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Characterization of the effect of oral rofecoxib treatment on PTZ-induced acute seizures and kindling

Robert J Claycomb, Sandra J Hewett*, and James A Hewett*

Department of Neuroscience, University of Connecticut School of Medicine, Farmington, CT 06030

Summary

Purpose—The goal of this study was to determine whether prophylactic prandial administration of rofecoxib, a selective cyclooxygenase (COX)-2 inhibitor, could alter seizure generation, kindling acquisition and/or kindling maintenance in the mouse pentylenetetrazole (PTZ) epilepsy model.

Methods—Male CD-1 mice were fed ad libitum with control chow or chow formulated to deliver 30 mg/kg/day rofecoxib. After five days, mice were treated with a single dose of 40 or 55 mg/kg PTZ (acute paradigm) or 40 mg/kg PTZ delivered daily (kindling paradigm). Seizure severity was scored on a 4 point behavioral scale and COX-2 expression was assessed in brain slices from a subset of mice 3 or 72hr after acute PTZ or following establishment of kindling.

Results—Hippocampal COX-2 expression was transiently up-regulated 3hr after an acute PTZ-induced convulsion and returned to baseline levels within 72hr, whereas it remained elevated for at least 72hr after the final seizure in the kindling paradigm. Despite this increase, chronic rofecoxib treatment did not attenuate the severity of acute PTZ-induced seizures and failed to alter kindling development or maintenance.

Discussion—The present study demonstrates that prophylactic prandial rofecoxib treatment lacks efficacy against acute PTZ-induced seizure generation and kindling acquisition, and does not reverse the kindled state once established.

Keywords

Cyclooxygenase-2; seizures; epileptogenesis; kindling; pentylenetetrazole; epilepsy

1. Introduction

Cyclooxygenases (COX) catalyze the rate-limiting reaction for the production of bioactive prostaglandins and thromboxane from arachidonic acid (Smith et al., 2000). There are two COX genes, *ptgs1* and *ptgs2*, which generate two different isoforms of the COX enzyme, COX-1 and COX-2, respectively. These two isozymes share similar structure and enzyme

*Direct correspondence to either author: Department of Neuroscience MC 3401, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-3401, USA., Tel: 860-679-4131; Fax: 860-679-8766, jhewett@nso1.uchc.edu; shewett@neuron.uchc.edu.

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kinetics; however, their cellular expression patterns are quite different. COX-1 is constitutively expressed in a wide variety of tissues, where it is thought to perform homeostatic functions, including regulation of blood flow, platelet aggregation, and gastric acid secretion. In contrast, COX-2 expression is undetectable in most tissues but can be induced by pro-inflammatory mediators, growth factors, and tumor promoters.

In the central nervous system (CNS), COX-1 protein is constitutively expressed in both astrocytes and neurons (Hewett et al., 2000). COX-2 is also constitutively present in the CNS, particularly in the cell soma and dendritic areas of glutamatergic neurons of the cortex, hippocampus, and amygdala (Yamagata et al., 1993; Breder et al., 1995). Within the hippocampus, basal COX-2 expression is observed in dentate granule cells and CA1-CA3 pyramidal neurons and this is markedly upregulated by excitatory synaptic activity associated with acute seizure activity (Yamagata et al., 1993; Adams et al., 1996). Additionally, selective COX-2 inhibitors suppress long-term potentiation (LTP) at the perforant path-dentate granule synapse (Chen et al., 2002; Cowley et al., 2008). This latter observation raises the intriguing possibility that COX-2 may be involved in neuroplastic changes that underlie learning and memory. Indeed, this notion is supported by evidence demonstrating that memory acquisition and consolidation and spatial learning (archetypical neuroplastic events) are impaired by selective COX-2 inhibition (Holscher, 1995; Teather et al., 2002; Cowley et al., 2008).

