SE onset (delayed treatment 2) significantly improved the 2-month survival from 48% to 83% ($P = 0.008$; Figs. 2A, B). However, treatments designed to provide brain exposure from 2 to approximately 11 h (truncated treatment), or beginning 21 h after SE onset (late treatment) had no effect on delayed mortality (Figs. 2A, B). Importantly, mortality was not simply delayed by transient treatment with TG6-10-1 because no further deaths occurred in the delayed treatment groups between 5 and 60 d after SE (Fig. 2A). During the week following SE mice first quickly lost as much as 20% of their body weights, then began to recover gradually (Fig. 2C). Delayed treatment with TG6-10-1 accelerated the regain of animal weight ($P < 0.05$ at day 3, $P < 0.01$ at day 4, $P < 0.001$ at day 6; Fig. 2C) when compared to vehicle-treated mice. About half of the mice (10 of 22) that received vehicle had additional weight loss from 1 d to 4 d after SE, whereas only 3 of 22 (14%) that received delayed treatment kept losing weight during the same period of time ($P = 0.045$; Fig. 2D). Truncated or late treatment failed to facilitate the weight regain after SE (Figs. 2C, D). The gradual development of the ability to construct good nests was recorded as an additional measure of functional recovery after SE because nesting deficits are often associated with brain damage especially in hippocampus (Deacon, 2006). Mice that received delayed treatment, but not truncated or late treatment, showed improved nesting activity following SE ($P < 0.001$ at days 3, 4, 5 and 6, $P < 0.01$ at day 7; Fig. 2E). For example, 4 d after SE, 17 of 22 (77%) mice that received delayed treatment began to build nests (nesting score > 1) and 16 of those built excellent nests (nesting score ≥ 4), whereas only 5 of 23 (22%) mice receiving vehicle were able to build nests during the same period of time ($P = 0.0003$; Fig. 2F). These results demonstrate that administration of EP2 antagonist TG6-10-1 beginning at 2 or 4 h after SE onset, but not 1 or 21 h, improved animal survival, facilitated recovery of weight loss, and improved functional recovery. Therefore, a therapeutic window for targeting EP2 receptor that opens at least 2 h after SE onset was identified (Fig. 2B).

COX-2 induction is an early event following SE

As the inducible isoform of COX, COX-2 is strongly upregulated by excessive neuronal activity, growth factors, or pro-inflammatory stimuli

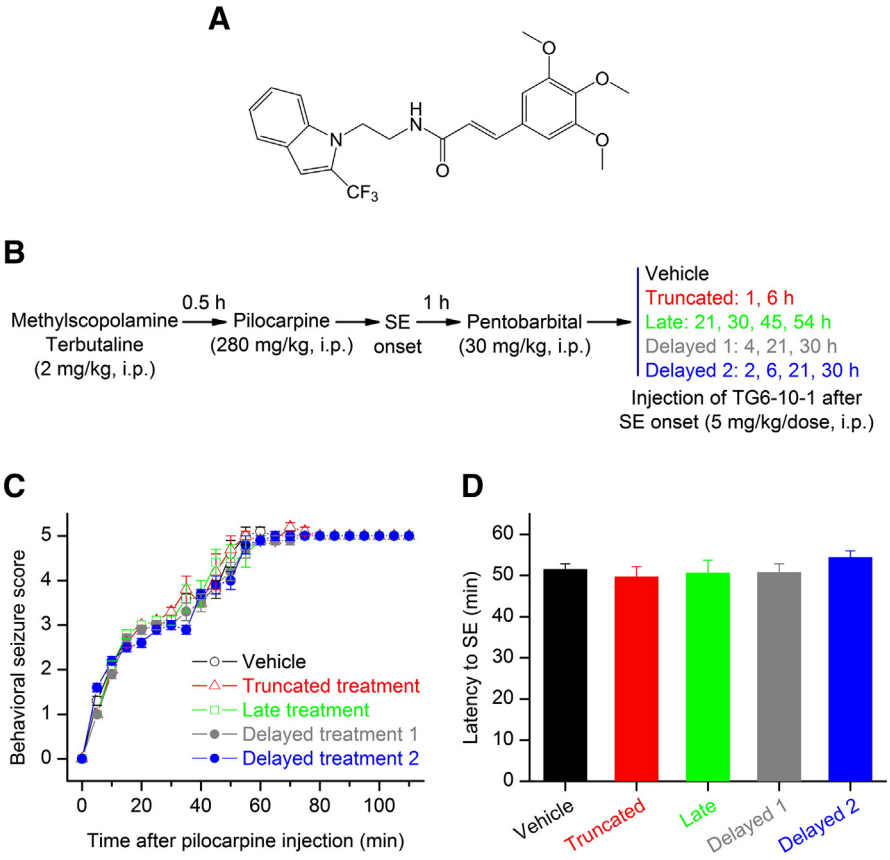

Fig. 1. Schematic of seizure induction and drug treatment paradigm. (A) Chemical structure of TG6-10-1. (B) Mice were pretreated with methylscopolamine bromide (2 mg/kg, i.p.) and terbutaline (2 mg/kg, i.p.), and 30 min later injected with pilocarpine (280 mg/kg, i.p.) to induce SE. The SE was allowed to persist for 60 min and terminated by pentobarbital (30 mg/kg, i.p.). Then mice were then randomly split into 4 groups and treated with vehicle or EP2 antagonist TG6-10-1 (5 mg/kg, i.p.) at different time points as indicated. The mice were checked daily for body weight, mortality and nesting behavior. (C) After pilocarpine injection, the behavioral seizure score was tabulated every 5 min until the seizure was terminated by pentobarbital injection 1 h after SE onset ($n = 9$–28 mice per group). Data are shown as mean ± SEM. (D) The latency to reach behavioral SE after pilocarpine injection ($n = 9$–28 mice per group). Data are shown as mean + SEM.

