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Immunomodulatory effect of Celecoxib on HMGB1/TLR4 pathway in a recurrent seizures model in immature rats

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ABSTRACT

Epileptic seizures constitute an important problem in pediatric neurology during the developmental period. The frequency and nosological significance of seizures, as well as their association with epileptogenesis, may be related to underlying mechanisms such as neuroinflammation. Those mechanisms of response activate inflammatory molecules induced in the neurons, activated glial cells and endothelial cells via the key HMGB1/TLR4 pathway. In this study, the drug celecoxib (CCX) was used as a blocker of the cyclooxygenase 2 (COX-2) and HMGB1/TLR-4 pathways. The experimental model was implemented in 10-day-old neonatal Sprague Dawley rats to induce recurrent seizures with kainic acid (KA, 1.4 mg/kg). Data were evaluated at early (14 PND) and late (30 PND) time points. The results showed that the CCX and CCX + pentobarbital (PB) groups exhibited a protective effect by significantly increasing the time latency of seizures compared to the KA group at both early ($p < 0.01$) and late ($p < 0.001$) times. When the CCX group was compared to the KA group, there was also a significant decrease in the number of HMGB1 and TLR-4 transcripts ($p < 0.05$) and in COX-2 protein expression ($p < 0.05$) in the most important areas for seizure generation (the hippocampus and cortex) at both the early and late time points. These results demonstrated that CCX treatment after epileptic seizures has a neuroprotective effect due to the inhibition of proinflammatory proteins and associated signaling pathways and reduces seizure susceptibility. Additionally, the timely intervention of inflammatory pathways will reduce the risk of developing epilepsy in adulthood.

1. Introduction

An immature brain has anatomical and physiological characteristics that facilitate the development of seizures (Jensen and Baram, 2000; Hernan and Holmes, 2016). The incidence of neonatal seizures according to US birth records from 2003 to 2013 was 0.26 per 1000 term births. Seizures are significant in pediatric neurology due to their frequency and nosological significance, despite having a low incidence (Berry et al., 2017). These seizures encourage the development of chronic epilepsy and the limited/ineffective response or potentiation of seizures during the use of antiepileptic drugs (Rennie and Boylan, 2007; Bonifacio et al., 2011). Recently, it was reported that US children have a lifetime prevalence of 10.2/1000 (95% confidence interval [CI]: 8.7–11.8) or 1% for epilepsy/seizure disorder (Russ et al., 2012).

After an early insult, acute seizure activity was triggered, a latency period appeared and subsequent spontaneous seizures appeared with more frequency and intensity (Jensen and Baram, 2000). Acute changes occurred during the first hours and weeks to generate an inflammatory process, induce neuronal death, decrease the balance between excitation and inhibition in the neurons, and activate the early genes involved in neural development and hyperexcitability processes, allowing seizure recurrence (Suchomelova et al., 2015; Feng and Chen, 2016). The experimental models with immature rats have contributed to an understanding of the electroencephalographic features and molecular, cellular, and pharmacological mechanisms observed during epileptogenesis (Holmes, 2005; Huneau et al., 2013; Huusko et al., 2015; Walker et al., 2016).

Kainic acid (KA) is a kainate receptor agonist and a subtype of ionotropic receptors to glutamate, which is found in the amygdala, entorhinal cortex, basal ganglia, cerebellum, and hippocampus (Ozawa

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et al., 1998). This compound is used in experimental models at early ages to induce seizures and status epilepticus (SE) or to promote epileptogenesis (Löscher, 2002; Ben Ari, 2006; De Araujo Furtado et al., 2012; Inostroza et al., 2012). The seizure activity may trigger a neuroinflammatory response. First, it results in the activation of microglia and monocyte infiltration, mediated by signaling pathways of proinflammatory molecules, including the IL-1R/TLR pathway and the subsequent expression of inflammatory proteins such as COX-2, TGF-β, HMGB-1 and TNF-α. These molecules participate in neuronal hyperexcitability, neurodegeneration and neuronal death, which make them an important target for new treatment strategies (Vezzani et al., 2013).

Cyclooxygenase-2 (COX-2) is an enzyme found in the glutamatergic neurons of the cortex and hippocampus. This enzyme catalyzes the synthesis of prostanoids, which regulate the signaling pathways related to plasticity and the excitability of the cellular membrane. COX-2 is strongly induced by infection, fever, inflammation, convulsive activity or brain lesions and is considered a major proinflammatory mediator (Maddrey et al., 2000; Katori and Majima, 2000). High-mobility group box-1 (HMGB1) is considered an endogenous danger signal protein of innate immunity by recognizing damage associated molecules DAMPs ligands. Furthermore, this molecule is released by immune cells, neurons, and glia in response to cell damage and participates in the evolution of diseases with inflammatory processes such as epilepsy, atherosclerosis, cancer or autoimmune diseases (Klune et al., 2008). HMGB1 activates TLR4 in neurons and astrocytes and has been proposed to be a critical event for decreasing seizure threshold and initiating brain inflammation (Maroso et al., 2010). Toll-like Receptor 4 (TLR4) belongs to the TLR family, a crucial component of innate immunity. In experimental models, lipopolysaccharide (LPS) administration increases the short- and long-term convulsive threshold that can be prevented with TLR-4 antagonists, considering that TLR4 is the LPS-sensing receptor (Leow-Dyke et al., 2012). Both TLR4 and HMGB1 are upregulated in the brain specimens of epilepsy patients and in the brain tissues of animals with chronic seizures (Maroso et al., 2010; Yang et al., 2017).

