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Ketogenic Diet Alters Dopaminergic Activity in the Mouse Cortex

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The work described in this paper has been carried out in accordance with the *Uniform Requirements for manuscripts submitted to Biomedical Journals*

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Abstract:

The present study was conducted to determine if the ketogenic diet altered basal levels of monoamine neurotransmitters in mice. The catecholamines dopamine (DA) and norepinephrine (NE) and the indolamine serotonin (5HT) were quantified postmortem in six different brain regions of adult mice fed a ketogenic diet for 3 weeks. The dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and the serotonin metabolite 5-hydroxyindole acetic acid (5HIAA) were also measured. Tissue punches were collected bilaterally from the motor cortex, somatosensory cortex, nucleus accumbens, anterior caudate-putamen, posterior caudate-putamen and the midbrain. Dopaminergic activity, as measured by the dopamine metabolites to dopamine content ratio – $([DOPAC] + [HVA])/[DA]$ - was significantly increased in the motor and somatosensory cortex regions of mice fed the ketogenic diet when compared to those same areas in brains of mice fed a normal diet. These results indicate that the ketogenic diet alters the activity of the meso-cortical dopaminergic system, which may contribute to the diet's therapeutic effect in reducing epileptic seizure activity.

Key words: Ketogenic diet, monoamines, dopamine, serotonin, HPLC, tissue punch

Introduction:

Epilepsy is a predominant neurological disorder which induces recurrent episodes of convulsive seizures, loss of consciousness, sensory disturbances and abnormal behavior. The ketogenic diet is a high fat, low protein and carbohydrate diet utilized as a non-pharmacological therapeutic alternative for individuals suffering from drug resistant epilepsy [1,2]. While the exact neuronal mechanism by which the ketogenic diet induces its therapeutic effect remains unclear, recent research suggests an involvement of monoamine neurotransmitters [3,4,5], adenosine [6,7,8] and glutamatergic transmitter systems [9,10] within the central nervous system.

In addition to affecting numerous chemical systems in the CNS, the ketogenic diet has been reported to selectively impact specific brain structures. Zarnowski et al. [11] have shown that the levels of the tryptophan metabolite kynurenic acid were increased in the hippocampus and striatum, but not the cortex of female rats fed a ketogenic diet for 21 days. Alternatively, Cantello et al. [12] reported that the ketogenic diet increased inhibition of neuronal activity in the cerebral cortex of non-epileptic humans. To evaluate if the ketogenic diet altered basal monoamine neurotransmitter content in a region specific manner, the present study measured the concentrations of norepinephrine (NE), dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole acetic acid (5HIAA) and homovanillic acid (HVA) in the brains of mice maintained on a three week ketogenic diet. The endogenous levels of these neuroactive compounds were determined in the motor cortex (MC), somatosensory cortex (SC), nucleus accumbens (NA), anterior and posterior caudate putamen (ACPu and PCPu, respectively) and the midbrain (MB) using micropunch sampling coupled with separation and quantification by high performance liquid chromatography with electrochemical and UV detection.

Material and Methods:

All animal care and use and surgical procedures were approved by the Institutional Animal Care and Use Committee of Trinity College and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Discrete brain region tissue collection and biogenic amine content determination was conducted as previously described [13,14]. Briefly, 8 week old mice were placed on either a three week chronic

ketogenic diet (#F3666 Bioserve, Frenchtown NJ USA) or control diet (LabDiet 5001, Pharmaserv, Framingham, MA USA). Changes in blood glucose and β -hydroxybutyrate levels between control and KD mice were observed (140.5 ± 22.2 mg/dL v. 78.8 ± 18.8 mg/dL and 0.26 ± 0.1 mM v. 2.8 ± 1.4 mM, respectively). Naïve mice were decapitated and whole brains were quickly dissected and collected into -20°C isopentane. Time from decapitation to immersion in isopentane was 30-40 seconds. To ensure similar dissection technique for all animals and treatment groups, all dissections were performed by the same experimenter. After freezing, brains were removed from isopentane and stored at -80°C until punch collection. Bilateral tissue punches (1 mm thick, 1 mm diameter) were acquired from the motor cortex, somatosensory cortex, nucleus accumbens, anterior caudate, posterior caudate, and the midbrain. Specific brain sites were identified using a standard mouse atlas [15]. The samples were homogenized in 400 μL of ethanol containing 10 μL of anti-oxidant solution [dihydroxybenzyl amine (DHBA; 0.01 mg/100mL; internal standard), EDTA (0.02 mg/100mL), sodium metabisulfite (0.1 mg/100mL) in 6mM HCl] and then centrifuged at $12,000 \times g$ for 10 minutes. The supernatant samples were filtered through a 0.2 μm nylon disposable syringe filter and centrifuged rotovaped to dryness (45°C and 0.1 bar; Centrivap Concentrator, Labconco, Kansas City, MO USA). Samples were then reconstituted in 1000 μL of phosphate buffer (pH= 7.4) and stored at -80°C until analyzed by HPLC.

