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CO-ADMINISTRATION OF SUBTHERAPEUTIC DIAZEPAM ENHANCES NEUROPROTECTIVE EFFECT OF COX-2 INHIBITOR, NS-398, AFTER LITHIUM PILOCARPINE-INDUCED STATUS EPILEPTICUS

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Abstract—*Rationale:* Seizures during status epilepticus (SE) cause neuronal death and induce cyclooxygenase-2 (COX-2). Pilocarpine-induced SE was used to determine if COX-2 inhibition with NS-398, when administered alone or with diazepam, decreases the duration and/or intensity of SE and/or reduces neuronal injury in the rat hippocampus.

***Methods:* Electroencephalogram (EEG) electrodes were implanted in male Sprague–Dawley rats. SE was induced with lithium-pilocarpine, and continuous EEG and video monitoring were performed for 24 h. Rats were divided into four groups (n = 8–14 rats/group) and received NS-398, diazepam, NS-398 and diazepam, or vehicle 30 min after the first motor seizure. Six hours later, NS-398 injection was repeated in the NS-398 and in the NS-398 + diazepam groups. The duration of SE (continuous spiking) and the EEG power in the γ-band were analyzed. FluoroJade B staining in the dorsal hippocampus at 24 h after SE was analyzed semi-quantitatively in the CA1, CA3 and hilus.**

***Results:* The duration and intensity of electrographic SE was not significantly different across the four groups. In rats treated with NS-398 alone, compared to vehicle-treated rats, neuronal damage was significantly lower compared to vehicle-treated rats in the CA3 (27%) and hilus (27%), but neuroprotection was not detected in the CA1. When NS-398 was administered with diazepam, decreased neuronal damage was further obtained in all areas investigated (CA1: 61%, CA3: 63%, hilus: 60%).**

***Conclusions:* NS-398, when administered 30 min after the onset of SE with a repeat dose at 6 h, decreased neuronal damage in the hippocampus. Administration of diazepam with NS-398 potentiates the neuroprotective effect of the COX-2 inhibitor. These neuroprotective effects occurred with no detectable effect on electrographic SE.**

Key words: cyclooxygenase-2 (COX-2), inflammation, neuroprotection.

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Abbreviations: COX-2, cyclooxygenase-2; EEG, electroencephalogram; PGE_2, prostaglandin E_2; SE, status epilepticus.

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INTRODUCTION

Status epilepticus (SE) is a neurologic emergency that requires prompt intervention to prevent long-term morbidity and mortality. Both human clinical studies and rodent models of SE indicate that prolonged seizure activity causes neuronal death via several pathways, and rapid treatment of SE reduces this injury and subsequent morbidity (Lowenstein and Alldredge, 1998; Alldredge et al., 2001; Klitgaard et al., 2002; Morimoto et al., 2004). Benzodiazepines, such as diazepam and lorazepam, are widely used as first-line agents to terminate seizure activity, but unfortunately, SE becomes progressively more refractory to these agents after 30–40 min (Walton and Treiman, 1988; Jones et al., 2002; Naylor et al., 2005). Thus, developing therapies for treating SE that more effectively terminate later stage seizure activity, enhance neuroprotection, or suppress the long-term consequences of SE is an important goal.

Inflammatory mediators contribute to the negative consequences of SE (Ravizza et al., 2011; Vezzani et al., 2012). The cyclooxygenase-2 (COX-2) pathway, in particular, has been studied in many animal models of SE and reportedly affects neuronal excitability (Chen and Bazan, 2005; Zhang et al., 2008), susceptibility to neuronal death (Ho et al., 1998; Kawaguchi et al., 2005), and influx of several antiepileptic drugs across the blood–brain barrier (Bauer et al., 2008; Schlichtiger et al., 2010; van Vliet et al., 2010), all of which may influence the severity of SE. COX-2 is an inflammatory enzyme involved in prostaglandin synthesis that is expressed under basal conditions in excitatory neurons from the cortex, hippocampus, hypothalamus and amygdala (Yamagata et al., 1993; Adams et al., 1996; Kaufmann et al., 1996). Increased synaptic activity associated with seizures markedly amplifies the expression of COX-2 within 30 min from the beginning of seizures, with the peak effect estimated to be about 24 h in different animal models of seizures (Voutsinos-Porche et al., 2004; Kawaguchi et al., 2005; Jung et al., 2006; Takemiya et al., 2006). COX-2 knockout mice showed decreased incidence of after-discharges, reduced after-discharge duration, and delayed induction of convulsive seizures compared to control mice after rapid kindling, suggesting that COX-2 facilitates the recurrence of seizures (Takemiya et al., 2003). The COX-2 pathway has also been implicated in regulating the transport of several antiepileptic drugs across the blood–brain barrier (Schlichtiger et al., 2010; van Vliet et al., 2010). Thus

hypothetically, COX-2 inhibitors could directly suppress SE, facilitate transport of antiepileptic drugs to brain tissue, and reduce subsequent damage to neurons. However, when attempts to study the effect of COX-2 inhibition on the severity of SE and resultant neuronal damage using primarily behavioral observation of seizure activity, blocking of the COX-2 pathway has given mixed results, with some studies showing neuroprotection (Kunz and Oliw, 2001b; Jung et al., 2006; Takemiya et al., 2006; Bauer et al., 2008; Polascheck et al., 2010), and others showing no neuroprotection (Holtman et al., 2009, 2010; Pekcec et al., 2009) or even exacerbation of neuronal death (Baik et al., 1999; Kim et al., 2008).

