Research Report

Fructose-1,6-diphosphate protects against epileptogenesis by modifying cation-chloride co-transporters in a model of amygdaloid-kindling temporal epilepticus

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Abstract

Fructose-1,6-diphosphate (FDP) shifts the metabolism of glucose from glycolysis to the pentose phosphate pathway and has anticonvulsant activity in several acute seizure animal models. In the present study, we investigated the anti-epileptogenic effects of FDP in an amygdaloid-kindling seizure model, which is an animal model of the most common form of human temporal lobe epilepsy. We found that 1.0 g/kg FDP slowed seizure progression and shortened the corresponding after-discharge duration (ADD). FDP increased the number of stimulations needed to reach seizure stages 2–5 and prolonged the cumulative ADD prior to reaching stages 3–5. It also shortened staying days and cumulative ADD in stages 4–5. However, it demonstrated no significant protective effect when administered after the animals were fully kindled. In hippocampal neurons, cation-chloride co-transporters (CCCs) are suggested to play interesting roles in epilepsy by modulating γ-aminobutyric acid (GABA)ergic activity through controlling GABAA receptor-mediated reversal potential. We examined the potential link between FDP and the hippocampal expression of two main members of the CCCs: the neuron-specific K⁺–Cl⁻ co-transporter 2 (KCC2) and Na⁺–K⁺–Cl⁻ co-transporter 1 (NKCC1). FDP inhibited the kindling-induced downregulation of KCC2 expression and decreased NKCC1 expression during the kindling session. Taken together, our data reveal that FDP may have protective activity against epileptogenesis, from partial to generalized tonic–clonic seizures. Furthermore, our findings suggest that the FDP-induced imbalance between KCC2 and NKCC1 expression may be involved in the neuroprotective effect.
1. Introduction

The high-fat and low-carbohydrate ketogenic diet (KD) has gained acceptance as a treatment for refractory epilepsy (Freeman et al., 2007). Our previous study demonstrated that KD significantly delayed seizure progression and has a neuroprotective effect against kindling (Jiang et al., 2012). However, even though it has been indicated that KD is well tolerated and does not produce any significant health concerns in KD-fed animals, it is still regarded as a difficult regime to follow (Auvin, 2012). This promoted us to investigate other potential agents that can mimic the protective effect of KD but with less adverse outcomes. Fructose-1, 6-diphosphate (FDP) inhibits glycolysis and diverts glucose into the pentose phosphate pathway, and has been shown to be neuroprotective in several acute chemical seizure models (Lian et al., 2007), and therefore can be considered an anticonvulsant. However, no investigations of the effects of FDP in seizure progression have been performed. The idea that “seizures beget seizures” was proposed some 130 years ago by Gowers (1881), and it is now widely accepted by contemporary epileptologists and neuroscientists. The animal model of amygdaloid kindling recapitulates many of the features of epilepsy progression (Garriga-Canut et al., 2006; Sharma et al., 2007) and has been used extensively to investigate temporal lobe epilepsy, the most common form of epilepsy. The present study was designed to explore the effects of FDP on epileptic progression in a chronic amygdaloid kindling model. The results shed light on the use of FDP as a potential alternative for KD.

Going one step further, recent evidence has revealed the importance of γ-aminobutyric acid (GABA)-mediated excitation in epileptogenesis, during which inhibitory GABAergic function can be dynamically changed to excitation (Koyama et al., 2012). Changes in plasmalemmal chloride ion-transport mechanisms, which are instrumental for the chloride ion channel function, and the regulation of chloride concentration have been suggested to play pivotal roles in GABA-mediated excitation. Among many KD-induced physiologic or biochemical changes in brain cell metabolism, an in vitro study showed that a KD may modify cation-chloride co-transporters (CCCs) and regulate chloride homeostasis (Rheims et al., 2009). Two major members of CCCs, K⁺–Cl⁻ co-transporter 2 (KCC2) and Na⁺–K⁺–Cl⁻ co-transporter 1 (NKCC1), control the electrochemical gradient of chloride across the neuronal plasma membrane, and they are secondary active transporters that drive net chloride extrusion or uptake by using the K⁺ and Na⁺ gradients (Loscher et al., 2013). To understand the mechanism underlying FDP’s action and its possible connection with KD through shared mechanisms, we investigated the effect of FDP on the regulation of KCC2 and NKCC1 mRNA expression levels.

2. Results

The electrodes were found in the right basolateral amygdala in 131 rats, and these animals were included in our study; 52 were investigated for seizure semiology and electroencephalogram (EEG), while 79 rats were killed by decapitation to collect samples for molecular analysis. The animals displayed no signs of abdominal cramps or other abnormal behavior after intraperitoneal (i.p.) injections of FDP.

