Epilepsy Research 120 (2016) 13–18

Contents lists available at www.sciencedirect.com

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

Ketogenic diet change cPLA2/clusterin and autophagy related gene expression and correlate with cognitive deficits and hippocampal MFs sprouting following neonatal seizures

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ARTICLE INFO

Article history:
Received 14 June 2015
Received in revised form 7 November 2015
Accepted 27 November 2015
Available online 3 December 2015

Keywords:
Ketogenic diet
Cpla2
Clusterin
Autophagy
Neonatal seizure

ABSTRACT

Because the ketogenic diet (KD) was affecting expression of energy metabolism- related genes in hippocampus and because lipid membrane peroxidation and its associated autophagy stress were also found to be involved in energy depletion, we hypothesized that KD might exert its neuroprotective action via lipid membrane peroxidation and autophagic signaling. Here, we tested this hypothesis by examining the long-term expression of lipid membrane peroxidation-related cPLA2 and clusterin, its downstream autophagy marker Beclin-1, LC3 and p62, as well as its execution molecule Cathepsin-E following neonatal seizures and chronic KD treatment. On postnatal day 9 (P9), 48 Sprague-Dawley rats were randomly assigned to two groups: flurothyl-induced recurrent seizures group and control group. On P28, they were further randomly divided into the seizure group without ketogenic diet (RS + ND), seizure plus ketogenic diet (RS + KD), the control group without ketogenic diet (NS + ND), and the control plus ketogenic diet (NS + KD). Morris water maze test was performed during P37–P43. Then mossy fiber sprouting and the protein levels were detected by Timm staining and Western blot analysis, respectively. Flurothyl-induced RS + ND rats show a long-term lower amount of cPLA2 and LC3II/I, and higher amount of clusterin, Beclin-1, p62 and Cathepsin-E which are in parallel with hippocampal mossy fiber sprouting and cognitive deficits. Furthermore, chronic KD treatment (RS + KD) is effective in restoring these molecular, neuropathological and cognitive changes. The results imply that a lipid membrane peroxidation and autophagy-associated pathway is involved in the aberrant hippocampal mossy fiber sprouting and cognitive deficits following neonatal seizures, which might be a potential target of KD for the treatment of neonatal seizure-induced brain damage.

1. Introduction

A ketogenic diet (KD) is a high-fat, low-carbohydrate and restricted-protein diet that is a world-widely used last resort for medically intractable epilepsy. Recent evidence also suggests that KD may have some beneficial effects in a lot of metabolic diseases such as obesity, diabetes, GLUT1 deficiency syndrome and pyruvate dehydrogenase (PDH) deficiencies (Gano et al., 2014; Veggiotti and De Giorgis, 2014). In animal studies, CD-1 mouse neonates whose mothers were fed a KD prior to and during gestation demonstrated altered maternal metabolic status as well as offspring physiological growth and brain structure (Sussman et al., 2013). These studies suggest that the metabolic pathway induced by the diet may afford its long-lasting seizure protection. However, the potential molecular mechanism remains poorly documented and evaluated.

We recently provided evidence that zinc/lipid transporters are involved in the neuroprotective effects of KD. Recurrent and prolonged neonatal seizure-induced long-term neurobehavioral toxicology and aberrant sprouting of mossy fibers in hippocampus were blocked by KD. In parallel, there was significant down-regulated expression of ZnT-3, MT-3, ApoE, clusterin and ACAT-1 in hippocampus when compared with non-KD treated seizure group (Tian et al., 2015.). In another study using the same animal model (Ni et al., 2012), the expression of phospholipid metabolism-related gene PRG-1 in both the acute and the long-term time point was investigated. At 1.5, 3, 6 and 24 h after the last seizures, there was no significant changes of PRG-1. However, the protein level of

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http://dx.doi.org/10.1016/j.eplepsyres.2015.11.021
0920-1211/

PRG-1 was significantly increased at the long-time point P35 after neurobehavioral analysis. Because PRG-1 is a new lipid phosphate phosphatase and because PRG-1 is located in the brain-specific lipid membrane which is associated with lipid membrane integrity (Brauer et al., 2003), we thus hypothesized that lipid phosphate phosphatase-associated membrane integrity may be associated with neonatal seizure-induced brain damage which might be therapeutic targets of KD.

Several genes have been recognized that play a role in lipid phosphate phosphatase-associated membrane integrity. Among these, cytosolic phospholipase A2 (cPLA2) and clusterin, along with their downstream autophagy related genes such as Beclin-1, microtubule-associated protein 1A/1B light chain 3 (LC3), sequestosome 1 (SQSTM1, p62) and the execution molecule cathepsin-E has recently been recognized to be important in regulation of neurotoxicity. cPLA2 KO mice exhibited altered brain phospholipid composition and subsequent structural differences of plasma membrane in cortical neurons which was associated with neurodegeneration in Alzheimer's disease and prion diseases (Leslie, 2015). Cortical neurons in cPLA2 KO mice also exhibited abnormal architecture including larger nuclei, increased number of nucleoli and aggregated intra-nuclear ribosomes suggesting the role of cPLA2 in regulating membrane structure and curvature (Qu et al., 2013). Clusterin has been implicated in a number of cellular processes such as lipid transport, membrane integrity and neurodegeneration (Wong et al., 2000). Autophagy stress was compromised in epilepsy-associated Lafora disease, supporting a possible role for autophagy in epileptogenesis (Knecht et al., 2010). Recently it was reported that autophagy process is involved in the neuronal plasticity of synapses and dendrites and in the regulation of the number of specific receptors using *Caenorhabditis elegans*, drosophila and cultured neurons (Nibuya et al., 2014).

