Epilepsy Research (2007) **74**, 232—236

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

SHORT COMMUNICATION

Evidence against enhanced glutamate transport in the anticonvulsant mechanism of the ketogenic diet

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Received 20 January 2007; received in revised form 4 March 2007; accepted 5 March 2007
Available online 9 April 2007

KEYWORDS
Ketogenic diet;
Glutamate;
GLT-1;
GLAST;
EAAC1;
Epilepsy

Summary Excessive glutamatergic neurotransmission is considered an underlying factor of epilepsy. Energy-dependent glutamate transporters clear extracellular glutamate to limit neuronal excitability. Evidence suggests that reduced expression and/or activity of glutamate transporters contribute to hyperexcitability and progressive seizure activity in rats. By comparison, treatment with the anticonvulsant ketogenic diet (KD) results in increased mRNA expression of the neuronal glutamate transporter EAAC1, elevated energy reserves, and an increased resistance to seizures in rats. The goal of the current study was to determine whether the expression and/or re-uptake activity of glutamate transporters were elevated in hippocampal tissue of rats after KD treatment. Rats were fed either a ketogenic- or control diet for 4—5 weeks. Western blot analysis showed that protein levels of EAAC1, GLT-1 and GLAST glutamate transporters were not changed in hippocampus, cerebral cortex, or cerebellum after KD. Electron microscopic evidence indicated that the KD did not affect hippocampal EAAC1 distribution. In addition, the re-uptake activity of ^{3}H-glutamate into hippocampal proteoliposomes was similar in both KD and control tissue extracts. These multiple studies suggest that the anticonvulsant nature of the KD does not stem from enhanced glutamate re-uptake.

Introduction

Epilepsy affects 1—3% of people worldwide and is defined symptomatically by recurring seizures. Excessive glutamatergic neurotransmission has been considered an underlying factor of epilepsy. Following synaptic release, energy-dependent glial (GLT-1 and GLAST) and neuronal (EAAC1) glutamate transporters clear extracellular glutamate to limit neuronal excitability. EAAC1, in particular, may

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doi:10.1016/j.eplepsyres.2007.03.002

have an important role in epilepsy. Prior work has shown that EAAC1-mediated glutamate re-uptake on GABAergic interneurons is important for GABA synthesis from glutamate and inhibitory function; antisense knockdown of EAAC1 in rats led to decreased hippocampal GABAergic inhibition, hyperexcitability, and spontaneous seizures (Sepkuty et al., 2002; but see, Peghini et al., 1997). In addition, progressive seizure activity was only observed in rats that displayed a chronic decrease in hippocampal EAAC1 protein levels (Gorter et al., 2002). Chronic elevations in EAAC1 expression may both enhance glutamate re-uptake and increase GABAergic inhibition to mitigate hyperexcitability and limit neurodegeneration (Zhang et al., 2004; Voutsinos-Porche et al., 2006).

The ketogenic diet (KD) is a long-standing treatment for difficult-to-control epilepsy. Clinical reports have shown that approximately half of all patients fed a KD exhibit $\geq$50% reduction in seizure frequency, irrespective of patient gender, age at diet onset, or seizure type (Freeman et al., 1998). Animal studies have confirmed an anticonvulsant role for the KD in both mice and rats (Stafstrom, 1999), but the underlying mechanisms remain unclear. Because KD treatment increases hippocampal EAAC1 mRNA expression, augments functional inhibition, decreases neuronal excitability, and elevates seizure threshold (Appleton and DeVivo, 1974; Bough et al., 2003, 2006), we hypothesized that enhanced expression and/or function of hippocampal glutamate transporters contribute to the anticonvulsant mechanism of the diet. Thus, our goal was to determine whether the expression and re-uptake activity of glutamate transporters were elevated in hippocampal tissue of rats after KD treatment.

Methods

Subjects and dietary treatment

Male Sprague—Dawley rats were fed either a KD (customized, AIN-93 high-fat diet, Bio-Serv #F-3666, Frenchtown, NJ) or normal, *ad libitum* diet beginning on post-natal day 37—41 (135—190 g). Normal animals were studied rather than 'epileptic' animals because the anticonvulsant actions of the KD do not occur as a result of pathophysiological changes associated the development of epilepsy (Appleton and DeVivo, 1974; Bough et al., 2003) and seizures could secondarily change the activity and/or expression of glutamate transporters. KD-fed animals were limited to ~85% of the recommended daily allowance for rats (0.3 kcal/g body weight/day). They were fed ~5 g of KD each day based on body weight, as previously described (Bough et al., 2006). Because the diet requires 1—2 weeks to become maximally effective (Appleton and DeVivo, 1974), diet treatment were maintained for 4—5 weeks. Water was provided *ad libit*um to both diet groups throughout. Blood levels of beta-hydroxybutyrate (BHB) or glucose were measured using a Keto-Site meter (GDS Technologies, Elkhart, IN) or a Precision Xtra meter (Abbot Labs, Alameda, CA), respectively. All experiments were performed in accordance with NIH guidelines for the care and use of laboratory and approved by Emory University's Institutional Animal Care and Use Committee.