2.1. Effect of FDP on kindling acquisition

This experiment included three groups of rats pretreated with 0.5 g/kg FDP (n=8), 1.0 g/kg FDP (n=10), or saline (n=9). FDP at the dose of 1.0 g/kg slowed seizure stage progression (P<0.05; Fig. 1A) and shortened the corresponding after-discharge duration (ADD; P<0.05, Fig. 1B). However, 0.5 g/kg FDP had no effect on either the progression of seizure stage or the ADD (Fig. 1A and B).

To further analyze the stepwise progression of kindling, we calculated the numbers of stimulations and cumulative ADD needed to reach each seizure stage or remain at generalized seizures (GS, stages 4–5). FDP at the dose of 1.0 g/kg increased the number of stimulations to reach stages 2–5 (P<0.05 to reach stages 2 and 4, P<0.01 to reach stages 3 and 5; Fig. 2A) and prolonged the cumulative ADD to reach stages 3–5 (P<0.05 to reach stages 4 and 5, P<0.01 to reach stage 3; Fig. 2B). FDP at 1.0 g/kg significantly reduced the number of days for which animals stayed in stages 4–5 (P<0.01, Fig. 2C) and shortened the corresponding cumulative ADD (P<0.01, Fig. 2D). However, FDP at the dose of 0.5 g/kg did not differ from the controls (Fig. 2A–D). In addition, we found that FDP at the dose of 1.0 g/kg prevented an after-discharge threshold (ADT) decrease at stage 5 compared to controls (P<0.05; Fig. 2E) but showed no such effect on stages 1–4.

2.2. Effect of FDP on fully kindled animals

We next examined the potential anticonvulsant effect of FDP when it was administered after the animals were fully kindled by an ADT current. FDP did not decrease the mean seizure stage or the incidence of GS, and it did not shorten the cumulative ADD or cumulative generalized seizure duration (GSD). There were no differences among the 0.5 g/kg FDP (n=9), 1.0 g/kg FDP (n=8), and control groups (n=8) (Fig. 3).

2.3. Hippocampal KCC2 and NKCC1 mRNA expression levels

Relative KCC2 mRNA expression in the hippocampus was increased after 4, 8, and 16 days of 1.0 g/kg FDP injection (P<0.01, Fig. 4A), while NKCC1 mRNA expression decreased after the 1st day (P<0.05, Fig. 4B) and also after 4, 8, and 16 days (P<0.01, Fig. 4B) of daily 1.0 g/kg FDP injections.

In the control rats, KCC2 expression decreased after 4, 8, and 16 days of stimulations (P<0.05 after 4 days and P<0.01 after 8 and 16 days, Fig. 4C). Compared with controls, rats pretreated with 1.0 g/kg FDP showed increased KCC2 mRNA expression after 4 (P<0.05), 8, and 16 (P<0.01) days of stimulations (Fig. 4C).

The expression levels of NKCC1 mRNA did not change significantly (Fig. 4D) during kindling in the control group. However, 1.0 g/kg FDP treatment induced a more significant
reduction in NKCC1 mRNA expression than control after the 1st day of stimulation, as well as after 4, 8, and 16 days of stimulations (P<0.05 after 1 day stimulation, P<0.01 after 4, 8, and 16 days of stimulation, Fig. 4D).

3. Discussion

A great deal of research has been done on KD, and some studies have identified molecular players in the anti-epileptic effects of the KD (Masino and Rho, 2012; Lutas and Yellen, 2013). No single mechanism has been identified to explain the seizure protection conferred by the KD (Politi et al., 2011), but its replacement for glucose as an energy source for brain has fascinated researchers. In addition to classic KD, different diets with carbohydrate levels below normal are also effective in the management of intractable epilepsy (Chang et al., 2013), which inspired studies to determine if approaches that specifically decrease glucose utilization through the glycolytic pathway can also protect against seizures (Lian et al., 2007). For instance, administration of the glycolytic-inhibitor 2-deoxy-D-glucose (2-DG) has shown anticonvulsant effects with increased ADT and delayed progression of epileptogenesis (Garriga-Canut et al., 2006). FDP, an intracellular metabolite of glucose, exerts potent feedback inhibition on the activity of phosphofructokinase-1, a rate-limiting enzyme in glycolysis, and is also thought to mimic the low-glucose availability associated with reduced carbohydrate intake in the KD (Stringer and Xu, 2008).