at sites of inflammation and injury (Bazan, 2001; Marcheselli and Bazan, 1996; Tu and Bazan, 2003). Elevated COX-2 activity can in turn promote an inflammatory storm that persists for days or weeks. Postnatal conditional ablation of the COX-2 gene limited to principal forebrain neurons blunts this SE-induced cytokine storm (Serrano et al., 2011), a finding that identifies a neuronal driver of largely glia-mediated inflammation after SE. The delayed therapeutic window after SE for targeting the EP2 receptor inspired us to examine the time-course of inflammatory mediator induction following prolonged seizures. We thus measured the mRNA levels of a host of inflammatory genes in mouse hippocampi by quantitative real-time PCR (qRT-PCR) after pilocarpine-induced SE. A 1-hour episode of SE rapidly induced COX-2 and IL-1β expression in hippocampus with a 7- to 8-fold induction by 30 min and the mRNA levels peaking 2–4 h after SE onset ($P < 0.01$; Fig. 3). The induction of COX-2 and IL-1β preceded the upregulation of all other inflammatory mediators examined including IL-6, TNF-α and the chemokines CCL2, CCL3 and CCL4 (Fig. 3). Membrane-associated PGE synthase-1 (mPGES-1), inducible nitric oxide synthase (iNOS) and the catalytic subunit of phagocyte NADPH oxidase (NOX-2 or gp91^{phox}) were also upregulated. Four days after SE, COX-2 and mPGES-1 mRNA expression had returned to or near basal level, whereas mRNAs encoding other inflammatory mediators still remained elevated (Fig. 3). A relatively delayed induction of TGF-β1 was observed at 16 h ($P < 0.01$; Fig. 3). TGF-β1 is also known for its anti-inflammatory signaling (Masli and Turpie, 2009), suggesting that the induction of pro-inflammatory mediators might be followed by anti-inflammatory responses after SE. We also examined other cytokines like IFN-γ and IL-10, but their expression levels in hippocampus were undetectable by qRT-PCR. Gliosis markers, GFAP and S100B for astrocytes and Iba1 and CD11b for microglia, were also induced in hippocampus by SE ($P < 0.01$; Fig. 3). Although glial-derived cytokine induction began within an hour or two of SE, the induction of mRNAs encoding proteins classically associated with astrogliosis (GFAP and S100B) and microglial activation (Iba1 and CD11b) was further delayed. These results confirm and extend the previous findings (Avignone et al., 2008; Jiang et al., 2013). Unexpectedly, prostaglandin receptor EP2 mRNA was initially modestly downregulated after SE onset, followed quickly by a moderate but long-lasting upregulation ($P < 0.05$; Fig. 3).

Next we examined protein expression by western blot analysis of mouse hippocampus after pilocarpine SE. Under normal conditions COX-2 protein was expressed at a very low basal level in hippocampus, but was rapidly induced by pilocarpine SE, beginning 1 h after SE onset. COX-2 protein expression continued to increase to over 60-fold by 4 to 24 h after SE ($P < 0.01$; Figs. 4A, B). Even 4 d after SE, COX-2 protein still remained more than 10-fold above its basal level ($P < 0.05$; Figs. 4A, B). Thus, compared to the rapid yet transient COX-2 mRNA induction, the induction of COX-2 protein was delayed about 30 min, but lasted longer (Figs. 3 and 4A, B). All three forms of IL-1β (pre-pro-, pro-, and mature) were also significantly increased after SE as mature IL-1β protein peaked 2 h after SE onset with a 6.5-fold increase ($P < 0.01$; Figs. 4A, B), and clearly remained above basal level even 4 d after SE. Astrocytic marker GFAP protein in hippocampus did not significantly increase until 1 d after SE ($P < 0.01$; Figs. 4A, B). In contrast to its moderate mRNA induction, EP2 protein level did not significantly change following pilocarpine SE (Figs. 4A, B).

