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The ketogenic diet changes metabolite levels in hippocampal extracellular fluid

Ramakrishna Samala [a], Jochen Klein [b], Karin Borges [c,*]

[a] Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter, Amarillo, TX 79106, United States
[b] Department of Pharmacology, Biozentrum N260, Campus Riedberg, Johann Wolfgang Goethe-University Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany
[c] School of Biomedical Sciences, Skerman Buildg 65, The University of Queensland, St. Lucia, QLD 4072, Australia

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ABSTRACT

Despite successful use of the ketogenic diet (KD) for the treatment of drug-resistant epilepsy, its mechanism of action is unclear. After KD-feeding, increased plasma D-beta-hydroxybutyrate (BHB) levels appear to be important for protection against seizures. We hypothesized that the KD leads to metabolic changes in the brain, which are reflected in the hippocampal extracellular fluid (hECF). CD1 mice were fed control or KD for 2–3 weeks since weaning. *In vivo* microdialysis of hECF was used to measure the levels of glucose, lactate, as well as BHB under basal conditions and during 30 min stimulation with 60 mM K^+, which was retrodialysed. The hECF BHB concentration in KD-fed mice was determined as 43.4 ± 10.1 μM using the zero-flow method and 50.7 ± 5.5 μM based on *in vitro* recovery. The total BHB concentration in brain homogenate from KD-fed mice was 180 nmol/g. The intracellular BHB concentration is therefore estimated to be about 3-fold higher than the extracellular level, which suggests that BHB in adolescent mouse brains may not be quickly metabolized. The basal hECF glucose concentration was 30% lower in KD-fed mice, indicating that glucose may be less important as an energy source. Lactate levels were similar in control and KD-fed mice. High potassium stimulation elevated lactate by 3–3.5-fold and decreased glucose by 40–50% in both diet groups, consistent with similar anaerobic and aerobic metabolism in both diet groups during high hippocampal activity. Overall, these data (1) defined the BHB concentration in the hippocampal extracellular fluid in KD-fed mice and (2) showed lower glucose metabolism compared to control diet-fed mice. This work will now enable other researchers to mimic the hippocampal extracellular environment in experiments aimed at deciphering the mechanisms of the KD.

1. Introduction

The ketogenic diet (KD) is a strict high fat, low protein, low carbohydrate diet, and is anticonvulsant in many drug-resistant epileptic children (Freeman et al., 2007; Neal et al., 2009). The KD is also effective in mice as previously reported (Uhlemann and Neims, 1972) and corroborated by us (Samala et al., 2008). The KD leads to the generation of ketone bodies D-β-hydroxybutyrate and acetoacetate) by the liver, reaching plasma levels of BHB in the millimolar range, 1–2 mM in our hands in mice (Samala et al., 2008) or 4 mM in children (Neal et al., 2009). BHB can enter the brain through the monocarboxylate transporters and can then be metabolized in the brain to acetyl-CoA (Nehlig, 2004). Several hypotheses have been proposed about the anticonvulsant mechanism of the KD, including changes in the extracellular milieu (Schwartzkroin, 1999). It was found that ketones directly reduce the spontaneous firing rate of neurons in the substantia nigra pars reticulata and this action was abolished by removal of K(ATP) channels (Ma et al., 2007). Yet, the physiological levels of extracellular ketones in the brain are still unknown. One of the main hypotheses is that the ketogenic diet is anticonvulsant by providing the brain with ketone bodies and thus additional ATP (DeVivo et al., 1978; reviewed by Schwartzkroin, 1999). So far, this hypothesis has been mainly addressed by measuring metabolite and neurotransmitter levels in tissue homogenates or in the cerebrospinal fluid. These measurements have pointed to some metabolic changes in energy and neurotransmitter metabolism (e.g. Bough et al., 2006; DeVivo et al., 1978; Yudkoff et al., 2005). However, total tissue or CSF levels of neurotransmitters and metabolites are not reflective of the neuronal microenvironment, the brain extracellular brain fluid (ECF), which ultimately determines neuronal and astrocytic energy supply and excitability. We showed previously that our 6:1 KD containing the same vitamin, mineral and antioxidant content per caloric density as the control diet, was repeatedly anticonvulsant in the 6 Hz test (Samala et al., 2008). In the present study, we determined the composition of the extracellular fluid in the hippocampus under basal conditions and after stimulation with high potassium levels.