The neuroactive compounds were separated on a reverse-phase HPLC system with electrochemical and UV detection configured in series. Separation was carried out on a 150 x 2.00 mm LUNA 5 μm C18 column (Phenomenex, Torrence, CA USA) using an acetonitrile/ phosphate buffer with ion pairing agent mobile phase (MD-TM; Thermo Scientific) delivered at a flow rate of 0.5 mL/min. Dual electrochemical detection (ESA Coulochem III; $E_{1\text{app}} = -150$ mV; $E_{2\text{app}} = +300$ mV; Thermo Scientific, Sunnyvale, CA USA) and dual wavelength UV detection ($\lambda_1 = 245\text{nm}$; $\lambda_2 = 280$ nm; BioAnalytical Systems, West Lafayette, IN USA) were used to quantify the compounds of interest using the internal standard calibration method. Chromatographic data was collected, stored, and analyzed using EZ Chrom chromatography software (Thermo Scientific). Protein content of tissue punches was determined by the Modified Lowry Protein Assay (Pierce; Thermo Scientific, Sunnyvale, CA USA).

Statistical Analysis

Differences in brain tissue levels of the biogenic amines were evaluated using a two-way ANOVA with post-hoc comparisons (n=6-9 brains per diet group; GraphPad Prism 4.0, GraphPad Software, Inc. San Diego, CA, USA).

Results

A three week KD regimen did not alter the tissue levels of NE, DA, 5HT, DOPAC, HVA, or 5HIAA in any of the brain regions analyzed (Table 1). The KD did result in a brain region-specific increase in the activity of DA neurons as measured by the metabolic ratio. Figure 1A shows that mice fed a ketogenic diet had a significantly higher dopaminergic metabolic ratio (the ratio of the tissue levels of the major dopamine metabolites, DOPAC and HVA, to the tissue level of DA) in the motor cortex and somatosensory cortex regions ($p < 0.001$ and $p < 0.05$, respectively). In the motor cortex, the ratio increased 71% , from a value of 3.66 to a value of 6.27, over controls. In the somatosensory cortex the ratio increased 151%, from a value of 1.14 to a value of 2.86, over controls. An increase was also observed in the midbrain region of mice fed a KD that approached significance (2.69 vs. 4.29; $p = 0.053$). There was no difference in the serotonin metabolic ratio ([5HIAA]/ [5HT]) between diet groups in any brain region sampled (Figure 1B).

Discussion

Numerous studies utilizing animal models to study the effect of the KD have recently been reported (see review [16]). However, little is known about the effects of the ketogenic diet on the neurochemical content in brain regions in these models. In the present study, the steady-state tissue levels of the major monoamine neurotransmitters NE, DA, and 5-HT were not changed in six different brain regions by a 3-week regimen of the low-fat, high-carbohydrate KD (Table 1). Similar results have recently been reported showing no change in tissue dopamine level in the striatum of mice fed a KD for 2 weeks [17]. Kynurenic acid, a metabolite of tryptophan (serotonin precursor), was shown to be increased in the hippocampus and striatum but not the cortex of rats fed a ketogenic diet for 21 days [11]. Similarly, in the present study, an

increase in the dopaminergic metabolic ratio was shown to be altered in a region-specific manner by the KD (Figure 1).

The dopaminergic system has been clearly identified as having seizure-modulating effects [18]. This modulating effect is very brain region and dopamine receptor subtype specific. It was recently reported that dopamine, acting through D₁ receptors, decreased neuronal activity in the rat cortex [19]. Local application of dopamine has been shown to inhibited spontaneous firing rates in pyramidal tract neurons in the rodent motor cortex [20]. In the current study, the ketogenic diet altered cortical dopamine activity as measured by an increase in the metabolite-to-transmitter ratio. An increase in this ratio is generally accepted to be representative of an increase in dopamine turnover produced by an increase in dopamine release. Increased inhibition of cortical neuronal activity as a result of increased dopamine release presents a possible mechanism for the KD's therapeutic effect. Using trans-cranial magnetic stimulation (TMS), Cantello et al. [12] have reported an increase in inhibition, as determined by an enhancement of measured intra-cortical inhibition (SICI), in the cerebral cortex of normal humans fed a KD. Inhibition of cortical neurons by dopamine has been reported in humans[21]. In the present study, a significant increase in dopaminergic neuronal activity, as measured by the metabolites-to-neurotransmitter ratio, was found in the motor and sensory cortex regions of mice fed a KD for three weeks (Figure 1A). Thus, it can be proposed that the KD acts directly on meso-cortical dopaminergic neurons and that subsequent increased cortical inhibition via increased meso-cortical DA activity may contribute to the anti-seizure properties of the KD.

