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Research paper

Effectiveness of ketogenic diet in pentylenetetrazol-induced and kindling rats as well as its potential mechanisms

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HIGHLIGHTS

- Antiepileptogenic effects of KD on electrographic and behavioral seizure thresholds were studied in PTZ-induced seizures.
- KD increased KCC2 protein expression levels without affecting NKCC1 expression in cortex after a monthly treatment without induced epilepsy.
- KD might play an antiepileptogenic role by regulating KCC2 and NKCC1 protein expression levels in PTZ-kindling model.

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ABSTRACT

The effects and mechanisms of ketogenic diets (KD) are unclear. In this study, we aimed to reveal electrographic and behavioral thresholds in responses to the KD in pentylenetetrazol (PTZ)-induced seizures, as well as its antiepileptogenic effects on PTZ-kindling rats. Additionally, we investigated the potential link between KD and expression levels of two cation chloride co-transporters: K^+–Cl^- co-transporter 2 (KCC2) and Na^+–K^+–Cl^- co-transporter 1 (NKCC1). The KD group had significantly higher electrographic thresholds than the control (ND) group for the first spike-and-wave, subcontinuous spike-and-wave, high amplitude spike-and-wave, and polyspikes both in the cortex and hippocampus. Compared to the ND group, the KD group had higher behavioral thresholds for behavioral absence, first jerk, first overt myoclonia, and generalized seizures. In the PTZ-kindling model, KD not only prolonged the latency of myoclonic and clonic convulsions, but shortened clonic and generalized duration. In addition, KD rats had higher KCC2 protein expression before kindling, during myoclonic jerks, and GTCS compared with ND rats. There were no significant differences in NKCC1 protein levels between both groups following the four-week dietary intervention without PTZ exposure (before kindling). Moreover, KD inhibited the upregulation of NKCC1 expression induced by kindling in myoclonic jerks and GTCS. Therefore, our findings demonstrated that KD had antiepileptic features in elevating thresholds to most electrographic and behavioral seizure patterns in PTZ-induced rats, as well as delaying the progression and alleviating the severity of seizure in PTZ-kindling model. The antiepileptogenic effects of KD may be attributed to its regulatory properties on KCC2 and NKCC1 protein expression.

1. Introduction

Ketogenic diet (KD) has been used in patients after multiple pharmaceutical interventions and treatments have failed, while the effects and mechanisms of action are unclear. Electroencephalogram (EEG) features of subjects subjected to KD are inconsistent [1,4,19]. It has been postulated that patients with epileptiform discharges in the temporal region are less likely to benefit from KD treatment [1]. Studies have reported that the frequency of interictal epileptiform discharges is significantly reduced and the EEG background rhythm is improved following three–six months of KD therapy in KD responders (have a more than 50% reduction in behavioral seizure frequency) [4,6].

Pentylenetetrazol (PTZ) is widely used to induce seizures and to assess the effectiveness of antiepileptic drugs [17,18]. Few studies have investigated the efficacy of KD on both the threshold of epileptiform discharges in different brain regions and behavioral performance. Therefore, in this study, we evaluated a wide

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spectrum of electrographic and behavioral thresholds in response to KD in PTZ-induced seizures.

Increasing evidence suggests that a shift in $GABA_A$-mediated excitation may be involved in epileptogenesis [12,14]. Cation-chloride co-transporters (CCCs) may play significant roles in epilepsy by modulating the efficacy of GABAergic inhibition and controlling the reversal potential of $GABA_A$ receptor-mediated current and voltage responses [14]. Two major CCC members regulate chloride homeostasis: K^+–Cl^- co-transporter (KCC2) and Na^+–K^+–Cl^- co-transporter (NKCC1). These CCCs are secondary active transporters that module Cl^- release or uptake via K^+ and Na^+ gradients [14,15]. KCC2 releases Cl^- from the neuronal soma while NKCC1 mediates Cl^- uptake. Recent studies have reported that KCC2 is down-regulated while NKCC1 is up-regulated under certain pathophysiological conditions, such as epilepsy and trauma [8,9]. Furosemide and bumetanide, two non-specific inhibitors of CCCs, may be beneficial in the treatment of epileptic disorders. It is possible that compounds affecting KCC2 or NKCC1 expression levels may be useful antiepileptic drugs [8,14]. However, it is not clear whether the antiepileptiogenic effects of KD are attributed to a regulation in KCC2 or NKCC1 protein expression levels. The repeated administration of subconvulsive electrical or chemical stimulus results in a progressive intensification of seizure activity, culminating in generalized seizures that closely resemble human refractory epilepsy. PTZ-induced kindling, which is the preferred experimental model for inducing seizures, allows the evaluation of epileptogenesis [22]. Therefore, this study was also designed to explore the regulatory effects of KD on KCC2 and NKCC1 protein expression in PTZ-kindling model.