The induction of COX-2 expression by acute seizure activity and its contribution to neuroplasticity have prompted much interest in the possibility that arachidonic acid metabolites of COX-2 may modulate seizure threshold and/or contribute to aberrant changes that predispose the CNS to development of epilepsy. In this regard, COX-2 expression and brain prostaglandin levels were shown to be increased in the hippocampus following status epilepticus or kindling (Chen et al., 1995; Sanz et al., 1997; Ciceri et al., 2002; Takemiya et al., 2003; Voutsinos-Porche et al., 2004; Kawaguchi et al., 2005; Jung et al., 2006; Lee et al., 2007; Holtman et al., 2009), commonly used models of epileptogenesis whereby animals develop spontaneous seizure activity or become chronically sensitized to convulsive stimuli, respectively (Leite et al., 2002)(Mason and Cooper, 1972; Morimoto et al., 2004). Interestingly, strong COX-2 expression was also found in the hippocampi of human patients with hippocampal sclerosis occurring secondary to temporal lobe epilepsy, suggesting that the changes demonstrated are not just an experimental epiphenomenon and that arachidonic acid metabolism via COX-2 may indeed serve a physiological or pathophysiological function in these epileptic patients (Holtman et al., 2009). If these changes in expression/activity contribute to epileptogenesis and/or epileptic seizure activity, this would raise the intriguing possibility that COX-2 could be a target for therapeutic intervention.

The possible relevance of COX-2 to epileptogenesis has been demonstrated by several studies (Takemiya et al., 2003; Tu and Bazan, 2003; Dhir and Kulkarni, 2006; Jung et al., 2006; Dhir et al., 2007). Specifically, kindling development—induced by a rapid electrical protocol — is attenuated in mice with genetically or pharmacologically altered COX-2 (Takemiya et al., 2003; Tu and Bazan, 2003). Modest effects on kindling scores were found with pharmacological COX-2 inhibition in a pentylenetetrazole (PTZ) -induced kindling model (Dhir and Kulkarni, 2006; Dhir et al., 2007). Finally, administration of celecoxib, a

selective COX-2 inhibitor, was shown to attenuate the likelihood of developing spontaneous recurrent seizures in the pilocarpine model of epileptogenesis (Jung et al., 2006). However, the evidence does not uniformly support this notion. Thus, COX-2 inhibition failed to modify epileptogenesis in rats that were either kindled electrically (Holtman et al., 2009) or via administration of pilocarpine (Polascheck et al., 2010).

Differences in the kindling models utilized, timing and mode of drug administration, as well as, inhibitor selectivity, could conceivably account for the discrepancies in the experimental outcomes, supporting the need for further experimentation. Hence, the primary goals of the current study were to determine 1) whether hippocampal COX-2 expression is upregulated in hippocampus after an acute PTZ-induced seizure or in PTZ-kindled mice, 2) whether prophylactic, prandial administration of the selective COX-2 inhibitor, rofecoxib, attenuates the severity of acute PTZ-induced seizures or affects PTZ-induced kindling acquisition and/or maintenance.

2. Materials and Methods

2.1 Animals

All experiments were performed on male CD-1 mice (Harlan; Indianapolis, IN) at 8-12 weeks of age. Mice (6-10 weeks) were housed three to four per cage on a 12hr light/dark schedule (6am/6pm) in a fully accredited Laboratory Animal Care facility. They were allowed to acclimatize to the facility for at least one week prior to any manipulations, during which time standard mouse chow and water were provided *ad libitum*. Five days prior to experimentation, daily mock injections were performed by inverting the mouse and rubbing the abdomen to familiarize the mice to the procedure. Animal use was conducted in accordance with the National Institute of Health guidelines for the use of experimental animals and was approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center, Farmington, CT (USA).

2.2 Rofecoxib Feeding Paradigm

Thirty-six mice were randomly divided into two groups. One group was fed control rodent chow (n = 18) and the other group (n = 18) was fed a chow containing 180mg/kg rofecoxib (Purina Mills International, St. Louis, MO). Based on a measured food intake rate of $\approx 5\text{g/day}$, the rofecoxib-containing food provided an approximate dose of 30mg/kg rofecoxib per day. This dose was chosen as we previously demonstrated it to be remarkably neuroprotective against hippocampal excitotoxic neurodegeneration *in vivo* (Hewett et al., 2006). The chow was provided to the animals in a coded fashion to ensure that the experiments were performed and analyzed blindly. Except for the extinction experiments (*vida infra*), diets were initiated five days prior to any PTZ injections (at the time of handling) to ensure steady-state drug levels were reached (Depre et al., 2000). Animals were maintained on their respective diets until sacrifice. Blood samples obtained upon completion of studies were assayed for rofecoxib levels via high-performance liquid chromatography (HPLC). The mice had a mean $\pm$ SEM rofecoxib plasma concentration of $400 \pm 60 \text{ ng/mL}$ ($1.3 \pm 0.2 \mu\text{M}$), which is consistent with previously reported data using the same rofecoxib delivery protocol (Hewett et al., 2006).

