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Ketone body metabolism and sleep homeostasis in mice

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ABSTRACT

A link has been established between energy metabolism and sleep homeostasis. The ketone bodies acetoacetate and β-hydroxybutyrate, generated from the breakdown of fatty acids, are major metabolic fuels for the brain under conditions of low glucose availability. Ketogenesis is modulated by the activity of peroxisome proliferator-activated receptor alpha (PPARα), and treatment with a PPAR activator has been shown to induce a marked increase in plasma acetoacetate and decreased β-hydroxybutyrate in mice, accompanied by increased slow-wave activity during non-rapid eye movement (NREM) sleep. The present study investigated the role of ketone bodies in sleep regulation. Six-hour sleep deprivation increased plasma ketone bodies and their ratio (acetoacetate/β-hydroxybutyrate) in 10-week-old male mice. Moreover, sleep deprivation increased mRNA expression of ketogenic genes such as PPARα and 3-hydroxy-3-methylglutarate-CoA synthase 2 in the brain and decreased ketolytic enzymes such as succinyl-CoA: 3-oxoacid CoA transferase. In addition, central injection of acetoacetate, but not β-hydroxybutyrate, markedly increased slow-wave activity during NREM sleep and suppressed glutamate release. Central metabolism of ketone bodies, especially acetoacetate, appears to play a role in the regulation of sleep homeostasis.

1. Introduction

Ketone bodies become major fuels in most tissues during starvation, prolonged exercise, or consumption of a high-fat, low-carbohydrate diet (Robinson and Williamson, 1980). Conditions of reduced glucose availability lead to increased ketone production (ketogenesis) and use. Ketones, such as acetoacetate (AcAc) and β-hydroxybutyrate (BHB), are generated from the breakdown of fatty acids (Fukao et al., 2004; Robinson and Williamson, 1980). Circulating levels of ketone bodies are determined by their rates of production and utilization (ketolysis). Although the liver is generally believed to be the major organ that supplies the brain with ketone bodies, it has been reported that astrocytes can also produce ketone bodies from fatty acids under conditions of glucose deprivation (Auestad et al., 1991; Blazquez et al., 1998). Sequential ketogenic reactions catalyzed by mitochondrial thiolase, 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2), and hydroxymethylglutaryl-CoA (HMG-CoA) lyase convert acetyl-CoA (Ac-CoA) to the ketone body AcAc (Cullingford, 2004; Fukao et al., 2004; Hegardt, 1999). AcAc can be reversibly reduced to BHB by mitochondrial BHB dehydrogenase (HBD) in an NAD^+/NADH-coupled redox reaction (Hegardt, 1999). The ratio of AcAc to BHB reflects the redox state within the mitochondrial matrix (Constantin et al., 2011; Katsuyama et al., 1999). In extrahepatic tissues, AcAc is activated to acetoacetyl-CoA (AcAc-CoA) by the mitochondrial matrix enzyme succinyl-CoA-3-oxoacid CoA transferase (SCOT), a mitochondrial CoA transferase in mammals (Fukao et al., 2004; Laffel, 1999; Williamson et al., 1971). Ac-CoA produced by the action of AcAc-CoA thiolase enters the tricarboxylic acid (TCA) cycle for terminal oxidation and provides fuel for ATP synthesis (Fukao et al., 2004; Laffel, 1999; Robinson and Williamson, 1980). The relationship between ketone body metabolism in the brain and neuronal activity continues to be investigated, and many mechanisms of ketone body action have been suggested (Cullingford, 2004; Guzman and Blazquez, 2001; Morris, 2005; Nehlig, 2004).

In recent years, a considerable number of studies using normal and obese or diabetic animals have shown that sleep/wake regulation and energy metabolism are closely intertwined (Laposky et al., 2008; Martins et al., 2008). High-fat feeding and food restriction paradigms, both of which are believed to enhance ketogenesis, can affect sleep/wake patterns (Alvarenga et al., 2005; Jenkins et al., 2006). In addition, a recent study in humans showed evidence that suppression of stages 3 and 4 of non-rapid