2. Materials and methods

2.1. Animals and diets

Male Sprague-Dawley rats (28 days of age, 104–122 g) were randomly divided into two weight-matched groups: KD and normal diet (ND) groups. Rats were housed in individual cages with 12-h light cycles. Experiments were carried out between 9:00 am and 4:00 pm in accordance with the ethical guidelines of the Animal Experimentation Committee of Zhejiang University and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used and their suffering.

The ND group was fed a regular rodent chow diet (Slac-M01; Slacom, Shanghai, China), consisting of a nutritionally balanced low-fat and high-carbohydrate diet that provides 3.52 kcal/g of gross energy. The KD group was fed a ketoCal diet (Nutricia, Shanghai, China), consisting of a nutritionally balanced, soy oil-based, high-fat, and low-carbohydrate diet that provides 7.3 kcal/g of gross energy and has a ketogenic ratio of 4:1. The animals had ad libitum access to water and food, which was prepared fresh each day. All animals were fasted for a period of 8 h prior to the dietary treatments. The rats continued to receive their specified diets in the whole study.

2.2. Surgery

Following four weeks of dietary treatments, rats (58 days of age) were anesthetized and mounted on a stereotaxic apparatus (512600; Stoelting, IL, USA). Electrodes were implanted into the right ventral hippocampus (bregma coordinates: AP = −5.4 mm, L = −5.2 mm, and V = −6.5 mm) and frontal cortex. The electrodes consisted of twisted stainless steel Teflon-coated (except for 0.5 mm at the tip) wires of 0.2 mm in diameter (A.M. Systems, WA, USA). The tip separation was 0.7–0.8 mm. Electrodes were connected to a receptacle, which was embedded in the skull with dental cement. Animals were allowed 7–10 days to recover after surgery.

2.3. Electrographic and behavioral thresholds of PTZ-induced seizures

To introduce an intravenous catheter, rats that had undergone surgery were immobilized in adapted Plexiglas® boxes and then waited for the injection. A freshly prepared 20 mg/ml PTZ solution was infused by a person blinded to the identity of the two groups at 6 ml/h through the tail vein using a 24-G catheter. Following catheter insertion, rats were transferred to cages and allowed to move freely to adapt to the environment. To avoid the occurrence of tonic seizures, PTZ infusion was stopped as soon as sustained typical clonic seizures began. EEG characteristics were recorded using a digital amplifier (RM-6240; Chengyi, China). With increasing time, we observed [18] (i) isolated spikes (first spike) and no behavioral motor; (ii) bursts of first spike-and-wave discharges (SWDs), usually matched with the first behavioral absence; (iii) subcontinuous SWDs (≥50% of each 20-s recording period) occurring concurrently with myoclonic seizures; (iv) recruiting SWDs activity of higher amplitude that matched the short clonic seizures of forelimbs, followed immediately by polyspikes in the cortex; and (v) polyspike discharges in the hippocampus, followed by major electromyographic artifacts, matching with GTCS.

Behavioral manifestations were observed [18]: (i) behavioral absence (the animal appeared to be in a “daze”); (ii) mild jerks; (iii) first overt myoclonia with slight elevation of forelimbs; and (iv) clonic seizure of limbs followed by a tonic axial seizure (generalized seizures). All thresholds were expressed as mg PTZ infused per body weight (kg).

2.4. Procedures for subconvulsive PTZ-induced kindling

After four weeks of dietary treatments, rats that had not undergone surgery were intraperitoneally injected with PTZ (35 mg/kg, i.p.) every other day on 13 occasions. Rats that showed more than three consecutive stage 4 episodes were considered to be fully kindled. Following each PTZ injection, convulsive behavior was observed for 30 min and classified into one of the following stages according to a modified Racine scale [22], 0 = no response; 1 = hyperactivity and vibrissae twitching; 2 = head nodding, head clonus, and myoclonic jerk; 3 = unilateral forelimb clonus; 4 = rearing with bilateral forelimb clonus; 5 = GTCS with loss of postural control. In the absence of corresponding seizures within 30 min, latency was considered to be 1800 s.

2.5. Western blotting

Rats were sacrificed 24 h following the last PTZ injection. Three quarters of the anterior cerebral cortex were rapidly dissected, flash-frozen in liquid nitrogen, and subsequently stored at −80 °C. Brain tissues and cells were lysed with a protein isolation kit (Thermo Pierce, USA). The resulting homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C. Protein concentration was determined with a BCA assay kit (Beyotime, China). Denatured samples (60 μg) were separated by SDS-PAGE at 80 V for 2 h, transferred to polyvinylidene difluoride membranes by a semidry method, and incubated with rabbit polyclonal antibodies against KCC2 and NKCC1 (1:500 dilution, Santa Cruz Biotechnology, USA). After incubation with the primary antibodies, membranes were incubated with appropriate secondary antibodies (1:5000 dilution; Thermo Pierce, USA). Chemiluminescent detection was performed with SuperSignal® West Dura Extended Duration Substrate (Thermo Pierce, USA). Gel bands were analyzed using BandScan