2.3 Pentylenetetrazole (PTZ) Dosing Paradigms

PTZ (Sigma Chemical Co.; St. Louis, MO) was dissolved in 0.9% saline, filter sterilized, and administered intraperitoneally in a volume of 0.2mL/0.03kg. **Acute Paradigm:** Mice were treated with a single dose of 40 or 55 mg/kg PTZ. Seizures typically had a rapid onset (<120sec), spontaneously resolved within 20 sec and were graded by an observer blinded to the experimental condition using an established scoring system: stage 0: no behavioral change; stage 1: hypoactivity and immobility; stage 2: two isolated, myoclonic jerks; stage 3: generalized clonic convulsions, with preservation of righting reflex; Stage 4: generalized clonic or tonic-clonic convulsions with loss of righting reflex (Ferraro et al., 1999).

Kindling Paradigm: Different strains of mice respond differentially to convulsants, including PTZ (Kosobud et al., 1992). As such, the convulsant threshold of PTZ was first identified in a dose-ranging study in which mice were treated acutely with a single dose of PTZ (30 to 70 mg/kg) and monitored for 15 min for seizure generation. A single dose of 40mg/kg PTZ elicited convulsive seizure (*vida infra*) in only 28% (2/7) of animals, with a median seizure score for the group being one (Supplemental Figure 1). Successive daily exposures of CD-1 mice to 40mg/kg PTZ resulted in a progressive reduction in PTZ seizure threshold (Supplemental Figure 2) and latency (data not shown). Mice were considered kindled after exhibiting four consecutive convulsive seizures [stage 3], after which daily PTZ injections were stopped. Ten and 20 days later, the permanence of the kindled state was assessed by re-challenging mice with 40mg/kg PTZ. A permanent state is confirmed by the presence of a convulsive seizure.

2.4 Extinction Paradigm

Sixteen mice were initially fed the control diet and subjected to the kindling paradigm using 40mg/kg PTZ as described above. Of these, 88% (14/16) became kindled. Once an animal reached the kindled state (*vida supra*), daily PTZ injections were discontinued. Mice were then separated from their original cage and randomly assigned to a new cage where they were fed either control or rofecoxib-supplemented diet. Ten and 20 days later, the mice were re-challenged with 40mg/kg PTZ and seizure severity scored as described above. Mice exhibiting extinction would, by definition, fail to have a convulsive seizure when re-challenged with the kindling dose of PTZ.

2.5 COX-2 Immunohistochemistry

Mice were deeply anesthetized (120mg/kg ketamine, 20mg/kg xylazine, i.p.) and transcardially perfused with ice cold 0.05M PBS followed by 4% paraformaldehyde (PFA). The cranial vault was entered by removal of the calvaria, the brain was removed and immediately placed in 4% PFA for 4-8 hr at 4°C. CNS tissue was sequentially equilibrated with 15% and 30% sucrose solutions, frozen on dry ice, and stored at -80°C. Frozen brains were cut serially (1.2 mm to 2.7mm posterior to bregma) into 30 µm coronal sections using a Leica Microsystems CM1900 cryostat. When applicable, cresyl violet and FluoroJade staining was performed on alternate sections as described below. Once the brain tissue was cut, individual sections were transferred to 0.05M PBS. These freely-floating sections were washed twice with 0.05M PBS. To quench endogenous peroxidase activity, sections were incubated in 0.6% H₂O₂ in 0.05M PBS for 20 min at room temperature with constant

rocking. Sections were washed thrice with 0.05M PBS and then incubated for 1 hr on ice in blocking solution (0.05M PBS containing 5% normal goat serum, 1% bovine serum albumin, 20% DMSO and 0.2% triton X-100), followed by addition of rabbit polyclonal anti-mouse COX-2 antibody (0.2µg/mL, Cayman Chemical; Cat# 160106) prepared in blocking solution. After incubating overnight at 4°C with constant rocking, sections were washed four times with 0.05M PBS and incubated for 1 hr at room temperature in 0.05M PBS containing biotinylated goat anti-rabbit polyclonal antibody (Vectastain Kit, Vector Labs). Sections were washed thrice with 0.05M PBS followed by incubation with ABC Reagent (Vector Labs) per the manufacturer's instruction. After washing with 0.05M PBS three times, sections were incubated with DAB (3,3'-diaminobenzidine) Reagent (Vector Labs) per manufacturer's instructions. Sections were floated onto slides and allowed to air dry overnight. Dry slides were washed by a 1 min water immersion, dehydrated with 1 min incubations of 75% ethanol followed by 100% ethanol, cleared with xylenes and mounted using Permount. Images of the sections were captured with an Olympus IX50 inverted microscope equipped with a CRX digital camera (Digital Video Camera Co). Images from individual studies were processed identically using Adobe Photoshop. Some sections were incubated with the secondary antibody in the absence of the primary antibody to assess for nonspecific secondary antibody binding. None was detected.