In this respect, in this study we investigated the effect of KD on cognitive deficits and hippocampal mossy fiber sprouting, as well if KD has any influence in the activity of cPLA2 and clusterin, as well as the downstream signaling of autophagy related genes Beclin-1, LC3, p62 and Cathepsin-E in Sprague-Dawley rats submitted to recurrent prolonged neonatal seizures.

2. Materials and methods

2.1. Animal preparation

Sprague-Dawley rats ($n = 48$) at postnatal day 8 (P8, weighing between 16.55 g and 23.52 g) were obtained from the Chinese academy of sciences, Shanghai experimental animal center, China. Rats were kept in an environment-controlled room which was away from bright light and noise under a 12 h/12 h light/dark cycle; they were housed with their littermates until weaning at P21. The animals were adapted for 1 day before the study and had free access to standard laboratory food and water ad libitum. The animals were treated in accordance with the guidelines set by the National Institutes of Health for the humane treatment of animals. Attempts were made to reduce animal suffering and the number of animals used. Two groups were randomly assigned: flurothyl (bis-2, 2, 2-triflurothyl ether; Sigma-Aldrich Chemical, WI, USA)-induced recurrent seizures group (EXP, $n = 24$) and control group (CONT, $n = 24$). Neonatal seizures were induced in EXP rats with volatile flurothyl (bis-2,2,2-triflurothyl ether, Aldrich-Sigma Chemical,WI,USA), a potent and rapidly acting central nervous system stimulant that produces seizures within 30 min of exposure once a day for consecutive 8 days (from P9 to P16), with a flow rate of 100 μl/min onto filter paper in the center of the container where it evaporated (Kadiyala et al., 2015). CONT rats were placed into the container for an equal amount of time to their counterpart without exposing to flurothyl.

At weaning day P21, rats were further randomly divided into four groups: the seizure group without KD (RS + ND, $n = 12$), the seizure plus KD (RS + KD, $n = 12$), the control group without KD (NS + ND, $n = 12$), the control plus KD (NS + KD, $n = 12$). At P21, rats in NS + KD and RS + KD groups received KD, while rats in NS + ND and RS + ND received normal diet. The formula of KD was reported in detail previously (Tian et al., 2015). KD (70% fat, 20% protein and no carbohydrate) and normal diet (4.5% fat, 20% protein and 50% carbohydrate) were obtained from Chinese academy of sciences, Shanghai experimental animal center, China. All rats were given food and water ad libitum for 3 weeks.

2.2. Morris water maze test

During the 5 consecutive days (P37–P43), rats ($n = 12$ each group) were tested in the Morris water maze to evaluate visual-spatial learning and memory ability. The procedure has been described previously (Ni et al., 2012). In brief, for the place navigation test, the escape latency (the duration for finding the platform) was automatically recorded by a video/compute system. For the spatial probe test, the platform was removed from the pool on alternate days after the navigation test (P43). Then each rat was placed in the water for 60 s and recorded the frequency (number of times) of passing through the platform quadrant to reflect the spatial memory ability.

2.3. Timm staining

On P43, a subset of rats were sacrificed by decapitation for Timm staining ($n = 6$/each group). The pyramidal/infra-pyramidal CA3 region and the inner molecular layer of the dentate gyrus of hippocampus were assessed on each section. Mossy fiber sprouting was analyzed using a semi-quantitative scale for terminal sprouting in the CA3 and the dentate gyrus. The person scoring the Timm staining was blind to treatment group.

2.4. Western blot analysis

Protein levels were detected by western blot method (Ni et al., 2012) on P43 from the rest part of rats after water maze test ($n = 6$/each group). Polyvinylidene fluoride membranes blots after blocking solution TBS-T were incubated with one of the following antibodies: a rabbit anti-cPLA2 polyclonal antibody (1:800, EnoGene Biotechnology), a rabbit anti-Beclin-1 polyclonal antibody (1:500, Santa Cruz), a goat anti-Clusterin polyclonal antibody (1:3000, Santa Cruz), a rabbit anti-LC-3 (1:400, abcam) or a rabbit anti-p62(1:2500, EnoGene Biotechnology) polyclonal antibody, and a rabbit anti-Cathepsin E (1:800, Santa Cruz) in Tris buffered saline containing 0.2% Tween-20 (TBST) and 5% non-fat dry milk overnight at 4 °C. The blot was then incubated with the secondary antibody for about 2 h at ambient temperature. Antibody reactions were exposed with Kodak X-ray film using the ECL detection system Amersham. The relative changes of the intensity of each immunoreactive band were evaluated with Sigma Scan Pro 5 and were normalized to a loading control GAPDH or β-actin.

2.5. Statistical analysis

Escape latency was analyzed using two-way ANOVAs (treatment as a between subject factor and training day as a within subject factor) for repeated measures. The frequency of passing through the platform quadrant of spatial probe test in the water maze, Timm staining and the protein levels were analyzed with one-factor ANOVA with post hocs. Data were presented as the mean ± SD, and statistical significance was considered as a $P < 0.05$.