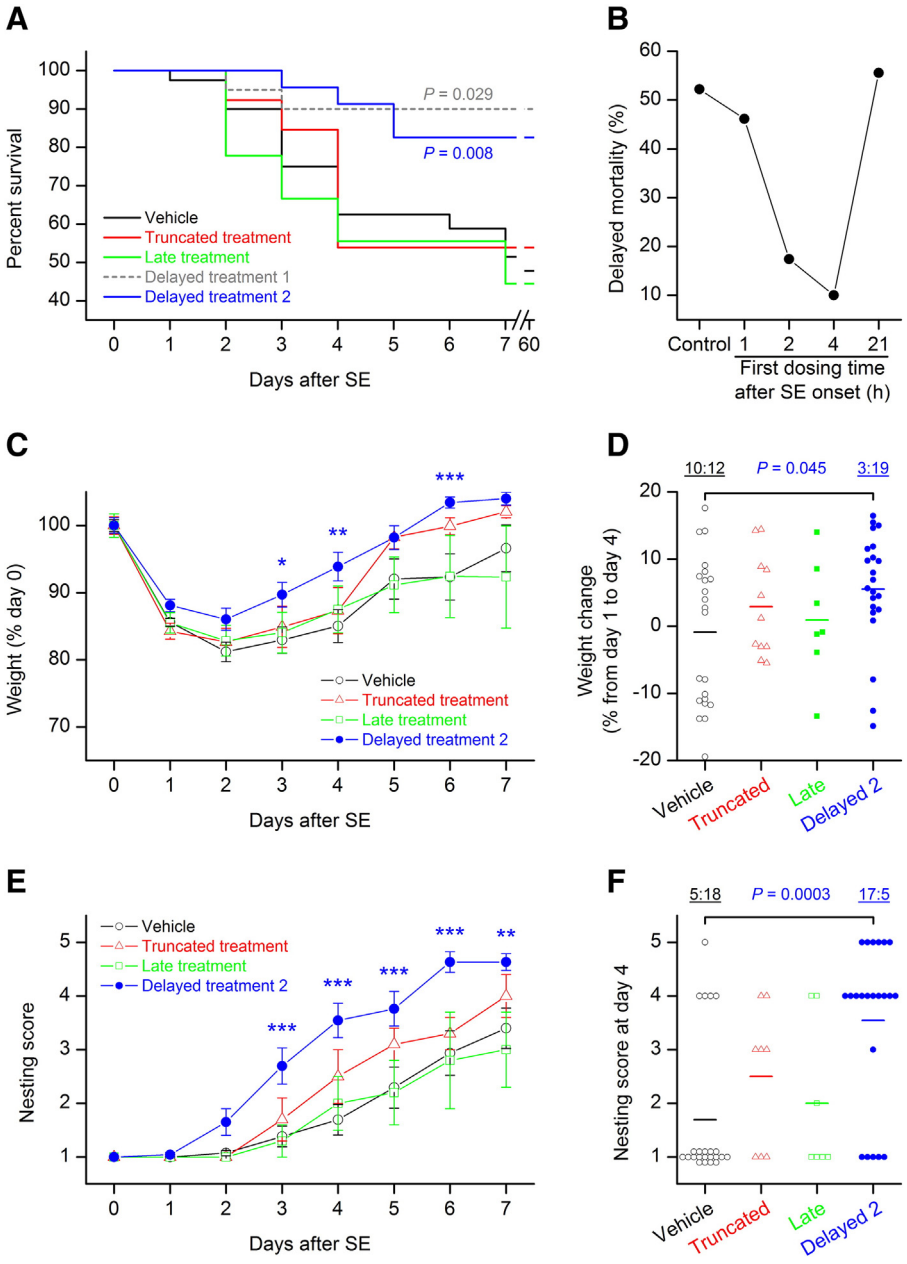

Fig. 2. Therapeutic window of blocking EP2 receptor to treat status epilepticus (SE). (A) Survival rates of animals that received vehicle or TG6-10-1 treatment ($n = 9$–28 mice per group) for up to 60 d after SE ($P = 0.008$ for delayed treatment 2 compared with vehicle group, Kaplan-Meier survival analysis; $P = 0.029$ for delayed treatment 1 as reported by Jiang et al., 2013). (B) Effect of the first dosing time after SE onset on animal 60-day delayed mortality. (C) Effect of TG6-10-1 on mouse body weight change after SE ($n = 9$–28 mice per time point, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle group, two-way ANOVA with post-hoc Bonferroni test). Data are shown as mean ± SEM. (D) Body weight change of individual animals from day 1 to day 4 after SE ($n = 7$–22 mice per group, $^{*}P = 0.045$, Fisher's exact test). The average weight changes from day 1 to day 4 after SE for each group are indicated by bars. (E) Effect of TG6-10-1 on mouse nesting behavior after SE ($n = 9$–28 mice per time point, $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle group, two-way ANOVA with post-hoc Bonferroni test). Data are shown as mean ± SEM. (F) Nesting score of individual animals at day 4 after SE ($n = 7$–22 mice per group, $^{***}P = 0.0003$, Fisher's exact test). The average nesting scores at day 4 after SE for each group are indicated by bars.

We then performed immunohistochemistry staining by fluorescence or 3,3′diaminobenzidine (DAB) to examine the localization of the COX-2 protein in mouse hippocampus after SE. Consistently, under normal conditions COX-2 protein was expressed at a very low basal level, but was substantially upregulated mainly in principal neurons of hippocampal subregions including dentate gyrus (DG), cornu ammonis area 1 (CA1) and CA3 after pilocarpine SE in mice (Figs. 5A, B). The SE-triggered COX-2 protein induction was obvious at 1 h, peaked between 4 and 12 h, and then gradually subsided by 3 to 4 d after SE onset (Figs. 5A, B). Interestingly, COX-2 protein in the CA3 region remained noticeably elevated even 10 d after SE (Fig. 5A). These findings suggest that following pilocarpine-induced SE, all tested inflammatory mediators including oxidative stress-related enzymes, cytokines, chemokines, and gliosis proteins were substantially upregulated, which might underlie the molecular basis of chronic inflammation after prolonged seizures. Among the pro-inflammatory mediators, the induction of COX-2 and IL-1β mRNAs and proteins is very rapid and robust and thus they are good candidates to drive seizure-provoked neuroinflammation.