2.6 Cresyl Violet and FluoroJade Staining

Potential changes in cell viability were assessed by cresyl violet and FluoroJade staining. Brain tissue and sections were obtained as described above. Sections were set onto gelatin-coated slides and allowed to dry overnight. The sections assigned to cresyl violet staining were initially dehydrated by 2 min incubations in 100% ethanol followed by 2 min in xylenes. The sections were then subjected to a descending ethanol series of 100%, 70% and 20% ethanol (2 min each) followed by 5 min in distilled water. Sections were then placed in a 1% cresyl violet (Sigma Chemical Co., St Louis, MO)/ 3% acetic acid solution for 30-45min. Sections were then washed in water followed by 2 sec incubation in 63% ethanol/1% acetic acid followed by 2 sec incubation in 90% ethanol/1% acetic acid. Sections were then dehydrated with 100% ethanol, cleared with xylenes and mounted using Permount. Sections assigned to FluoroJade staining were initially incubated with 100% ethanol for 3 min followed by 70% ethanol for 1min and a 1min wash in distilled water. Sections were then placed in 0.06% potassium permanganate for 15 min, washed in a continuous stream of distilled water and then immersed for 25-30 min in 0.1% acetic acid containing 0.001% FluoroJade B (Sigma Chemical Co., St. Louis, MO). Sections were washed with a continuous stream of distilled water, dehydrated with 100% ethanol, cleared with xylenes and mounted with Permount. All images were captured with an Olympus IX-50 inverted microscope equipped with epifluorescence and a CRX digital camera (Digital Video Camera Co) using standardized settings. Images from individual studies were processed identically using Adobe Photoshop.

2.7 Statistical Analysis

All statistical analyses were performed using GraphPad Prism, Version 4.03 (GraphPad Software, Inc.). Data are reported as either the median alone or mean $\pm$ SEM seizure scores. A two-tailed Mann-Whitney U-test was used to compare responses between animals

administered rofecoxib and the control diet. A student's t-test was used to compare seizure latency and animal weights between animals administered rofecoxib and the control diet. Data sets representing proportions were analyzed using a two-tailed Fisher's exact test. Curves depicting the percentage of mice kindled as a function of time were compared using a logrank test. Significance was set at $p < 0.05$.

3. Results

3.1 COX-2 protein expression is increased in murine hippocampi following PTZ-induced acute and kindled seizures

The modest expression of COX-2 protein detected in control brains (Figure 1A, B and C) is markedly but transiently enhanced following acute convulsive seizures generated via systemic administration of pentylenetetrazole (PTZ) (Figure 1). Three hours post-seizure, there is a dramatic enhancement in COX-2 protein expression within the hippocampi of animals that exhibited a convulsive seizure (score of 3 or 4, Figure 1G, H and I), whereas COX-2 levels in the hippocampi of mice exhibiting non-convulsive seizures (score of 2, Figure 1D, E and F) are similar to that measured in saline-treated control mice (Figure 1A, B and C). Increased expression occurs within all areas of the hippocampus, although the most striking enhancement is demonstrated in the granule cells of the dentate gyrus, where basal COX-2 immunoreactivity in saline-treated control animals is normally minimal or absent (Figure 1A, B and C). The increased expression in animals exhibiting convulsive seizures is transient, returning to baseline levels by 72 hr (Figure 1J, K and L). In contrast, the hippocampi and dentate gyri of fully kindled animals was increased and maintained for at least 72 hr (Figure 2G, H and I). This increase was most prominent in the CA3 region and dentate granule cell layers (Figure 2G, H and I).

3.2 Increase in COX-2 protein expression following PTZ treatment is not associated with neuronal cell death

Interestingly, this increase in COX-2 immunoreactivity is not associated with neuronal cell loss assessed 72 hr after an acute (Figure 3E-H) or kindled seizure (Figure 3I-L). Saline-treated animals are shown for comparison (Figure 3A-D). Hippocampi of kindled mice or mice that experienced an acute PTZ-induced seizure had no overt neurodegeneration as evidenced by lack of pyknotic neuronal nuclei (Figure 3E-L). The architecture of the principle cell layers of the hippocampus (CA1 and CA3) and the dentate gyrus were intact and devoid of any obvious defects (Figure 3E-L). In confirmation of these findings, no dead or dying cells in any brain region 72 hr after an acute or kindled seizure were detected using the anionic fluorochrome FluoroJade B (Figure 4A,B). This is in striking contrast to what one sees following kainic acid- induced status epilepticus (Figure 4C,D).