Western blot

Following diet treatment, animals were sacrificed and hippocampi, the frontal, cerebral cortex, and cerebellum were dissected on ice and solubilized. We focused on the hippocampus based upon previous work demonstrated that the hippocampal expression of EAAC1 mRNA increased after KD; the other brain regions served as controls. After determination of the protein concentration of lysates by Bradford assay, samples were resolved by SDS-PAGE (4—20% Tris—glycine gels) and subjected to Western blot analysis with mouse anti-EAAC1 (1:500), guinea pig anti-GLT1 (1:5000), and guinea pig anti-GLAST (1:10,000) antibodies (Chemicon, Temecula, CA). Immunoreactive bands were resolved by enhanced chemiluminescence detection (Pierce, Rockford, IL) with HRP-conjugated goat anti-mouse (1:4000, Amersham Biosciences, Little Chalfont, UK) and goat anti-guinea pig (1:2000, Chemicon). The relative amount of protein was determined by densitometric scanning of immunoreactive bands and analysis using the Li-Core system and software. Scanned values for KD and control animals were expressed as a percent of mean controls and analyzed by ANOVA.

Electron microscopy

Sub-cellular changes in protein expression were assessed by electron microscopy. Following 5 weeks of diet treatment, animals were sacrificed and transcardially perfused. Coronal sections were incubated in rabbit anti-EAAC1 (1:2000; Chemicon, Temecula, CA) for 24 h at 25 °C and processed for electron microscopy. Electron micrographs of the dentate/hilar region of the hippocampus were taken randomly at 20,000× magnification at the surface of the blocks where antibody penetration was optimal. Data from four KD-fed animals (160 micrographs representing 2683 μm^2) and five controls (184 micrographs representing 3085 μm^2) were analyzed as described previously (Peters et al., 1991). EAAC1-labeled processes were scored qualitatively (presence/absence) on a per-process basis. The number of labeled processes was statistically compared among the groups on a per unit area basis using ANOVA. Ultrastructural features described in Peters et al. (1991) were used to categorize immunoreactive and non-immunoreactive elements. Elements that lacked clear, identifiable ultrastructural features or contained excessive DAB labeling, which confounded classification, were scored as ''unidentified'' and discarded from subsequent analyses.

Glutamate transport activity

A separate cohort of KD ($n = 10$) and control ($n = 10$) animals was maintained on diet treatment for 28 days, lightly anesthetized with isoflurane, and decapitated. Hippocampi were dissected rapidly over ice and flash frozen. Proteoliposomes were prepared from hippocampal homogenates (Hassel et al., 2001) and used to determine whether there was a functional increase in glutamate transport, which would not have been detected by immunoblotting. Proteoliposomes were incubated in a solution containing valinomycin (1 μmol/L), glycerol (1%), and NaCl (150 mmol/L), the latter of which drove sodium-dependent [^{3}H]-glutamate uptake (Danbolt et al., 1990). Preparations treated with 1 μM nigericin were used as blanks. The uptake of [^{3}H]-glutamate was determined at 30 °C in triplicate with blanks run in duplicate for each animal; blank values were typically <5% of sodium-dependent uptake.

Results

The KD induces persistent ketonemia when effective (Appleton and DeVivo, 1974). After diet treatment, BHB levels in KD-fed animals (0.97 ± 0.13 mM) were increased over controls ($0.11 + 0.2$ mM; $p < 0.005$, t-test), whereas, glucose levels tended to diminish (KD = $107.3 + 11.5$ mg/dL, control = 162.2 ± 21.2 mg/dL; $p = 0.05$, t-test). This indicated that the diet-treated animals were indeed ketonemic at the time of tissue harvest.