Celecoxib (CCX) is a non-steroidal sulfa anti-inflammatory drug (NSAID) that selectively inhibits COX-2. However, its inhibitory effect is inconsistent among the models of seizures, inhibitor type, dose and management time and the expression/inhibition of inflammatory molecules such as HMGB-1 and TLR-4 (Kim et al., 2007; Gobbo and O'Mara, 2004).

Although pharmacological studies in experimental models suggest the prevention of epileptogenesis by the inhibition of the COX-2 pathway, their products and members of the HMGB1/TLR-4 pathway, it is still not clear that a timely intervention with CCX can reduce the expression of inflammatory molecules associated with damage and reduce the susceptibility to seizures.

2. Materials and methods

2.1. Animals

Twenty pregnant Sprague Dawley females were selected, balancing the litters of 5 to 8 males to guarantee a homogeneous weight, 10-day-old rats of 20 to 25 g were used. The litters were allowed unlimited maternal feeding for up to 21 days of age and after the lactation period were fed with Formulab Diet # 5008 on demand and maintained in a light/dark cycle of 12 × 12 h and a regulated temperature of 20 to 22 °C.

2.2. Induction of seizure activity

Convulsive activity was induced by the administration of 1.4 mg/kg of kainic acid (K0250, Sigma, USA) by intraperitoneally via (i.p.) dissolved in saline solution, pH 7.4, every 24 h for 5 days from 10 DPN and repeating a single dose (1.4 mg/kg) to 30 PND. The dose and administration via were selected based on previous works (Tremblay and Ben-Ari, 1984; Sayin et al., 2015).The rats were recorded for 1 h after the administration of kainic acid to later return with their mothers to be hydrated (Figueiredo et al., 2016). The mothers were conditioned (manipulation by the researcher), so as not to experience the rejection of the offspring and ensure their survival.

2.3. Experimental groups

Sixty-four animals divided into 4 groups:

- SH group (N = 16): animals received the same volume of vehicle in which celecoxib was dissolved (saline solution).
- KA group (n = 16). Animals received kainic acid (1.4 mg/kg, i.p.) for 5 days del 10–14 PND and al 30PND.
- CCX + KA group (n = 16). Animals received the same treatment as the KA group and celecoxib (20 mg/kg). One hour later, the celecoxib (1098504, Sigma USA) dissolved in saline solution was administered by esophagus via (e.v.) of a stock solution that was previously dissolved in carboxymethylcellulose.
- PB + KA group (n = 16). Animals received the same treatment as the KA group. One hour later, the pentobarbital (10 mg/kg, i.m.) was administrated.
- CCX + PB + KA group (n = 16): animals received the same treatment as the KA group. One hour later, celecoxib (20 mg/kg, e.v.) as well as pentobarbital (10 mg/kg, i.m.) were administrated.

Eight animals of each group were used for PCR-RT studies and remaining rats were used for western blot studies at each evaluation time.

2.4. Evaluation of motor behavior

The evolution of motor behavior generated by the AK administration on day 10, 14 and 30 PND was evaluated. Each control and experimental group received treatment, and seizure occurrence was evaluated for 1 h after the administration of Kainic acid i.p. (KA). Motor behavior latencies were recorded for 60 min and assessed via the following scale: (Ben Ari, 2006; Raol et al., 2009; Sayin et al., 2015).

- Phase 0: No response.
- Phase I: Facial clonus.
- Phase II: Head movements and chewing.
- Phase III: Unilateral or bilateral clonic movements.
- Phase IV: Bilateral clonus with attempts to join.
- Phase V: Bilateral clonus with falls and attempts to join. When phase 5 was identified, the presence of status epilepticus (SE) was considered.

2.5. PCR amplification of TLR-4 mRNA and HMGB1 mRNA

Brain cortex and hippocampus tissues were frozen and processed with Trizol reagent (Thermo Scientific, USA) for total RNA extraction according to the manufacturer's protocol. RNA samples suspended in molecular biology grade water were processed to obtain cDNA using the turbo DNA system (Thermo Scientific, USA) per the manufacturer's recommendations. The amplification of TLR-4 and HMGB1 transcripts was performed from 1 µl of cDNA in 10 µl of total reaction volume, consisting of 0.2 µl of Taq polymerase 10× reaction buffer (Thermo Scientific, USA), 0.6 µl of 25 mM $MgCI_2$, 0.2 µl of dNTP, 0.2 µl of each flanking primer at 10 mM, and 0.2 µl of Taq polymerase recombinant (Thermo Scientific, USA). The primers used for TLR4 were Forward (Fw) 3′-TGCAGAAAATGCCAGGATGA-5′ and Reverse (Rv) 3′ACACCT GGATAAATCCAGCCA-5′ to obtain a 277 bp fragment, and for HMGB1, they were Fw 3′-TCAACTAAACATGGGCAAAGG-5′ and Rv 3′-AGGCA GCAATATCCTTCTAT-5′ to obtain a 494 bp fragment. The amplification was done in a DNA engine system® thermocycler (Bio-Rad, USA) with a