3.3 Treatment with rofecoxib does not modify PTZ-induced acute seizures

Oral administration of rofecoxib for 5 days prior to PTZ administration neither increased nor decreased the incidence or severity of seizures in mice induced by either 40 or 55 mg/kg PTZ (Figure 4). The median seizure score in response to a 40mg/kg PTZ challenge was identical in both treatment groups (stage 1), as was the number of animals exhibiting a convulsive (stage 3) seizure (1:18; 5.6%). Rofecoxib treatment also failed to modify

seizures generated by 55mg/kg PTZ; median seizure scores of animals receiving the control (stage 2.5) or rofecoxib diet (stage 3) did not differ statistically (Figure 5). Additionally, the percentage of animals in both groups responding to 55mg/kg PTZ with a convulsion [50% (4/8) and 60% (3/5) of animals fed the control diet or rofecoxib-treated diets, respectively] was not different (Fisher's exact test, $p = 1.0$). Finally, the latency to convulsive seizures induced by 55mg/kg PTZ administration in either treatment group was nearly identical (108 ± 31 and 106 ± 77 sec for control diet and rofecoxib-treated mice, respectively; $p > 0.05$, Student's t-test).

3.4 Kindling acquisition is unaffected by rofecoxib treatment

To assess the effect of rofecoxib on the acquisition of PTZ-induced kindling, mice were provided either a control rodent chow or rofecoxib supplemental chow *ad libitum* for five days prior to and then continuously throughout kindling protocol. A progressive and gradual increase in seizure score was seen in mice irrespective of diet with rates of kindling being similar between groups (Figure 6A and B, $p = 0.96$, logrank test). Additionally, a similar percentage of animals in each group became kindled by the end of the study [72 vs. 78% of the mice fed the control vs. rofecoxib diets, respectively]. Of these, the mean day at which the control and rofecoxib-treated mice kindled was identical (13 ± 1 day; mean $\pm$ SEM) (Figure 6C). Mortality associated with the paradigm was low [11% (2/18) and 6% (1/18), control and rofecoxib-treated, respectively] and all mice exhibited a steady weight gain of approximately 2g. The final weight of all the kindled mice did not differ significantly from non-kindled mice [34 ± 3 g and 33 ± 2 g, respectively; $p > 0.05$, unpaired t-test] nor did it differ between treatment groups [33 ± 3 g and 34 ± 2 g, for the control and rofecoxib-supplemented diets, respectively; $p > 0.05$, unpaired t-test], indicating that the regimen was well-tolerated.

3.5 Kindling maintenance is unaffected by rofecoxib treatment

Mice were treated each day with PTZ (40mg/kg) until they exhibited four consecutive convulsive seizures, at which time they were considered kindled and the daily PTZ injections were stopped. Animals were then randomized to receive either control rodent chow or chow supplemented with rofecoxib. The kindling latencies of the mice assigned to the control chow and the rofecoxib-containing food were 11 ± 1 and 10 ± 2 days (mean $\pm$ SEM), respectively. Ten and 20 days later, mice were re-challenged with 40mg/kg PTZ. Convulsions occurred in all members of both groups except one. This indicates that these mice retained their kindled state regardless of the dietary presence of rofecoxib (Figure 7). Additionally, the seizure latencies between the control and rofecoxib fed mice were not statistically different during either re-challenge trial (Ten days: 130 ± 20 sec and 110 ± 30 sec; 20 days: 120 ± 30 and 110 ± 30 sec, respectively). Finally, both dietary groups had similar weights at the end of the experiment 33 ± 3 g and 32 ± 4 g, (mean $\pm$ SEM, control and rofecoxib-supplemented diets, respectively). Hence, prandial rofecoxib treatment did not extinguish kindling.