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eye movement (NREM) sleep, which are accompanied by reduced slow-wave activity (SWA, power density of the electroencephalogram (EEG) delta band between 0.5 and 2.0 Hz in humans), resulted in decreased insulin sensitivity and glucose tolerance, and the risk of type 2 diabetes was therefore increased (Tasali et al., 2008). SWA in NREM sleep is believed to be a variable of sleep depth and homeostatic need for sleep because it is enhanced after sleep deprivation (SD) (Borbely et al., 1981; Dijk et al., 1990). Since ketone bodies are elevated in patients with diabetes, and conversely, treatment with ketone bodies was shown to improve insulin sensitivity in type 2 diabetic rats (Laffel, 1999; Park et al., 2011), disturbances in SWA are likely related to metabolic impairment due to alteration of ketogenesis and/or ketolysis. Thus, ketone bodies may be involved in sleep/wake regulation, but this has never been studied.

Ketogenesis is modulated by the activity of peroxisome proliferator-activated receptor alpha (PPARα), one of three PPAR subtypes (α, β, and γ). PPARα is expressed in the liver, muscle, and brain, and controls transcription of many genes involved in fatty acid metabolism including those involved in ketogenesis in response to fasting (Cullingford, 2004; Desvergne and Wahli, 1999). We recently demonstrated that treatment with bezafibrate, a PPAR agonist, increased SWA in NREM sleep in mice over 24 h, accompanied by increased expression of genes encoding ketogenic enzymes such as *Hmgcs2* in the liver (Chikahisa et al., 2008). In that study, bezafibrate-treated mice showed increased AcAc and decreased BHB in plasma, resulting in a very high ketone body ratio (AcAc/BHB). However, it was not clear whether ketone body metabolism in the brain influenced sleep homeostasis.

In this article, we investigated the role of ketone body metabolism in sleep homeostasis by measuring plasma ketone body levels in sleep-deprived mice, and by evaluating SWA during NREM sleep in mice centrally injected with ketone bodies.

2. Materials and methods

2.1. Animals

All experiments were performed using male Jcl/ICR mice (Slc Inc., Shizuoka, Japan). Eight-week-old mice were fed *ad libitum* and maintained on a 12-h light–dark (L/D) cycle (lights on at 0900) at a controlled ambient temperature (23 ± 1 °C). The Animal Study Committee of Tokushima University approved these experiments, and we performed them in accordance with Guidelines for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

2.2. Sleep recording and analysis

EEG/electromyogram (EMG) implantation surgery for sleep recording and telemetry (TA10TA-F20; Data Sciences Int., USA) for recording of body temperature and locomotor activity were performed as previously described (Chikahisa et al., 2009). Off-line sleep scoring was done on the computer screen by visual assessment of EEG and EMG activity using the Spike2 analysis program (CED, Cambridge, UK). Vigilance states were based on data binned in 6-s epochs and classified as wakefulness, rapid eye movement (REM) or NREM sleep. The EEG power spectrum in the epoch determined to represent NREM sleep was calculated by Fast Fourier Transform using the Spike2 analysis program. The EEG delta frequency band was set at 0.5–4.0 Hz. The delta power was normalized as a percentage of the total power (0.5–50 Hz). Body temperature, locomotor activity, time spent sleeping and awake, and EEG delta power were averaged for hourly intervals.

2.3. Procedure for SD and food deprivation (FD)

Ten-week old mice were sleep-deprived for 6 h between *Zeitgeber* Time (ZT) 0 and ZT6 using a small soft brush to touch the back of the mouse several times when it appeared to become sleepy. At ZT6, SD ended and half of the sleep-deprived mice were euthanized, while the other half had a 6-h period of uninterrupted recovery sleep and were euthanized at ZT12. The control (non-SD) mice were also euthanized at ZT6 and ZT12. For FD, food pellets were carefully removed from the recording cages at the onset of the light period (ZT0), and returned again to the cage at ZT6.

2.4. Pharmacological treatments and injection procedure

Cannulae were implanted intracerebroventricularly (icv) at the time of surgery for EEG/EMG, as previously described (Chikahisa et al., 2009). Ketone bodies, including AcAc (lithium acetoacetate, Sigma Chemical Co., St. Louis, MO, USA) and BHB (sodium 3-hydroxybutyrate, Sigma Chemical Co.), were injected (icv) into 10-week-old mice at ZT0 or ZT12. AcAc and BHB (50 μg and 200 μg) were dissolved in 1.0 μl Ringer solution and injected slowly over 1 min using a Hamilton microsyringe.