Abbreviations: BHB, D-β-hydroxybutyrate; hECF, hippocampal extracellular fluid; KD, ketogenic diet.

* Corresponding author. Tel.: +61 7 3365 3113; fax: +61 7 3365 1766.
E-mail address: k.borges@uq.edu.au (K. Borges).

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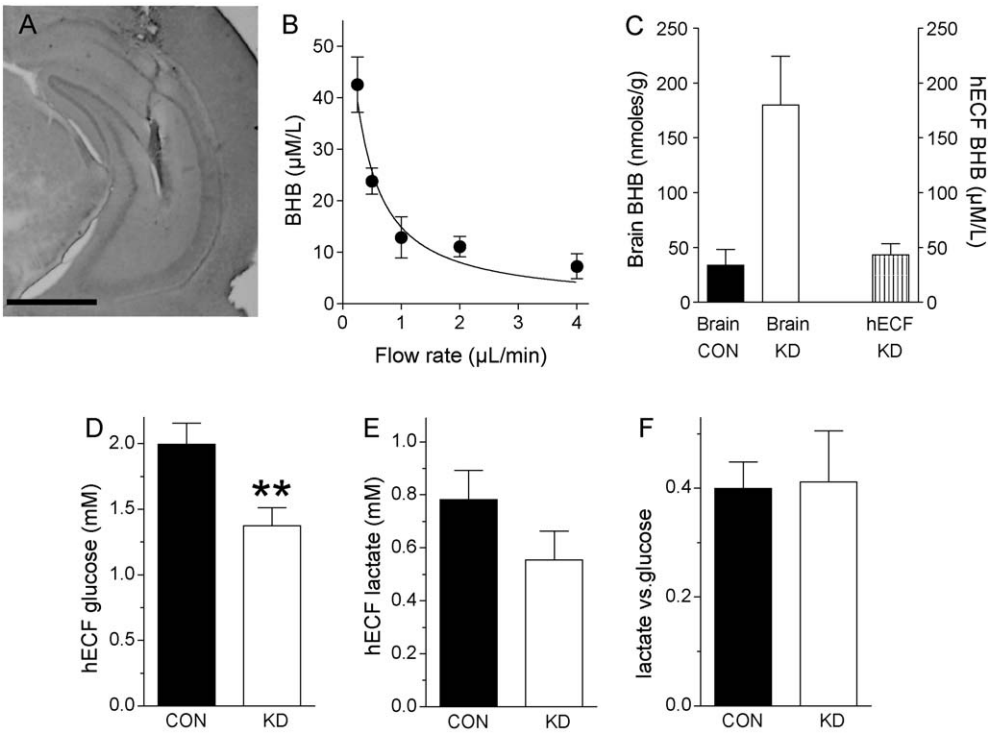

Fig. 1. Metabolite concentrations in extracellular hippocampal fluid under basal conditions determined by *in vivo* microdialysis. (A) Probe location. (B) Zero-flow method to determine intrahippocampal extracellular concentrations of BHB. The concentration of hECF BHB was determined by fitting the curve to the equation $Y = Y_0 \times (1 - \exp(-K/X))$. (C) The total brain BHB concentration is compared for control and KD-fed mice (μM/g wet weight, $p = 0.02$) and compared to that of the hECF in KD-fed mice (μM/L). The concentration of hECF glucose (D) and lactate (E) was determined based on the in vitro recovery for these metabolites. F shows the lactate vs. glucose ratio. Only the glucose levels are statistically significantly lower in KD-fed mice ($p = 0.0098$).

We expected to observe a reduced metabolism of glucose in the KD-fed mice, as BHB can be used as an alternative substrate for energy metabolism.