EP2 receptor contributes to prolonged seizure-induced neuroinflammation

As a major prostaglandin product of COX-2, PGE_2 plays a significant role in inflammatory responses after SE through its EP2 receptor (Jiang

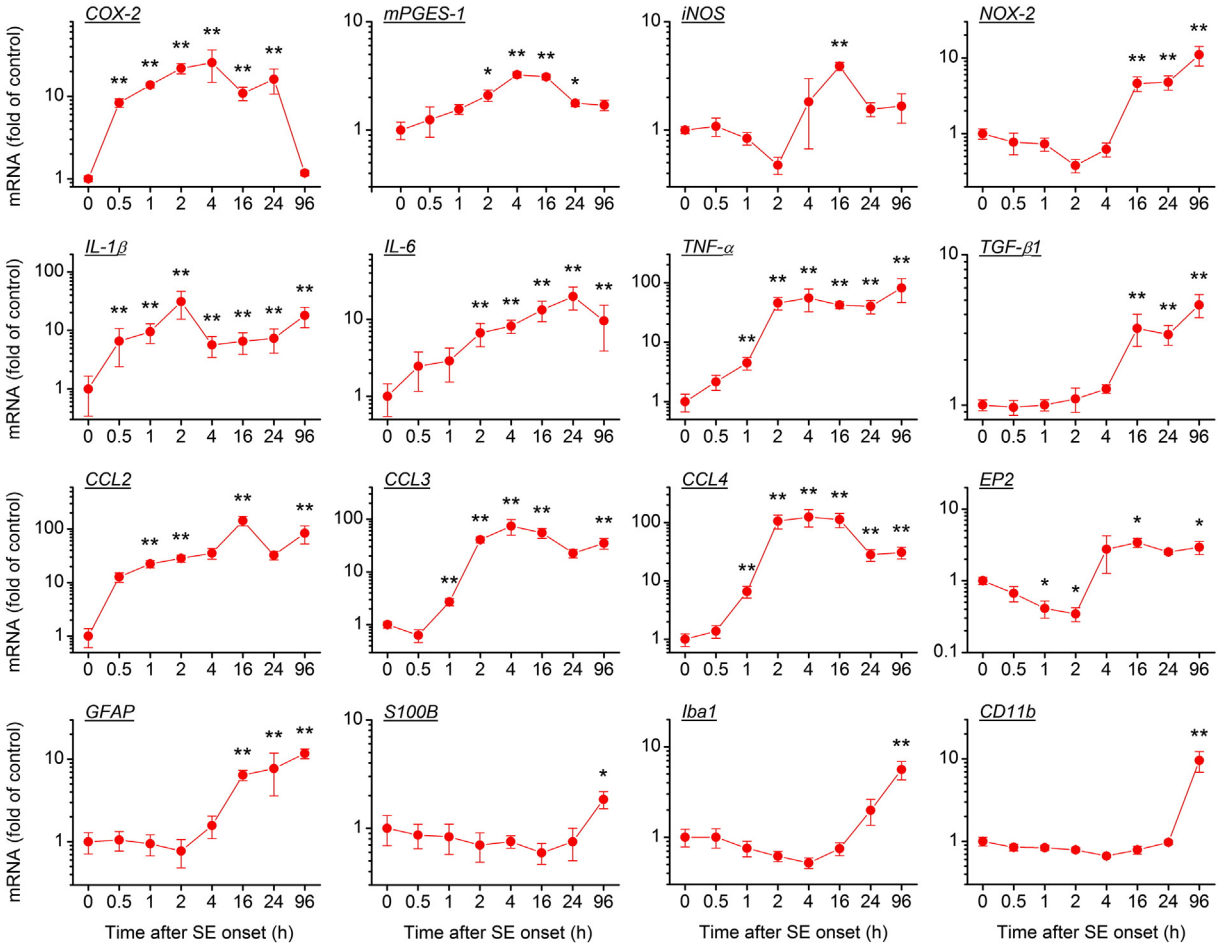

Fig. 3. Inflammatory genes are upregulated following pilocarpine-induced SE. Quantitative real-time PCR (qRT-PCR) was performed to measure the time-course of changes in mRNA levels of a number of inflammatory mediators and markers in mouse hippocampi after pilocarpine-induced SE. These molecules include enzymes: COX-2, mPGES-1, iNOS and NOX-2 ($gp91^{phox}$); cytokines: IL-1β, IL-6, TNF-α and TGF-β1; chemokines: CCL2, CCL3 and CCL4; EP2 receptor; and gliosis markers: GFAP, S100B, Iba1 and CD11b. All tested genes were substantially induced by SE with COX-2 and IL-1β temporally leading many others ($n = 6$–8 mice per time point, $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control mice, one-way ANOVA and post-hoc Dunnett's test of the ΔΔCT values from the qRT-PCR). Data are shown as mean ± SEM.

et al., 2012, 2013). In conditions of chronic inflammation and neurodegeneration, activation of the EP2 receptor may lead to secondary neurotoxicity by promoting the formation of pro-inflammatory cytokines and enzymes producing reactive oxygen and nitrogen species (Jiang et al., 2013; Quan et al., 2013). Thus, we next examined by qRT-PCR the effect of pharmacological inhibition of the EP2 receptor on mRNAs encoding oxidative stress-related enzymes, pro-inflammatory cytokines and gliosis-related proteins in the hippocampi from mice after SE. Hippocampal RNA samples were prepared from eight groups of mice ($n =$ 5–8 per group). In the first experiment mice were injected with saline or pilocarpine, then treated with TG6-10-1 or its vehicle (5 mg/kg, i.p.) at 4 and 21 h after SE onset, and sacrificed at 24 h for hippocampal RNA extraction and qRT-PCR. With this treatment regimen there was a trend towards attenuation of COX-2, NOX-2 and IL-6 mRNA production by TG6-10-1 but overall there was no effect of EP2 inhibition on the induction of pro-inflammatory cytokines and gliosis markers ($P = 0.216$; Fig. 6A). In the second experiment mice received three injections of TG6-10-1 at 4, 21 and 30 h after SE and were sacrificed 4 d after SE. With this more prolonged EP2 inhibition, induction of these pro-inflammatory genes in the hippocampus was decreased by an average of 34% ($P = 0.036$; Fig. 6B). Because COX-2 and iNOS mRNAs peaked 4–16 h after SE onset and returned near basal level thereafter, the effect of EP2 inhibition on induction of COX-2 and iNOS 4 d after SE was insignificant (Figs. 3 and 6B). These results confirm and extend our previous studies with a smaller number of inflammatory mediators (Jiang et al., 2013), and suggest that transient EP2 activation sets in motion a process that results in a delayed cytokine burst 4 d later, indicating that PGE_2 signaling via EP2 is a major COX-2 downstream pathway that contributes to prolonged neuroinflammation following prolonged seizures.