4. Discussion

Numerous studies have demonstrated that generalized seizure activity, whether it be elicited via systemic administration of kainic acid (Yamagata et al., 1993; Adams et al., 1996; Tocco et al., 1997), pilocarpine (Turrin and Rivest, 2004) (Jung et al., 2006), or electrical stimulation (Takemiya et al., 2003; Tu and Bazan, 2003), enhances neuronal COX-2 expression. These models induce seizures by directly amplifying excitatory circuits and, in the cases of kainic acid and pilocarpine, can cause excitotoxic neuronal cell death. COX-2 expression was also found to be increased in brains of genetically seizure-susceptible *El+* mice following vestibulomotorily-induced seizures (Okada et al., 2001). Results in the present report confirm and extend these observations in the acute PTZ seizure and kindling epileptogenesis models, whereby excitatory neuronal activity is enhanced indirectly via antagonism of GABAergic inhibition (Huang et al., 2001).

The role of COX-2 in acute seizure responses appears to be complex. For example, upregulation of neuronal COX-2 expression after pilocarpine- or kainate-induced status epilepticus, a condition of sustained acute seizure activity, is temporally and anatomically associated with subsequent neuronal degeneration (Tocco et al., 1997) and selective COX-2 inhibitors diminished neuronal cell loss in rats when administered after the convulsive stimulus (Kunz and Oliw, 2001b; Kawaguchi et al., 2005). Further, kainate-treated rats performed better in both spatial and non-spatial learning behavioral paradigms when treated with celecoxib after seizure induction, an effect associated with a decrease in hippocampal neuronal cell death (Gobbo and O'Mara, 2004). While these studies demonstrate that postictal delivery of COX-2 inhibitors is neuroprotective in models of status epilepticus, pretreatment with COX-2 inhibitors appears to have the opposite effect. Thus, pharmacological inhibition of COX-2 prior to status epilepticus increased seizure severity and mortality, with surviving animals demonstrating an enhancement of neuronal cell death (Baik et al., 1999; Kunz and Oliw, 2001a; Gobbo and O'Mara, 2004). In agreement with these results, seizure severity and neuronal cell death elicited by NMDA were also reported to be enhanced in COX-2 deficient mice (Toscano et al., 2008). One possible explanation for these contradictory results is that arachidonic acid metabolites derived from basal COX-2 expression at the onset of seizure activity serve an anti-convulsive role, whereas products generated from induction of COX-2 expression after status epilepticus promotes cytotoxicity.

In the present study, five day, chronic, prophylactic treatment with rofecoxib delivered in mouse chow failed to alter seizure severity induced by acute PTZ exposure in CD1 mice. In contrast, others have reported that acute administration of rofecoxib given via oral gavage just 45 min prior to PTZ injection delayed the onset and decreased the severity of acute clonus (Dhir et al., 2006a) and increased PTZ seizure threshold (Akula et al., 2008) in albino (Laka) mice. Hence, the effect of selective COX-2 inhibitors on acute PTZ-induced seizures may also be influenced by the timing and method of administration of inhibitors and potentially the strain of mouse employed. Nevertheless, the findings in the current study are supported by the observation that seizures induced by lindane, which possesses GABA_A receptor antagonistic properties similar to PTZ, were unaffected by deletion of the COX-2

gene (Toscano et al., 2008). Additionally, 4 week treatment with indomethacin also failed to alter seizure threshold or duration in the EI+ mice (Okada et al., 2001).

Despite the marked increase in COX-2 protein expression after acute PTZ-induced convulsive seizures, no evidence of neuronal cell death was observed in this model. This is noteworthy given the evidence suggesting that new COX-2 expression contributes to the neurodegeneration associated with models of status epilepticus (*vida supra*). It indicates that seizure-induced COX-2 expression is not necessarily a harbinger of death, a notion that is in accord with studies demonstrating that up-regulation of COX-2 after electroconvulsive seizures and spreading depression are not associated with cell death (Miettinen et al., 1997; Newton et al., 2003). Hence, it is possible that, in the absence of overt neurodegeneration, COX-2 metabolic products participate in adaptive responses, thus begging the question as to whether neuronal COX-2 gene induction is a key signaling event in epileptogenesis.

