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Short communication

Metallothionein expression in the rat brain following KA and PTZ treatment

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ABSTRACT

Epilepsy is a neurological disorder that has been associated with oxidative stress therefore epilepsy models have been develop such as kainic acid and pentylenetetrazol are usually used to understanding of the molecular mechanisms of this disease. We examined the metallothionein expression in rat brains of treated with kainic acid and pentylenetetrazol. Increase in metallothionein and nitrotirosyne immunoreactivity of both seizures epilepsy models was observed. Moreover, we show a significant increase on levels of MT expression. These results suggest that the increase of metallothionein expression is related with kainic acid and pentylenetetrazol treatments as response to damage mediated by oxidative stress.

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1. Introduction

Epilepsy is defined by a condition in which exists an increased and abnormal synchronization of neuronal activity; the temporal lobe epilepsy (TLE) is the most common form of partial seizures and is characterized by recurrent spontaneous seizures in adults (Cardenas-Rodriguez et al., 2013). Some works founded that temporal lobe epilepsy in humans and animal models is associated with neuronal loss in hippocampus during epileptogenesis (Majores et al., 2007; Méndez-Armenta et al., 2014).

Epilepsy has been associated with oxidative and nitrosative stress due to prolonged neuronal hyperexcitation and loss neurons during seizures. Experimental animals models report level ATP diminished, increase on lipid peroxidation, catalase activity and altered glutathione levels in brain regions rats treated with Kainic Acid (KA) and Petylenetetrazole (PTZ) (Erakovic et al., 2003; Liang and Patel, 2006; Agarwal et al., 2011). Oxidative stress and mitochondrial dysfunction occur as a result of intense seizure activity such as the produced in animal models, can lead to cytotoxic effects mediated by oxidative stress; therefore it is regarded as a possible mechanism in the pathogenesis of epilepsy (Waldbaum and Patel, 2010; Torres et al., 2012).

Metallothioneins (MT I–II) are expressed in astrocytes, whereas that the MT-III is expressed only in neurons (West et al., 2008). An increase on MT-I,-II levels in macrophages/microglia and astrocytes in a number of different neuropathological conditions including traumatic injury, neurotoxicity, brain ischemia and epilepsy have been reported in human and experimental animals (Dalton et al., 1995; Carrasco et al., 2000; Kim et al., 2003; Stankovic et al., 2007; Peixoto-Santos et al., 2012). Functionally MT-I and -II are indeed multipurpose proteins involved in the metal zinc homeostasis, acting as cellular defense proteins mainly how efficient scavengers of free radicals and protecting against neuronal damage producing by oxidative stress (West et al., 2008).

A better understanding of the molecular mechanisms associated with seizure development include the study of animal models of epileptic seizures, the systemic administration with KA a rigid analog of glutamate, produces a series of behavioral signs culminating in Status Epilepticus (SE) with neuropathological changes that are similar to human epilepsy (Ben-Ari and Cossart,

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2000). PTZ is an antagonist of gamma-aminobutiric acid receptors (GABAr); in this model repeated injection of sub-convulsive dose of PTZ causes gradual development of seizure culminating to generalized-tonic–clonic seizures (Löscher, 2011). The purpose of the current study was examines the immunohistochemical expression of the MT-I and MT-II in rat brains treated with KA and PTZ.

2. Material and methods

2.1. Animals

All experiments were performed on adult male Wistar rats. They were housed under standard conditions at 25 ± 2 °C, with a 12-h light/dark cycle, and were allowed free access to water and a standard diet. All of the animals were handled according to the National Institutes of Health (USA) Guidelines for the Care and Use of Laboratory Animals, and this protocol was approved by the Bioethics Committee of the National Institute of Neurology and Neurosurgery of Mexico.

2.2. Experimental procedure

The rats ($n = 8$) per group were administered a single i.p. dose of 10 mg/kg KA (Ben-Ari and Cossart, 2000). Eight animals treated with saline vehicle (control group) were sacrificed immediately after i.p. injection to serve as time 0 control. The animals develop during the first 20–30 min "have staring" spells. The following 30 min, the animals develop had head nodding and several wet dog shakes and finally they have a recurrent limbic motor seizure evolving to a full motor limbic SE. The animals with extensive tonic–clonic seizures were included in the time course study and were sacrifice at 1, 2, 6 and 24 h after injection of KA. For PTZ kindling, a subconvulsant dose of PTZ 25 mg/kg body weight was injected i.p. route daily and the animals were sacrificed after 3, 6, 13, 18 and 21 consecutive injections. The PTZ injections induced generalized clonic or tonic seizures consistent with Racine scale, whose classification is as follows: 0. – no reaction (0 day after injection aprox.); 1. – stereotypic mounting, eye blinking, and/or mild facial clonus (3–6 days after injection aprox.); 2. – head nodding and/or multiple facial clonus (6–9 days after injection aprox.); 3. – myoclonic jerks in the forelimbs (9–13 days after injection aprox.); 4. – clonic convulsions in the forelimbs with rearing (13–18 days after injection aprox.); and 5. – generalized clonic convulsions and loss of balance 818–21 days after injection aprox.). The latency and duration of seizure was observed behaviorally (Löscher, 2011; Velišek, 2006).