Discussion

In this study we identified a therapeutic window for a brain-permeable EP2 antagonist, TG6-10-1, to suppress delayed mortality and functional deficits following pilocarpine-induced SE in mice. The window opens between 2 and 4 h after SE begins and appears to coincide with the time-course of COX-2 induction after SE, taking into account the plasma half-life of the compound. The delayed induction of mPGES-1, the inducible PGE_2 synthase (Fig. 3), likely also contributes to the EP2 window. Comparing the temporal profile of COX-2 and mPGES-1 induction (Figs. 3 and 4) with the brain exposure profile of TG6-10-1 predicted from pharmacokinetics (Jiang et al., 2013), it is likely that the delayed dosing paradigms employed here led to therapeutic gaps during which breakthrough EP2 activation occurred. Whether a slow release formulation of TG6-10-1 that provides gapless exposure during the time of COX-2 and mPGSE-1 induction could further benefit animals after SE is an important topic for future study.

A number of inflammatory mediators including oxidative stress-related enzymes, cytokines and gliosis proteins were rapidly and persistently induced by SE with COX-2 and IL-1β induction temporally leading many others, suggesting that neuronal COX-2 induction and IL-1β secretion might be leading events driving SE-induced neuroinflammation.

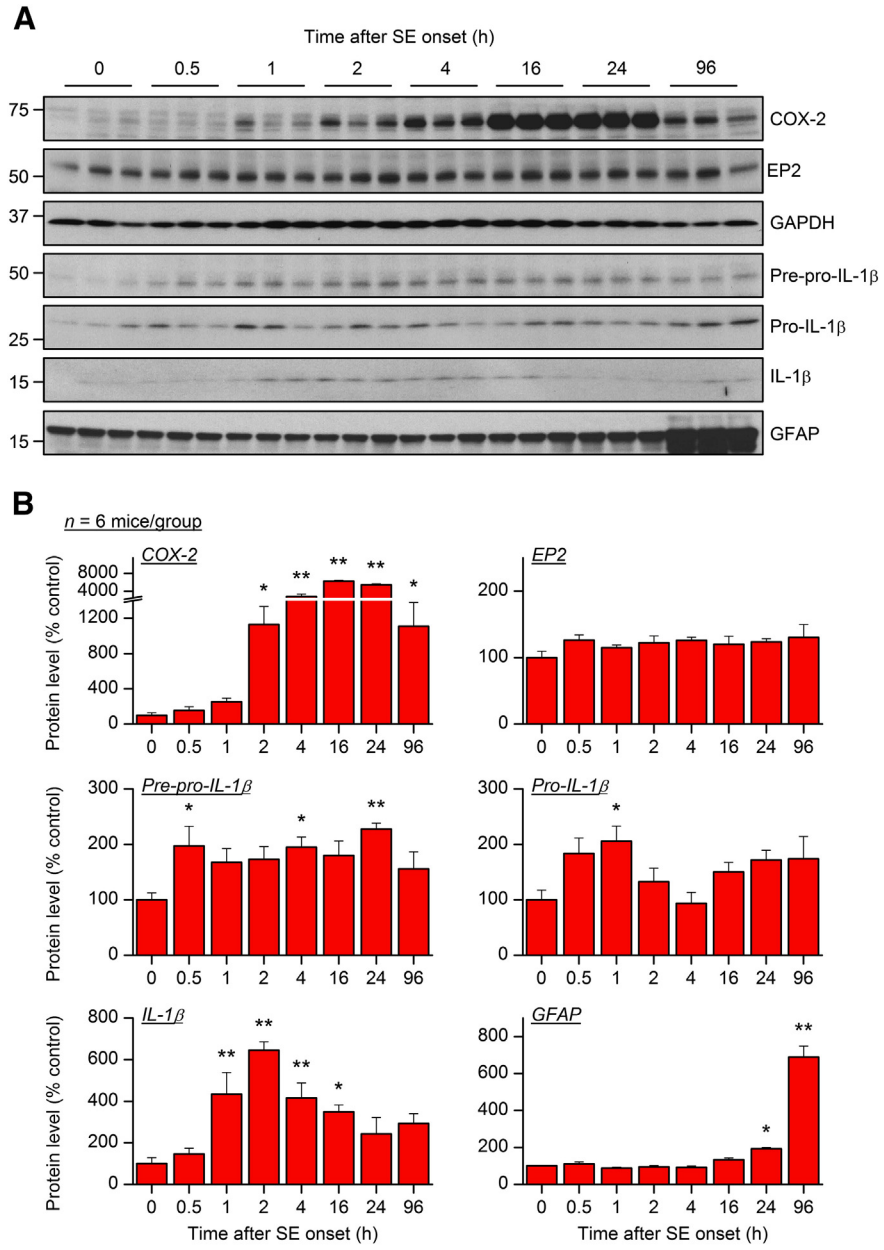

Fig. 4. COX-2 and IL-1β proteins are rapidly induced by SE. (A) The COX-2, EP2, IL-1β and GFAP protein levels in mouse hippocampi after SE were measured by western blot analysis with GAPDH as the loading control. Three representative samples from each time point after SE onset are shown on the blots. (B) The blots were scanned and the band intensities were assessed by ImageJ. The relative protein levels of COX-2, EP2, IL-1β (pre-pro, pro-, and mature forms) and GFAP were normalized to their mean basal levels. COX-2, IL-1β and GFAP protein levels, but not EP2, were rapidly and substantially upregulated in mouse hippocampi after pilocarpine-induced SE ($n = 6$ mice per time point, $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control mice, one-way ANOVA and post-hoc Dunnett's test). Data are shown as mean + SEM.