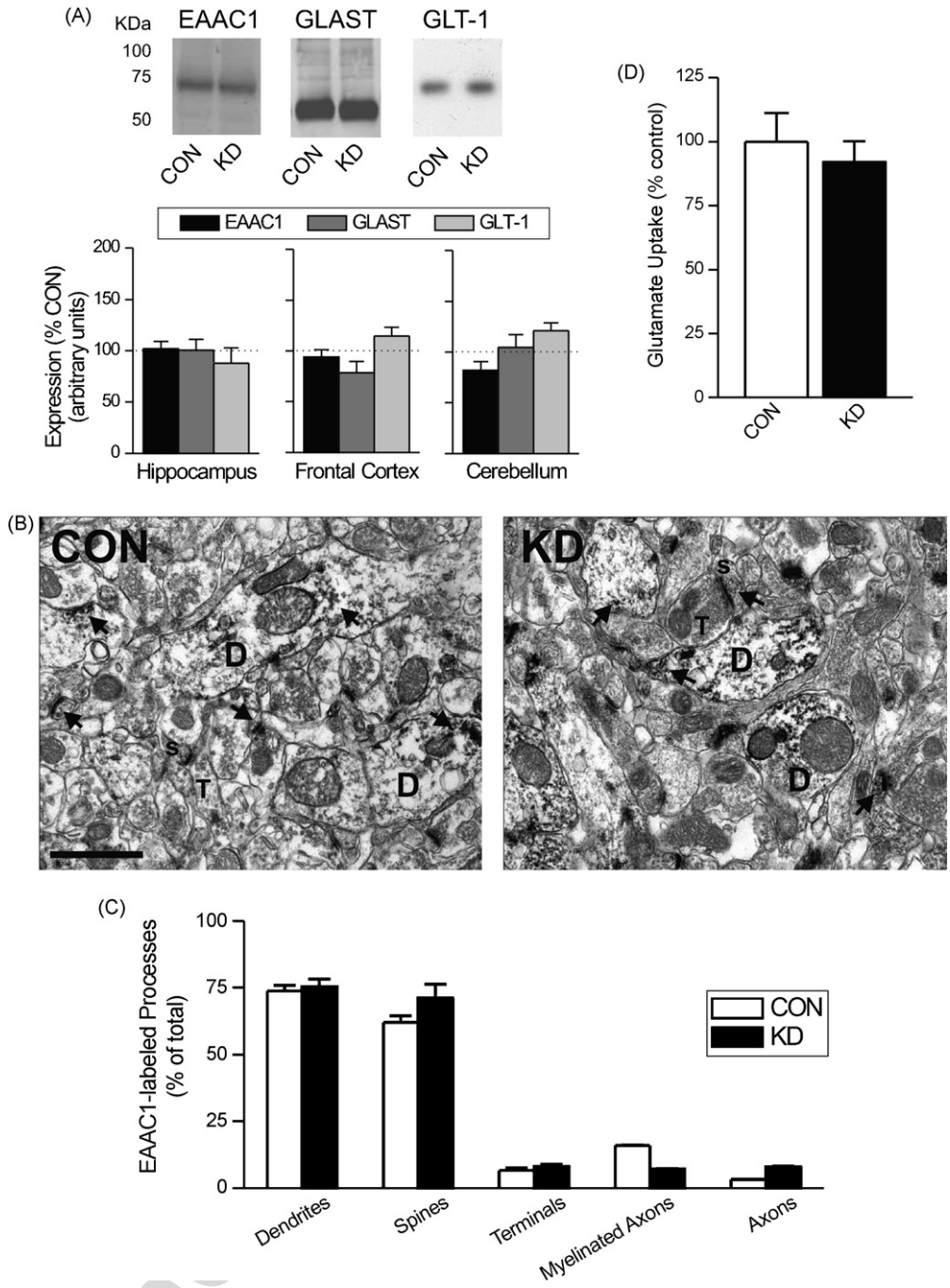

Figure 1 Glutamate transport is not altered in rat hippocampus after ketogenic diet (KD). (A) Upper panel, representative immunoblots show the monomeric forms of EAAC1, GLT-1, and GLAST in hippocampus following chronic treatment with a KD or control diet. Lower panel, Summary of immunoblot protein expression data for these three transporters collected from hippocampus, frontal cortex, and cerebellum. Each bar represents the mean percent of control expression and S.E.M. (KD = 6, CON = 4). (B) Representative electron micrographs of EAAC1-immunopositive (arrows) neuronal processes taken from the dentate/hilar region of the hippocampus; S, spines; D, dendrites; T, terminals; CON, control; KD, ketogenic diet. Magnification was 20,000×. Scale bar = 1 μm. (C) Summary of electron microscopic data for EAAC1-labeled processes. Processes were identified as described previously (Peters et al., 1991). Data are expressed as the percent of EAAC1-labeled processes found within the dentate/hilar region of the hippocampus. Each bar represents the mean and S.E.M. (KD = 4, CON = 5). (D) Effect of KD on uptake of glutamate into proteoliposomes prepared from hippocampal homogenates. GLT-1 was reconstituted into proteoliposomes and sodium-dependent uptake of [^{3}H] glutamate was measured in triplicate for each animal (KD = 9, CON = 9). Data are expressed as mean percent of control activity + S.E.M.