In the present study, we demonstrated that 1.0 g/kg FDP significantly delayed seizure progression induced by amygdaloid kindling, which shortened the corresponding ADD. In contrast, the effect of FDP at a relatively lower dose (0.5 g/kg) was much weaker. These data confirm that the anti-epileptogenesis function of FDP is dose related, which is similar to the anticonvulsant activity in rat models of acute generalized motor seizures induced by chemical convulsants, such as pilocarpine, kainic acid, or pentylenetetrazole (Lian et al., 2007). A previous study suggested that oral administration FDP may protect against generalized tonic–clonic seizures but not absence seizures (Lian et al., 2008). The amygdaloid-kindling seizure model enabled us to analyze epileptogenesis in graded stages. FDP at a dose of 1.0 g/kg exerts anti-epileptic action not only in partial seizure stages (stages 2–3) but also delayed epileptogenesis from partial to generalized seizures (stages 4–5). At the

Fig. 1 – Effects of FDP on amygdaloid-kindling acquisition (n=9, control group; n=8, 0.5 g/kg group; n=10, 1.0 g/kg group). (A) Behavioral stage. (B) After-discharge duration (ADD). Values are expressed as mean ± S.E.M. *P<0.05 compared with control.
same time, it attenuated the ADT, preventing rats from reaching stage 5.

Our study on how FDP affects the balance between KCC2 and NKCC1 expression is driven by the evolving role of GABAergic function in epileptogenesis and the recognized complexes of ion-channels/co-transporters involved in chloride transport. GABA is the principal inhibitory neurotransmitter in the brain, producing inhibitory postsynaptic potentials in both feedforward and feedback circuits. A decrease in GABA release could account for the neuronal hyperexcitability observed in epilepsy (Melo et al., 2010). According to a traditional amino acid hypothesis (Dahlin et al., 2005), KD suppresses seizures by increasing the level of GABA in the brain. However, this may not be the case because Hartman et al. (2007) reported that GABA levels showed no significant elevation in KD-fed animals. Remarkably, recent studies have shown the importance of GABA-mediated excitation in epileptogenesis. Intracellular chloride concentration, $\text{Cl}_i$, determines the polarity of $\text{GABA}_A$-induced neuronal $\text{Cl}^-$ currents. In neurons, $\text{Cl}_i$ is relies on a balance between NKCCs, such as NKCC1, which physiologically accumulate $\text{Cl}^-$ in the cell, and KCC2, which extrudes $\text{Cl}^-$ from the neuronal soma. Alterations in the balance between NKCC1 and KCC2 activities may determine whether the effects of GABA are hyperpolarizing or depolarizing (Munoz et al., 2007). Accumulating evidence has correlated epileptogenesis with altered functional expression of NKCC1 and KCC2 co-transporters. A study on human epileptic tissues also revealed that NKCC1 is up-regulated, while KCC2 is down-regulated in subicular cells adjacent to the hippocampus in patients with epilepsy (Huberfeld et al., 2007). FDP has been shown to shift the metabolism of glucose from the glycolytic pathway to the pentose phosphate pathway and modify different signal pathways (e.g., BDNF/TrkB in our previous study) (Wheeler and Chien, 2012), and it ultimately affects the GABA neurotransmission system. In the present study, we found that FDP can prevent kindling-induced KCC2 downregulation and decreased NKCC1 expression; this finding suggests that FDP may decrease the intracellular $\text{Cl}^-$ level by modulating the balance between KCC2 and NKCC1 expressions, and thus contributes to a reduction of GABA-mediated neuronal excitability.

Conversely, oxidative stress is involved in the pathogenesis of kindling seizures (Aguiar et al., 2012), and it can result in a rapid decrease in KCC2 expression in vitro (Wake et al., 2007). FDP diverts glucose into the pentose phosphate pathway, increasing intracellular NADPH production,
which preserves cellular glutathione levels and decreases ROS production (Park et al., 2004). Therefore, FDP may also regulate the change in KCC2 levels through ROS. Thus, the FDP-induced increase in KCC2 expression following kindling seizures may also be related to the antioxidant property of FDP.

In conclusion, we demonstrated the dose-dependent anti-epileptogenic effects of FDP in amygdaloid-kindling acquisition; however FDP had no effect on fully kindled seizures. Additionally, FDP inhibited the kindling-induced downregulation of KCC2 and decreased hippocampal expression of NKCC1, suggesting that reduced GABA-mediated excitation induced by alteration of intracellular chloride level may also play a role in the anti-epileptogenic function of FDP. Because the mechanisms of current clinical anti-epileptic drugs are unrelated with glucose metabolism, these findings are of great importance because they illuminate novel approaches for controlling seizures and may lead to the development of a small molecule replacement for the complex KD treatment.

4. Experimental procedures

4.1. Animals and surgery

Male Sprague Dawley rats (260–300 g, Grade II, Certificate No. SCXK2003-0001; Experimental Animal Center, Zhejiang Academy of Medical Science, Hangzhou, China) were housed in individual cages with a 12-h light–dark cycle (lights on from 0800 to 2000). All experiments were carried out in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Water and food were provided ad libitum. Experiments were carried out between 1000 and 1700.