COX-2 and IL-1β appear to be reciprocally regulated in that neuronal COX-2 ablation reduces IL-1β expression after SE (Serrano et al., 2011), whereas interruption of IL-1β signaling prevents COX-2 induction (Hou et al., 2011; Samad et al., 2001). Pharmacological inhibition of the EP2 receptor by TG6-10-1 blunted SE-triggered induction of pro-inflammatory genes in hippocampus. To the best of our knowledge, this is the first identification of a therapeutic window for an anti-inflammatory therapy targeting COX-2 downstream signaling pathways. Front-line treatment for SE attempts to stop the seizures; the delayed treatment window of an EP2 receptor antagonist lends itself well to an adjunctive treatment strategy.

Inflammatory reactions in the brain are often associated with neurodegeneration and cognitive deficits (Lucas et al., 2006; Varvel et al., 2015; Vezzani et al., 2011); however the underlying molecular and cellular mechanisms by which inflammation persists in the brain and may cause neuronal injury remain largely unknown. Excessive COX-2 activity might be a candidate mechanism, as COX-2 is upregulated and perpetuates neuroinflammation in virtually all chronic neurological disorders (Minghetti, 2004). Recent studies in animal models of epilepsy revealed that pharmacological inhibition or genetic ablation of COX-2 reduced neuroinflammation and conferred neuroprotection after prolonged seizures (Jung et al., 2006; Polascheck et al., 2010; Rojas et al., 2014; Serrano et al., 2011; Takemiya et al., 2006), suggesting an essential role for COX-2 in neuroinflammation and secondary neurodegeneration. In this study, COX-2 was robustly upregulated in mouse hippocampus within an hour after SE onset (Figs. 3–5). Genetic ablation of neuronal COX-2 reduced cytokine induction in the pilocarpine model of SE, implicating a neuronal driver of inflammation (Serrano et al., 2011). As an early inflammatory response to seizures, neuronal COX-2 induction is thus in a position to facilitate or modulate the upregulation of