We initially determined whether the protein levels of glutamate transporters were increased in brain after KD. As shown in Figure 1A, diet treatment did not appreciably increase protein levels of EAAC1, GLT-1 or GLAST glutamate transporters in hippocampus, frontal cortex, or cerebellum ($p > 0.05$ for all, ANOVA).

Next, we investigated whether the sub-cellular distribution of EAAC1 was modified following KD in the dentate/hilar region of the hippocampus by electron microscopy. EAAC1 was not differentially distributed in the dentate gyrus of KD-fed animals. In both control and KD-fed animals, EAAC1 immunoreactivity was associated with both the cytoskeleton and the plasma membrane of neuronal profiles (Figure 1B, arrows) and largely confined to post-synaptic dendrites and spines (Figure 1B and C). For example, 74% of the control and 79% of the KD dendritic profiles were labeled with anti-EAAC1 (Figure 1C); by contrast, only 7% of presynaptic terminals in both KD and control animals were EAAC1 positive.

Astroglial sodium-dependent glutamate transporters such as GLT-1 are responsible for ~80% of glutamate transport following synaptic release (Sepkuty et al., 2002). Thus, we also determined whether GLT-1-mediated glutamate re-uptake activity was enhanced after KD. We found that the uptake of ^{3}H-glutamate was similar in both KD and control hippocampal tissue extracts (Figure 1D).

Discussion

We found no evidence to indicate that the anticonvulsant KD improves glutamate handling. Despite the KD-induced elevation in EAAC1 mRNA expression (Bough et al., 2006), EAAC1 protein level was not increased after KD (Figure 1A—C). In addition, there were no changes in protein levels for either GLT-1 or GLAST (Figure 1A). Even though KD treatment did not alter protein levels, it remained possible that glutamate re-uptake activity could still be enhanced. Glutamate transport activity, however, was unaffected by diet treatment (Figure 1D). The lack of enhanced GLT-1 protein expression and/or activity is somewhat surprising given that KD treatment significantly elevated glutamine concentration in hippocampus (Bough et al., 2006) and cerebral cortex (Melo et al., 2006). This finding suggests that KD treatment does not enhance the clearance of synaptically-released glutamate by glia (Figure 1A and D).

One of the most prominent anticonvulsant mechanistic theories for the KD relates to a functional enhancement of GABAergic inhibition via an enhanced synthesis of GABA from glutamate (Yudkoff et al., 2001a). Prior work has shown that EAAC1-mediated glutamate re-uptake on GABAergic interneurons is important for GABA synthesis and inhibitory function (Sepkuty et al., 2002); knockdown of the EAAC1 led to hippocampal hyperexcitability and seizures in rats. Although they do not preclude the possibility of a KD-induced enhancement in the metabolic production of GABA *de novo* (Yudkoff et al., 2001b; Melo et al., 2006), these data do not support the notion that KD enhances EAAC1 glutamate re-uptake as a means to increase GABA synthesis in hippocampus (Figure 1A—C). This result is in accordance with previous work showing a lack of increase in hippocampal GABA concentration after chronic administration of a KD (Bough et al., 2006).

It is plausible that KD-induced elevations in the expression and/or function of glutamate transporters only occur during or after the development of epilepsy (i.e., in 'epileptic' animals). For example, the lack of increase in EAAC1 protein level observed here (Figure 1A—C), despite the increase in EAAC1 mRNA (Bough et al., 2006), may suggest that EAAC1 translation is up-regulated only in response to hyperexcitability and abnormally elevated glutamatergic 'load'. However, non-epileptic, KD-fed animals exhibit diminished hippocampal excitability, augmented paired-pulse inhibition, and an increased resistance to limbic seizures (Appleton and DeVivo, 1974; Bough and Eagles, 1999; Bough et al., 2002; Bough et al., 2003). This indicates that the pathophysiological changes associated with the development of spontaneous seizures are not required to induce at least some of the KD's anticonvulsant actions. Therefore, we conclude that the lack of changes noted here suggests that improved glutamate handling is not part of the observed anticonvulsant mechanism of the KD.

Acknowledgments

This work was supported by the Charlie Foundation (KJB) and the NIH—NINDS (RJD, YSS, and RAH). We thank David A. White, PhD for helpful comments during the preparation of this manuscript.

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