Alternatively, the observed increase in cortical dopaminergic activity may have been generated through an indirect pathway involving elevated adenosine and the glutamate-dopamine synapse within the ventral tegmental area (VTA). Recently an increase in adenosine levels in neuronal tissue and subsequent increase in A₁ receptors has been proposed as a mechanism responsible for altered cortical activity following a KD [7, 22]. Adenosine was shown to selectively inhibit mGluR IPSPs in VTA dopamine neurons via A₁ receptor antagonism [23]. The authors suggest that this would result in more effective burst firing, thereby resulting in an increase in dopamine neuronal activity. The KD could therefore produce its therapeutic effect through an increase in adenosine and subsequent stimulation of A₁ receptors on glutamatergic afferents which would result in the disinhibition of the VTA dopaminergic neurons.

Interestingly, the aspartate-glutamate homeostasis in cerebellar neurons was disrupted when β -

hydroxybutarate was used as an energy source for the neurons, resulting in a decrease in neuronal glutamate content [24]. This decrease in glutamate release as a result of the energy-source change produced by the KD would also result in a disinhibition of the VTA dopaminergic neurons and the observed increase in cortical dopaminergic metabolic ratio.

It is to be noted that the KD used in this study contained a significantly higher amount of the antioxidant vitamin E (244 IU/kg compared to 42 IU/kg). Sharma and Nehru [25] recently reported that rats administered vitamin E (100 IU/kg/day i.m.) for 35 days had increased levels of dopamine in the midbrain. Future investigations to elucidate the mechanism responsible for the therapeutic effect of the KD will have to include the impact of increased dietary antioxidants.

To our knowledge, the current report is the first to evaluate the effect of a chronic KD on tissue levels of monoamine neurotransmitters in multiple brain regions of experimental animals. While no change in the tissue content of neurotransmitters or metabolites was observed after three weeks on the KD, the observed increase in the metabolic ratio for the dopaminergic system within the motor and somatosensory cortex suggests involvement of the meso-cortical dopaminergic system in the anti-convulsive effect of the KD. Evaluation of KD-induced cellular metabolism changes and the above mentioned neurotransmitter systems within this localized region may provide insight into the therapeutic differences seen between the KD and standard anti-epileptic pharmacological treatments in humans.

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This work is consistent with the International Committee of Medical Journal Editors guidelines for ethical publication

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Table 1 – Neurochemical content from selected brain regions of mice fed either a normal chow (*Control*) or a ketogenic diet (*Ketogenic*). Data are reported as ng/mg protein (S.E.M.) for $n = 6$ -8 animals.

Brain Structure	NE		DA		5HT		DOPAC		HVA		5HIAA	
	Control	Ketogenic	Control	Ketogenic	Control	Ketogenic	Control	Ketogenic	Control	Ketogenic	Control	Ketogenic
MC	29.6 (4.1)	38.5 (10.4)	33.1 (9.3)	20.5 (9.6)	32.0 (5.4)	32.5 (4.4)	8.7 (2.1)	12.1 (3.8)	62.9 (7.8)	90.6 (30.7)	17.4 (3.0)	17.2 (5.6)
SC	35.5 (9.4)	38.4 (9.4)	65.4 (10.6)	61.8 (24.5)	35.9 (7.1)	45.7 (12.7)	18.9 (5.0)	18.4 (5.5)	64.3 (7.7)	113.5 (38.0)	17.7 (3.9)	22.7 (6.9)
NA	31.2 (8.5)	27.6 (3.6)	231.9 (31.5)	233.8 (48.7)	33.7 (5.5)	38.9 (7.5)	30.5 (4.8)	37.5 (8.7)	57.3 (12.1)	75.9 (14.1)	18.4 (4.0)	17.0 (3.8)
ACPu	32.4 (11.1)	22.6 (42)	289.9 (54.6)	281.2 (45.4)	24.6 (7.8)	29.7 (6.5)	37.2 (7.4)	35.7 (9.1)	88.8 (21.9)	92.9 (27.4)	18.7 (5.5)	14.1 (2.6)
PCPu	20.8 (4.3)	31.2 (8.2)	132.3 (16.8)	206.9 (63.8)	30.2 (5.9)	39.1 (8.3)	15.5 (4.3)	27.5 (4.4)	52.1 (11.8)	93.1 (18.5)	15.8 (4.0)	19.5 (4.1)
MB	47.0 (81)	61.9 (8.3)	25.2 (5.1)	20.1 (4.3)	37.9 (7.7)	35.1 (7.6)	12.5 (2.1)	16.4 (3.5)	51.5 (8.1)	76.6 (13.9)	22.7 (3.5)	26.2 (4.0)