2.3. Histopathological evaluation

The animals were deeply anesthetized with pentobarbital and perfused transcardially phosphate-buffered saline (PBS), followed by 10% w:v buffered formalin solution at 4 °C. The brains were removed from the skull cut with a matrix (coronal rodent brain matrix, EMS) at 2 mm thick. The section between −3.14 and −4.16 mm posterior to the bregma suture containing the dorsal hippocampus were selected using a Paxinos and Watson stereotaxic atlas (2007). The section tissue was paraffin embedded and cut 5 μ thick and finally stained with cresyl violet and examined using a light microscope.

2.4. Immunohistochemistry

Briefly, for heat-induced epitope retrieval, sections were boiled in citrate buffer (pH 6 or 9) in a microwave oven for 2 × 10 min. The sections were preincubated with 0.3% hydrogen peroxide in PBS for 30 min. After the sections were incubated with monoclonal antibody against Metallothionein 1:20 (DAKO, Carpinteria, CA); and Nitrotyrosine (NITT) 1:100 (NITT, Santa Cruz Biotechnology) for localize the sites of oxidative damage. The sections were incubated with secondary biotinylated antisera and then immersed in avidin–biotin–peroxidase complex; (LSAB System HRP, DAKO, Carpinteria; CA). The immune reaction resulted in the oxidation of the 3,3′-diaminobenzidine by peroxidase (Liquid DAB, DAKO Carpinteria CA) into an insoluble brown precipitate.

2.5. Western blotting

For Western blot analysis, samples (40 μg of cytosolic fraction of whole hippocampus) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 15% gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes (GE Healthcare Limited Buckinghamshire, UK). After transfer, membranes were incubated at room temperature for 2 h in TBS containing 3.5% fat-free milk. Primary and secondary antibodies were diluted in 3% nonfat milk, 0.05% Tween-20 in TBS.

Membranes were incubated with the polyclonal rabbit anti-Metallothionein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and clone FL-16 diluted 1:1000. Following multiple washes, membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Detection of HRP-conjugated antibody was performed using West Pico Supersignal (Thermo Fisher Scientfc, Walthman, MA, USA). Chemiluminescence was detected with high performance film (GE Healthcare Limited). Images were digitalized using the BioDoc-ItSystem (UVP) and then analyzed densitometrically using the LabWorks TM 4.0 Image Acquisition and Analysis software (UVP). Densitometry results were expressed as arbitrary Optical Density (O.D.) units.

2.6. Data analysis

Statistical analyses were performed using U-Mann–Withney to determine differences between control and experimental groups; p values of less than 0.05 were considered significant.

3. Result

3.1. Behavioral observations

KA group showed high scores of seizure (SE) and 90% of them spontaneous seizures. The animals showed progressive behavioral seizures characterized by important clinical signs such as hypersalivation, wet dog shake, varying of continuous tremors, continuous clonus and limbic seizures. The rats treated with PTZ shown initial slight symptoms consisting of short-term shaking in the head and face; with the appearance of the mild forelimb clonus, loss of balance and falling; finally tonic–clonic seizures involving all four limbs (data not shown).

3.2. Histopathological examination

Light microscopic study shown the neuronal populations within the brain heterogeneously, with normal appearance of pyramidal cells and neuropil on hippocampus of control rats (Fig. 1A 0 h). Neurons with pyknotic nuclei, condensed hypercromatic neuroplasm and interstitial brain edema with swelling of perineuronal and perivascular astroglia resulting in parenchymal necrosis of the hippocampus were found in KA treated rats at 6 h (Fig. 1A) and 24 h (Fig. 1A). Loss of neurons in hippocampal subfields CA1 and CA3, degeneration of neuronal elements and dendritic swelling region were also observed (Fig. 1A). Sites of oxidative damage were detected mediates NITT antibody; in groups treated with KA at 1