Interestingly, COX-2 mRNA and protein expression are upregulated in the hippocampus following electrical (Takemiya et al., 2003; Tu and Bazan, 2003) and chemical (Turrin and Rivest, 2004) (Jung et al., 2006) (this study) kindling development. Additionally, the selective COX-2 inhibitor, nimesulide, attenuated both behavioral and electrical parameters of kindling acquisition (Tu and Bazan, 2003). The electrically-induced kindled state was also reduced in mice lacking the COX-2 gene (Takemiya et al., 2003). It should be noted that the effect of pharmacological or genetic disruption of COX-2 on electrical kindling in the above-mentioned studies was rather modest, suggesting that additional factors may act in parallel to elicit the maximal kindled phenotype. In the current study, however, chronic treatment with rofecoxib provided *ad libitum* 5 days prior to and for the duration of the PTZ-kindling paradigm failed to modify kindling acquisition. Additionally, rofecoxib treatment initiated after the completion of the kindling paradigm failed to reverse the kindled phenotype, suggesting that persistent COX-2 expression as demonstrated herein does not contribute to maintenance of the kindled phenotype. Conceivably, differences between the electrical and PTZ kindling paradigms could account for the discrepancies between the two models with regards to selective COX-2 inhibitor efficacy. However, it should be noted that a modest reduction in PTZ-induced kindling acquisition has been reported when animals received rofecoxib via oral gavage once per day 45 min prior to each PTZ injection (Dhir et al., 2006b; Dhir et al., 2007). The main differences between this and our own study are the mode (gavage vs. chow) and timing (acute vs. chronic) of drug administration, as well as the strain of mouse used (Laka vs. CD1). In any case, the results reported herein are consistent with previous results demonstrating that chronic oral administration of selective COX-2 inhibitors fail to alter the evolution of spontaneous seizure activity following electrically (Holtman et al., 2009) or pilocarpine-induced (Polascheck et al., 2010) status epilepticus in rat as well.

Finally, it is unlikely that the inability of rofecoxib to affect either PTZ-induced acute seizures or kindling in the current study is because the drug failed to reach an effective concentration in the brain. Rofecoxib readily crosses the blood brain barrier (Halpin et al., 2000; Dembo et al., 2005), has a half-life of ≈ 17 hr (Depre et al., 2000), and has been shown by numerous investigators to effectively decrease prostaglandin biosynthesis in rodent brain (Candelario-Jalil et al., 2003; Teismann et al., 2003; Xiang et al., 2007).

Directly pertinent to the dosing paradigm employed herein, chronic administration of rofecoxib (20 mg/kg/day) to mice in chow effectively reduced brain prostaglandin levels in both wild-type and human COX-2 over-expressing animals (Xiang et al., 2007). Additionally, chow delivering 30 mg/kg rofecoxib per day, the dose used herein, provided significant neuroprotection against hippocampal excitotoxic neurodegeneration in mice engendered by direct NMDA microinjection *in vivo* (Hewett et al., 2006). Finally, COX-2 protein expression levels in the hippocampi of kindled mice receiving the rofecoxib diet were higher than in kindled mice that were maintained on the control diet, showing direct CNS effects of rofecoxib in this study (Supplemental Figure 3). This finding is consistent with reports in the literature showing increased COX-2 protein levels in cells after NSAID treatment (Callejas et al., 1999; Ferguson et al., 1999). COX-2 protein is degraded by two pathways (Mbonye et al., 2008) and our own unpublished observations suggest that this effect of rofecoxib on CNS COX-2 expression may be due to blockade of normal protein degradation subsequent to activity-dependent suicide-inactivation.

In sum, the present study demonstrates that prophylactic, prandial rofecoxib treatment of moderate to chronic duration neither affects acute seizure generation, kindling acquisition nor reverses the kindled state in a PTZ-induced model of epilepsy. Overall, these data suggest that upregulation of COX-2 may be a secondary response to seizure activity in this model and plays no significant role in the process of PTZ-induced epileptogenesis. In a broader sense, this study—taken together with other studies demonstrating that prophylactic suppression of COX-2 might actually be deleterious to the epileptic brain (*vida supra*) — suggests that prophylactic treatment with COX-2 inhibitors would be an ineffective therapeutic strategy for the treatment of epilepsy or for the prevention of epileptogenesis in “at risk” populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PTZ enhances hippocampal COX-2 immunoreactivity in a seizure-dependent manner Mice were administered (i.p.) saline (A-C), 40mg/kg PTZ (eliciting a Stage 2 seizure, D-F) or 55mg/kg PTZ (eliciting a Stage 4 seizure, G-L) and were sacrificed either 3 hrs (A-I) or 72 hr (J-L) afterward. Brains were removed, sectioned (30 μ m) and processed for COX-2 immunohistochemistry. Photomicrographs were acquired from a representative coronal section showing the entire hippocampal formation between -1.2 and -1.4 bregma (A, D, G and J, 8x) and the dentate gyrus (B, E, H and K, 20x) and CA3 (C, F, I and L, 20x) subregions. N = 4-5 mice per treatment group from two independent experiments.