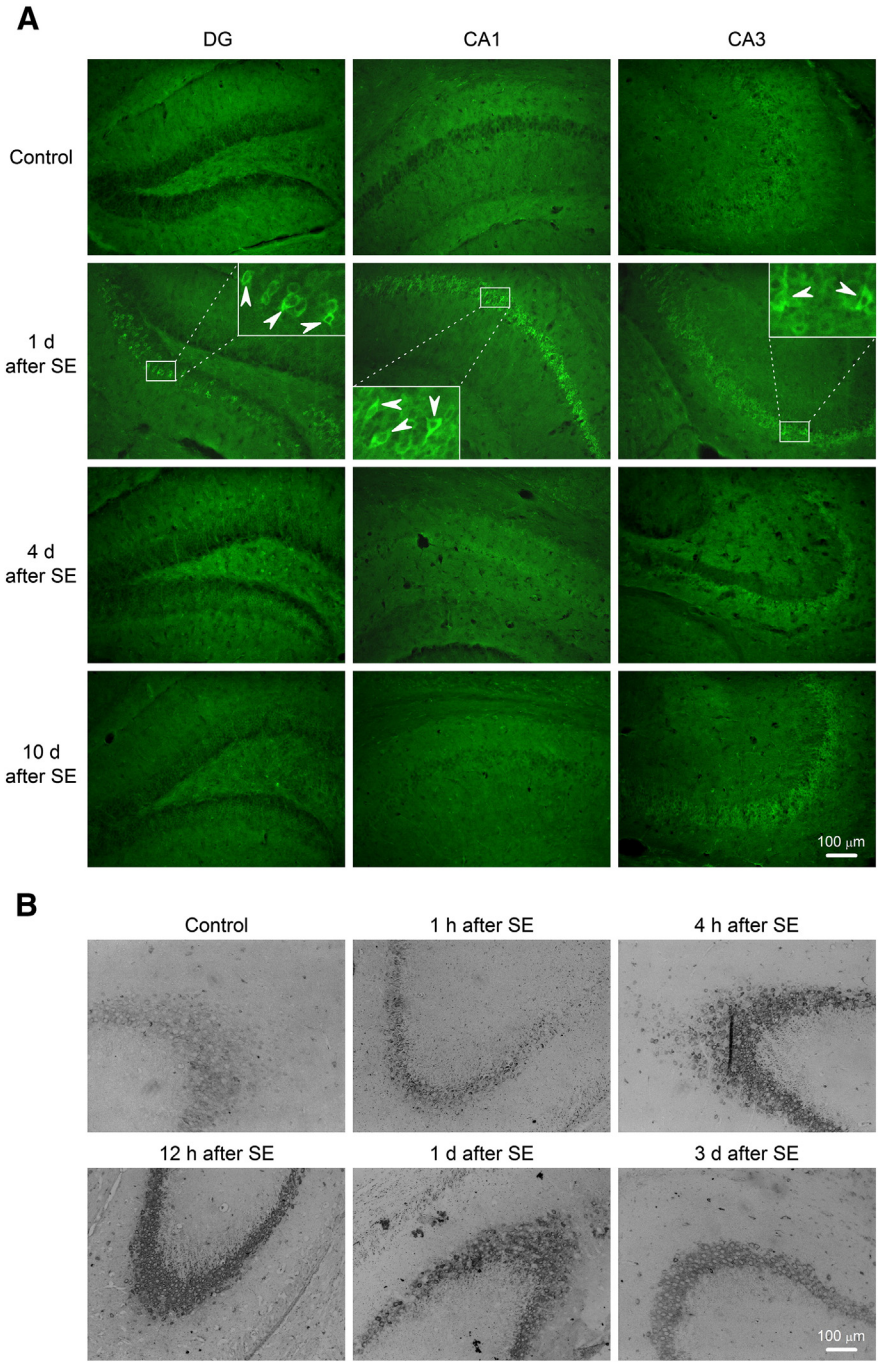

Fig. 5. Immunohistochemistry of COX-2 in hippocampus after pilocarpine-induced SE in mice. (A) Immunostaining was performed to examine COX-2 protein expression and location in hippocampal subregions: dentate gyrus (DG), cornu ammonis area 1 (CA1) and CA3, 1 d, 4 d or 10 d after pilocarpine-induced SE in mice, visualized by fluorescence. Scale bar = 100 μm. (B) Time-course expression of COX-2 protein in mouse hippocampal CA3 subregion, 1 h, 4 h, 12 h, 1 d or 3 d after SE onset, assessed by 3,3′-diaminobenzidine (DAB) staining. Note that SE induced COX-2 protein expression in hippocampal principal neurons, beginning within 1 h after SE onset. The COX-2 protein induction peaked by 4 to 12 h, and subsided by 3 d after SE. Scale bar = 100 μm.

other inflammatory molecules in glia. Some pro-inflammatory mediators can in turn induce COX-2 itself. For example, neuronal nitric oxide synthase (nNOS), via its PDZ domain can bind COX-2, which then is directly S-nitrosylated by NO with consequent increased catalytic turnover rate (Tian et al., 2008). In addition, pro-inflammatory cytokines IL-1β and IL-6 also can induce COX-2 via an NF-κB pathway and, thus increase PGE_2 level in the brain (Chikuma et al., 2009; Samad et al., 2001). Direct EP2 activation by butaprost has been shown to upregulate COX-2 in activated microglia (Quan et al., 2013; Yang et al., 2006). This self-reinforcing cycle of COX-2 activation might underlie a molecular mechanism of chronic inflammation and injury in epilepsy and perhaps other chronic degenerative brain diseases.

Temporally, in response to prolonged seizure stimuli, COX-2 and IL-1β were quickly induced and accompanied by many pro-inflammatory enzymes and cytokines, then TGF-β1, a conventional anti-inflammatory cytokine, was induced beginning 16 h after SE, and its level continued to increase even 4 d later (Fig. 3). Interestingly, TGF-β1 is proposed to contribute to cortical dysfunction and hyperexcitability by facilitating albumin uptake into astrocytes (Ivens et al., 2007). In addition to its well-established pro-inflammatory activities, the immunosuppressive