2.5. Real-time RT-PCR analysis

Tissues used for molecular analysis were dissected immediately after decapitation, frozen in liquid nitrogen, and stored at −80 °C until use. Mice were euthanized at ZT6 and ZT12. Tissue preparation and analysis were performed as previously described (Chikahisa et al., 2008). We used pre-designed, gene-specific TaqMan probes and primer sets (Applied Biosystems, Foster City, CA) to assess expression of the following genes: Pparα (Mm00440939_m1), Hmgcs2 (Mm00520236_m1), Scot (Mm00499303_m1), and acetoacetyl-CoA synthetase (Aacs; Mm00513427_m1, a cytosol ketolysis enzyme which catalyzes the synthesis of acetoacetyl-CoA from AcAc). Real-time RT-PCR was carried out using an Applied Biosystems 7900HT real-time RT-PCR system and TaqMan universal PCR Master Mix (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For endogenous quantity control, we normalized values to those for the housekeeping gene *β-actin* (Mm00607939_s1).

2.6. Measurement of plasma ketone body and glucose levels

Trunk blood was collected from each group at the time of decapitation for real-time RT-PCR analysis. The plasma ketone bodies AcAc and BHB were measured enzymatically using an automatic analyzer system (JCA-BM12; JEOL, Tokyo, Japan) and reagents for enzymatic measurement of ketone bodies (Kainos Laboratories, Tokyo, Japan). Glucose was detected using a glucose biosensor (LifeScan, Inc., Milpitas, CA, USA).

2.7. In vivo microdialysis

The microdialysis cannula was implanted into the left lateral ventricle (AP -0.5 mm; ML 1.2 mm; V 1.5 mm to bregma), and the cannula for drug injection was implanted obliquely into the right lateral ventricle (AP -2.2 mm; ML 0.9 mm; V 2.5 mm, Angle 30° relative to bregma) of mice under general anesthesia with a cocktail of ketamine (100 mg/kg) and xylazine (25 mg/kg). After 2 weeks of recovery, a microdialysis probe with a 2 mm-long semipermeable membrane (Eicom, Kyoto, Japan) was inserted into the lateral ventricle 3–4 h prior to the experiment. The microdialysis lines were continuously perfused with Ringer's solution at a rate of 1 μl/min. Dialysate was collected from conscious mice every 30 min from 60 min before to 150 min after drug injection. After sampling the baseline for 60 min, AcAc and BHB (200 μg, icv) were injected into control (non-sleep deprived) mice at ZT6.

Glutamate content in the cerebrospinal fluid was determined using an Eicom high-performance liquid chromatography-electrochemical detector (HPLC-ECD) system. Dialysate samples (30 μl) were injected onto a column (Eicompak SC-5ODS, 150 mm × 3.0 mm i.d.) with a pre-column (CA-ODS, 4 mm × 3.0 mm i.d.). The glutamate was eluted with 0.1 M potassium phosphate at a flow rate of 0.5 ml/min, post-labeled with o-phthalaldehyde under alkaline conditions, and detected with an ECD-300 detector (Eicom, Kyoto, Japan).

2.8. Statistics

Results are expressed as means ± SEM. Plasma ketone bodies and glucose levels were analyzed using Student's *t* test. Real-time RT-PCR data were analyzed using one-way analysis of variance (ANOVA) followed by Scheffe's *post-hoc* test. Changes in sleep architecture, SWA, body temperature, and locomotor activity were analyzed by repeated measures of two-way ANOVA followed by Scheffe's *post-hoc* test. $p < 0.05$ was assumed to indicate statistical significance.

3. Results

3.1. SD increases blood ketone bodies

Six-hour SD significantly increased NREM sleep and SWA during subsequent NREM sleep in mice (Fig. S1). Based on this general finding and our previous findings (Chikahisa et al., 2008), we hypothesized that SD would alter ketone body levels, inducing the homeostatic response to sleep loss. Six-hour SD induced a marked increase in both plasma AcAc and BHB (Fig. 1A and B; ZT6). Ketone body ratio was also increased because the rise in AcAc was greater than that of BHB after SD (Fig. 1D; ZT6). These changes in ketone body content returned to control levels after a 6-h recovery period