After deep anesthesia with chloral hydrate (400 mg/kg, i.p.), rats were mounted on a stereotaxic apparatus (512600; Stoelting, IL, USA). Electrodes were implanted into the right basolateral
Amygdala (coordinates from the bregma: AP = −2.4 mm, L = −4.8 mm, and V = −8.8 mm). The electrodes were made of twisted stainless steel wires with a diameter of 0.2 mm (A.M. Systems, USA) and Teflon coated except for 0.5 mm at the tip. The distance between tips was 0.7–0.8 mm. The electrodes were connected to a miniature receptacle, which was embedded in the skull with dental cement. Animals were allowed to recover from surgery over a 10-day period before testing.

4.2. Procedure for kindling and threshold measurement

EEGs of the right amygdala were recorded with a digital amplifier (RM-6240, Chengyi, China). The ADT of each subject was determined with a constant current stimulator (YC-2; Chengyi, China). An initial current of 50 μA was used and subsequently increased stepwise was 20-μA increments. Consecutive trials were separated by at least 30 min. The ADT was defined as the lowest current required elicited an after-discharge lasting for at least 5 s on EEG. All animals were subjected to once-a-day kindling stimulation with the same current intensity as their own ADT. The daily kindling stimulus consisted of a 1-s train of monophasic, 1-ms square-wave pulses at 60 Hz. Animals that exhibited three consecutive stage 5 seizures were considered to be fully kindled. Seizure severity was classified according to a modification Racine classification and scored as follows (Racine et al., 1972): (1) facial movement; (2) head nodding; (3) unilateral forelimb clonus; (4) bilateral forelimb clonus and rearing; and (5) bilateral forelimb clonus, rearing, and falling. Stages 1–3 were considered to be focal seizures, while stages 4 and 5 were considered GS. In addition to seizure stage, the ADD and GSD were recorded.

4.3. Effect of FDP on kindling acquisition and fully kindled animals

In experiment 1, rats were divided into three groups matched for ADTs. Based on pharmacokinetics in a previous study (Xu and Stringer, 2008), FDP (47810; Sigma, USA) was intraperitoneally administered 1 h before kindling stimulation. FDP...
was administered once daily at 0.5 and 1.0 g/kg doses; an equivalent volume of saline (vehicle) was administered in the control group. Animals were stimulated daily at ADT intensity until the control group animals were fully kindled.

In experiment 2, rats were stimulated until they were fully kindled (without any FDP treatment during kindling) and were then divided into three groups in the same fashion as experiment 1. On the next day, three groups received stimuli with pre-kindling ADT intensity after pretreatment with saline or 0.5 or 1.0 g/kg FDP. All rats were pretreated and stimulated daily for 5 consecutive days, and the seizure stage, ADD, and GSD were measured by the same procedures used for kindling seizures.

4.4. Gene expression study

The rats were killed by decapitation, and the brains were removed within 1 h of the last stimulation. The ipsilateral hippocampus was dissected and flash frozen in liquid nitrogen. Total RNA was extracted from the hippocampal tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out using the SYBR® Green Realtime PCR Master Mix (QPK-201; Toyobo, Japan) on a LightCycler® 2.0 Instrument (Roche Diagnostics Corporation, Germany) according to manufacturer’s instructions. Primers were designed and synthesized by Invitrogen Biotechnology Co. Ltd. (Shanghai, China). The primers sequences were as follows: GAPDH forward: 5′-GTGACCCTCATGGCTCTACATC-3′ and reverse: 5′-GCCCTCTCTTGCTGCTGATC-3′; KCC2 forward: 5′-ATCGAGATCCTGCTGGCTTA-3′ and reverse: 5′-ACTTGACGGCCACAAAAACT-3′; NKCC1 forward: 5′-GAAGCAAAGGCTCAGATCGT-3′ and reverse: 5′-ATCGGATCTGCCTGTGCCTC-3′.

4.5. Histology

Upon completion of the behavioral experiments, electrode placements were histologically examined by staining the brain sections with toluidine blue O. Data from animals in which the electrodes were placed within the right basolateral amygdala according to the atlas of Paxinos and Watson (2006) were included in the statistical analysis. In the case of animals used in biochemical experiments, basolateral amygdala electrode placement was visually examined and confirmed in the process of tissue separation.

4.6. Statistical analysis

Data are presented as mean±standard error of the mean (S.E.M.). Statistical evaluations of group differences in kindling acquisition were performed with two-way analysis of variance (ANOVA). Other tests were performed with one-way ANOVA when the data were normally distributed and the variances were homogeneous; otherwise, nonparametric Mann–Whitney U tests were used. All analyses were two-sided, and P<0.05 was considered statistically significant.

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References


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