Figure 2. COX-2 protein expression is enhanced in the hippocampi of kindled mice

Mice were treated (i.p.) once daily with either saline (A-C) or 40mg/kg PTZ (D-I). Upon becoming kindled, the daily PTZ injections were discontinued and mice were sacrificed either 3 (D, E and F) or 72hr (G, H and I) after their last kindled seizure. Additionally, saline-treated mice (A-C) were sacrificed 3hr after their last saline injection. Brains were removed, sectioned (30 μ m) and processed for COX-2 immunohistochemistry. Photomicrographs were acquired from a representative coronal section showing the entire hippocampal formation between -1.2 and -1.4 bregma (A, D and G, 8x) and the dentate gyrus (B, E and H, 20x) and CA3 (C, F and I, 20x) subregions. N = 3-4 mice per treatment group from two independent experiments.

Figure 3. Lack of hippocampal neuronal cell death after acute and kindled seizures induced by PTZ

Brains were harvested from animals treated in Figures 1 and 2. Representative photomicrographs of cresyl violet stained tissue showing the entire hippocampal formation between -1.2 and -1.4 bregma (A, E and I, 8x) and the dentate gyrus (B, F and J, 20x) CA3 (C, G and K, 20x) and CA1 (D, H and L, 20x) subregions from saline-treated mice (A-D) or from mice 72 hr after an acute (E-H) or kindled (I-L) stage 4 PTZ-induced seizure.

Figure 4. Representative photomicrographs of cresyl violet and Fluoro Jade-stained sections from post-ictal mice receiving either PTZ or kainic acid

Mice were administered convulsive doses of either PTZ (A and B) (55 mg/kg) or kainic acid (C and D) (40 mg/kg titrated over 150 min) and were sacrificed 72 hr afterward by transcardial perfusion and fixation with 4% PFA. Brains were removed and serial coronal sections (30 μ m) were stained using either 1% Cresyl Violet (A and C) or 0.001% Fluoro Jade B (B and D). Photomicrographs show the entire hippocampal formation between -1.2 and -1.4 bregma. Images were captured with an Olympus IX-50 inverted microscope equipped with epifluorescence and a CRX digital camera (Digital Video Camera Co) and processed identically using Adobe Photoshop.

Figure 5. PTZ-induced acute seizure severity is unaffected by prandial rofecoxib treatment
Mice were fed control rodent chow (filled circles) or chow standardized to deliver 30mg/kg/day rofecoxib (open circles) *ad lib* five days prior to administration of 40mg/kg (n = 18/group) or 55mg/kg (n = 5-8/group) PTZ i.p. The horizontal bar represents the median seizure score for each group. Data were pooled from eight independent experiments and a two-tailed Mann-Whitney U-test revealed no significant differences between the control and rofecoxib diet-treated animals at either PTZ dose (p = 0.66 and 0.72 for 40 and 55mg/kg PTZ, respectively).

Figure 6. Rofecoxib does not affect PTZ-induced kindling acquisition

Animals received either control (filled circles, solid lines, $n = 18$) or rofecoxib-containing rodent chow (open circles, dashed lines, $n = 18$) *ad lib* five days prior to the start of the kindling paradigm (once daily PTZ (40 mg/kg, i.p.) and were maintained on their respective diets throughout the kindling protocol (next 24 days). A) Mean seizure score $\pm$ SEM at each day of the kindled protocol. There are no between-group differences in seizure score at any point during the kindling paradigm as determined by Kruskal-Wallis one-way ANOVA ($p > 0.05$). B) Percentage of mice kindled on each day. The kindling rates of the mice fed the control diet vs. the rofecoxib diet did not statistically differ, as determined by a logrank test ($p = 0.96$). C) Day each individual animal was kindled is depicted by symbols while the horizontal line depicts the mean day kindled per group, which did not differ statistically (unpaired t-test, $p = 0.87$). $n = 13$ - 14 /group pooled from four independent experiments.

Figure 7. Dietary administration of rofecoxib does not reverse an established PTZ-induced kindled state

Mice were fed control chow and administered 40mg/kg PTZ (i.p.) once a day until kindled at which time they were either maintained on the control diet (n = 6, filled circles) or switched to the diet containing rofecoxib (n = 8, open circles). Ten and 20 days later mice were re-challenged with 40mg/kg PTZ (i.p.). Each circle represents the individual seizure score while the horizontal lines represent the median seizure score of the group. A two-tailed Mann-Whitney U-test revealed no between- group differences in the median seizure scores at either time point. Data were pooled from two independent experiments.