The aim of the present study was to investigate the effect of COX-2 inhibition, both alone and in the presence of diazepam, on the severity of SE and on neuronal damage. Brain electrical activity was monitored continuously for 24 h with subdural electroencephalogram (EEG) electrodes, thus allowing analysis of the effect of COX-2 and diazepam on the electrographic SE, not just behavioral seizure activity. Neuronal damage in the hippocampus was assessed by FluoroJade B staining of brain sections obtained 24 h after induction of SE. We hypothesized that inhibition of COX-2 decreases the duration and/or intensity of electrographic SE, possibly through decreased pharmacoresistance to benzodiazepines, with resultant neuroprotection.

EXPERIMENTAL PROCEDURES

Experimental animals

Adult male Sprague–Dawley rats (Charles River, USA) weighing 250–400 g were kept under 12-h light/dark conditions with free access to food and water. All procedures were approved by the University of Utah Animal Care and Use Committee.

Electrode implantation

The animals were anesthetized with isoflurane (2%) and placed in a stereotaxic device. Bipolar electrodes (MS333-3-B, Plastics One, Roanoke, VA, USA) were used for subdural recordings. Two holes (500 μm) were drilled on the right side of the midline under the bregma, and one lead was placed into each of the craniotomies to provide differential recordings. A third lead was placed in a third craniotomy left of the midline to be used as the ground electrode. Three additional screws were implanted in the skull, then the electrodes were fixed in place with dental cement and the skin was sutured around the skull. After surgery, the animals were put on a 12-h light/dark cycle and fed standard rat chow *ad libitum*. After recovery from the surgery ($\geqslant$7 days), the animals were treated with pilocarpine.

Video and EEG recording

Implanted animals were placed into cages with commutators (Plastics One, Roanoke, VA, USA) and connected to cables via their skull caps for EEG recordings. Signals were amplified using EEG100C amplifiers (high-pass filter, 1 Hz; low-pass filter, 35 Hz; 5000× gain), digitized at 500 Hz using an MP150 digital–analog converter, and acquired with AcqKnowledge acquisition software (BioPac Systems, Santa Barbara, CA, USA). While the rats were tethered in these cages, they were continuously video monitored using eight color cameras linked to a multiplexer to allow for eight animals to be recorded on one DVD. Recordings were obtained for 24 h across three DVD recorders (DMR-ES20, Panasonic, Newark, NJ, USA), each recording for an 8-h period.

SE induction

Animals were connected to the video-EEG recording system and pretreated with LiCl (127 mg/kg, i.p., 18 h before pilocarpine injection, Fig. 1A). Scopolamine bromide (1 mg/kg, i.p.) was administered 30 min before the injection of pilocarpine (50 mg/kg, i.p.). For one set of experiments, animals were grouped into two categories: (1) animals receiving a vehicle injection, 0.5% methylcellulose, (vehicle group, $n = 14$), and (2) animals receiving COX-2 inhibitor (NS-398, 10 mg/kg, i.p., Cayman Chemical, Ann Arbor, MI, USA, NS-398 group, $n = 11$) 30 min after the first convulsive seizure. The injections were repeated after 6 h. This concentration of NS-398 has been reported to significantly reduce COX-2 activity in the brain as measured by prostaglandin E_2 (PGE_2) activity in a rat model of kainate-induced SE (Takemiya et al., 2006). Based on our experience with pilocarpine-induced SE model, the mortality rate in the vehicle group is about 35%, so we used a higher number of animals in this group than in the NS-398 group. In a second set of experiments, three different groups of rats received: (1) vehicle ($n = 14$), (2) diazepam (10 mg/kg, $n = 8$), and (3) diazepam + NS-398 (10 mg/kg each, $n = 9$) 30 min after the first convulsive seizure. NS-398 injection was repeated at 6 h in diazepam + NS-398 group, while the other groups received vehicle. EEG-video recordings were obtained for 24 h after induction of SE (Fig. 2). During this period of time, the mortality rate was: 29% and 36% in vehicle groups, 9% in NS-398 group, 0% in diazepam group, and 11% in diazepam + NS-398 group.

Analysis of electrographic SE

An algorithm that extracts the energy in the γ-band was used to evaluate the efficacy of NS-398 and/or diazepam on electrographic SE (Lehmkuhle et al., 2009). This program filters the EEG to reduce the movement artifacts (<20 and >70 Hz), and models the energy with an eighth-order polynomial to estimate the effect of the drug over a 10-h period with 10-min resolution. This approach allowed statistical comparisons to be made across treatment groups at selected time points. To normalize the electrographic seizure data across animals, the time of drug administration was considered 100% and data are presented as percent change in power. The duration of electrographic SE was considered to be the period of time in which the animal displayed continuous spiking activity without periods of inactivity between the spiking (Fig. 1B).