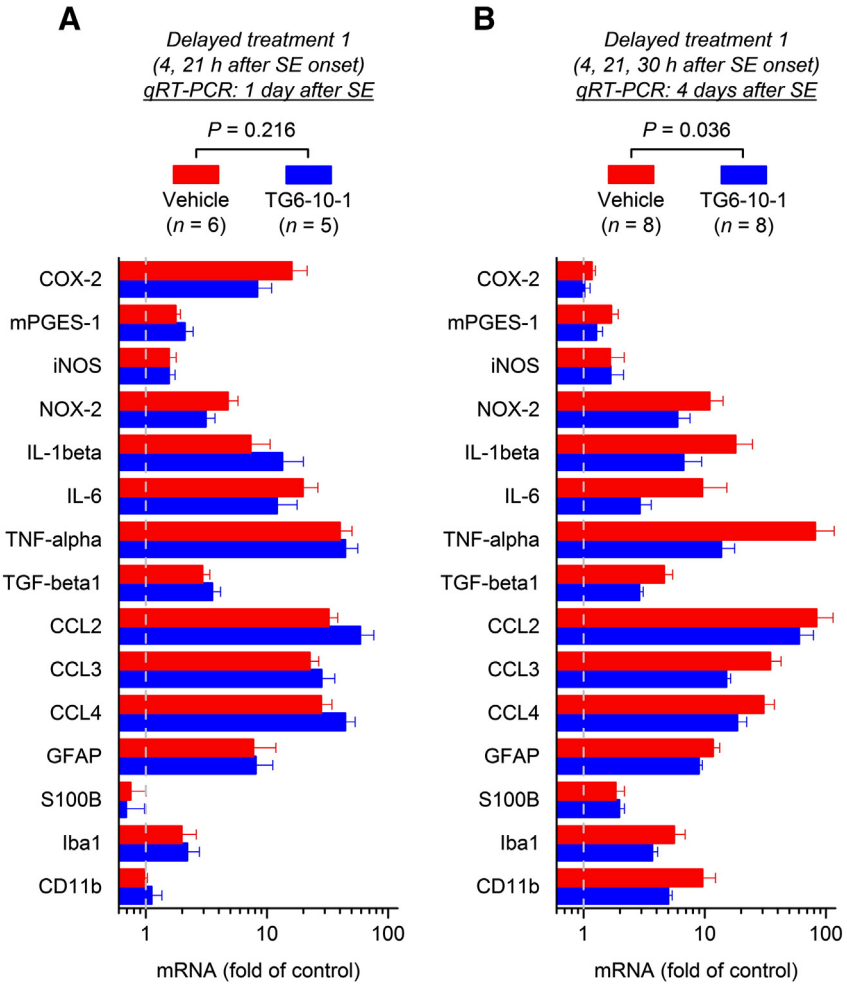

Fig. 6. EP2 receptor mediates expression of pro-inflammatory genes after SE. The mRNA levels of an array of pro-inflammatory mediators or markers were measured by qRT-PCR in mouse hippocampi after pilocarpine SE, including oxidative stress-related enzymes: COX-2, mPGES-1; iNOS, NOX-2; pro-inflammatory cytokines: IL-1β, IL-6, TNF-α, TGF-β1, CCL2, CCL3, CCL4; and gliosis markers: GFAP, S100B, Iba1, CD11b. mRNA induction of each gene was shown by comparing with its basal level in mice not treated with pilocarpine. (A) Administration of EP2 antagonist TG6-10-1 (at 4 and 21 h after SE onset) had no effect on mRNA induction of most pro-inflammatory genes, measured 1 d after SE ($n = 5$–6 mice per group, $P = 0.216$, two-tailed paired t test). Data are shown as mean + SEM. (B) Administration of EP2 antagonist TG6-10-1 (at 4, 21 and 30 h after SE onset) significantly reduced SE-triggered pro-inflammatory gene induction, measured 4 d after SE ($n = 8$ mice per group, $P = 0.036$, two-tailed paired t test). Data are shown as mean + SEM.

role of TNF-α has recently been appreciated in many inflammatory conditions (Masli and Turpie, 2009). TNF-α can also mediate both pro- and anti-seizure effects through its receptors TNFR1 and TNFR2, respectively (Balosso et al., 2013; Weinberg et al., 2013). A major source of TNF-α and TGF-β1 in the CNS is astrocytes and microglia although other cell types might also contribute. Thus, together with TGF-β1, TNF-α might help regulate and limit the extent and duration of the inflammatory response days after SE. The delicate balance of pro- and anti-inflammatory reactions suggests that a therapeutic window for quelling seizure-triggered inflammation might be tightly regulated.

The EP2 receptor is a major contributor to COX-2-mediated inflammation in both the periphery and the brain (Jiang and Dingledine, 2013a). EP2 receptor plays essential roles in the regulation of inflammatory cytokine and chemokine expression in many different cell types including macrophages, microglia, and tumor cells (Jiang and Dingledine, 2013b; Johansson et al., 2013; Quan et al., 2013). We previously reported that intraventricular administration of an EP2 agonist immediately after SE brought a moderate neuroprotection of hippocampal CA1 pyramidal cells in pilocarpine-treated rats (Serrano et al., 2011). This seeming incongruity could reflect opposing roles for EP2 receptor activation following acute brain injuries—early neuroprotection and later neurotoxicity. Therefore, the therapeutic window for quelling neuroinflammation via EP2 inhibition may vary with injury types and perhaps species.

Pilocarpine is a non-selective muscarinic receptor agonist and among the most widely used chemoconvulsants in animal models of epilepsy. The mechanism by which pilocarpine elicits SE proceeds through activating the M1 muscarinic receptor subtype in that M1 receptor knockout mice are resistant to seizures following injection of pilocarpine but not kainate (Hamilton et al., 1997). A recent study showed that intravenous administration of IL-1 receptor antagonist (IL-1ra) can prevent pilocarpine-induced seizures (Marchi et al., 2009), endorsing the long-held concept that systemic inflammation involving immune cells and molecules increases seizure susceptibility (Galic et al., 2008; Marchi et al., 2011; Riazi et al., 2008; Sayyah et al., 2003). Whether the EP2 antagonist also targets peripheral inflammation caused by pilocarpine is not known and requires follow-up study. However, the beneficial effects of EP2 blockade are probably attributed mostly to an action on EP2 receptors in the CNS because the consequent reductions in delayed mortality, weight loss, neuronal injury, neuroinflammation and blood–brain barrier opening were fully recapitulated in mice bearing a conditional knockout of COX-2 limited to principal forebrain neurons (Jiang et al., 2013; Levin et al., 2012; Serrano et al., 2011).

We previously showed that two different EP2 antagonists: TG4-155 and TG6-10-1, when administered beginning 2 or 4 h after SE onset, afford profound neuroprotection in mice after pilocarpine SE (Jiang et al., 2012, 2013). Inflammatory responses of brain tissues are well-known to aggravate neuronal cell loss; in turn, neuronal injury can trigger glial

activation and exacerbate neuroinflammation (Vezzani et al., 2011). In pilocarpine-treated mice evidence of neuroinflammation appears well before injury to most neurons (except perhaps those of the dentate hilus (Borges et al., 2003)), suggesting that in this model the initial cytokine burst elicited by seizures is not caused by neuron loss. In the future it would be worthwhile to explore pharmacological inhibition of EP2 in other models of neuronal injury such as ischemia, AD, PD and ALS. Our findings pave the way to target EP2 rather than block the entire COX-2 cascade to counteract brain inflammation and injury, recognizing the undesirable effects from COX-2 inhibitors that have emerged in the past decade.

Author contributions

J.J. conceived and performed experiments, analyzed the data, and wrote the manuscript; M.Y., Y.Q., P.G. and T.G. helped perform experiments; R.D. helped experimental design, data analysis and writing.

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