2. Materials and methods

2.1. Diets and mice

All experiments were approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center and conducted in accordance with its guidelines. Every effort was made to reduce animal suffering. All mice were housed under a 12 h light/dark cycle with free access to food and water. Male CD1 mice (Charles River) were placed onto either a standard diet (TD.06316) or 6.3:1 KD as used by (Samala et al., 2008) immediately after weaning for 2–3 weeks. The amounts of vitamins, minerals, antioxidants and protein match the newest nutritional standards and were equal among all diets relative to their caloric densities.

2.2. Microdialysis experiments

Microdialysis was performed similarly as described earlier (Kiewert et al., 2010). Mice were anesthetized with isoflurane (induction dose, 4%; maintenance dose, 1–2%, v/v) in oxygen and placed in a stereotaxic frame. Self-made, I-shaped, concentric dialysis probes with an exchange length of 2 mm were implanted in the right ventral hippocampus using the following coordinates (from bregma): anterior–posterior, 6 mm; lateral, 1.5 mm; dorso-ventral, 4 mm. Mice were allowed to recover overnight in a box with food and water provided in dishes. Experiments were carried out on two consecutive days after probe implantation in freely moving animals in the same box.

On the experimental days, the microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, and 1.2 mM $MgCl_2$). The perfusion rate was 0.25–4 μl/min, and efflux from the microdialysis probe was collected in intervals of 10–30 min, depending on flow rate. For the zero-flow experiments, we collected samples at the flow rates of 0.25 μl/min on the first day of microdialysis and 0.5, 1, 2 and 4 μl/min on the second day. Dialysate was collected after equilibration to different flow rates. For the potassium stimulation experiments, 60 mM NaCl was replaced with 60 mM KCl 20 min after determining baseline levels. After 30 min KCl stimulation the perfusion fluid was switched back to aCSF, and mice were further monitored for a period of at least 2 h. After microdialysis, animals were sacrificed with an overdose of pentobarbital (100 mg/kg, i.p.) and brains were placed in 4% paraformaldehyde overnight. Brains were sectioned into 40 μm slices using a vibratome and stained with hematoxylin. The position of the microdialysis probe was verified to be in the ventral hippocampus before metabolite quantification.

2.3. Whole brain homogenisation

Mice were decapitated after isofluarane anesthesia and forebrains (brains without cerebellum) were removed within less than 1 min and homogenized in ice cold 2 ml 7% perchloric acid with a polytron. After spinning for 10 min at 1000 × *g*, the supernatant was adjusted to pH 7 using potassium hydroxide. After another 10 min centrifugation at 14,000 × *g* at 4 °C the supernatant was collected and frozen for later analysis.

2.4. Metabolite quantification in hECF

Glucose and lactate concentrations in the microdialysis samples were determined by a CMA/Microdialysis (Solna, Sweden) 600 microanalyzer using a kinetic photometric assay as described by the manufacturer.

BHB levels in the microdialysate and whole brain homogenates were measured using the Autokit-3HB from Wako Diagnostics, which is based on the measurement of NADH by enzymatic cycling after conversion of BHB to acetoacetate by β-hydroxybutyrate dehydrogenase. The measurements at different flow rates were fitted to the equation $Y = Y_0 \times (1 - \exp(-K/X))$, in which Y_0 is the extracellular tissue concentration (Jacobson et al., 1985).

2.5. Ketone quantification in plasma

Trunk blood was collected in heparinized tubes and centrifuged at 6000 × *g* for 5 min to obtain plasma. Plasma D-BHB levels were measured using a kit from Pointe Scientific. This kit employs the conversion of β-hydroxybutyrate and NAD to acetoacetate and NADH. The NADH produced is converted to color using INT and diaphorase.

2.6. Statistics

All data points are presented as averages ± standard error of the mean (S.E.M.). Unpaired two-tailed student *t*-tests were employed to assess whether metabolite levels were significantly different between ketogenic and control diet-fed mice. GraphPad Prism (version 5) was used for two way ANOVAs to compare glucose and