

Neuronal–glial interactions in rats fed a ketogenic diet

Torun Margareta Melø^{a,*}, Astrid Nehlig^b, Ursula Sonnewald^a

^a Department of Neuroscience, Norwegian University of Science and Technology (NTNU), N-7489 Trondheim, Norway

^b INSERM 666, Faculty of Medicine, 67085 Strasbourg, France

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Abstract

Glucose is the preferred energy substrate for the adult brain. However, during periods of fasting and consumption of a high fat, low carbohydrate (ketogenic) diet, ketone bodies become major brain fuels. The present study was conducted to investigate how the ketogenic diet influences neuronal–glial interactions in amino acid neurotransmitter metabolism. Rats were kept on a standard or ketogenic diet. After 21 days all animals received an injection of [1-¹³C]glucose plus [1,2-¹³C]acetate, the preferential substrates of neurons and astrocytes, respectively. Extracts from cerebral cortex and plasma were analyzed by ¹³C and ¹H nuclear magnetic resonance spectroscopy and HPLC. Increased amounts of valine, leucine and isoleucine and a decreased amount of glutamate were found in the brains of rats receiving the ketogenic diet. Glycolysis was decreased in ketotic rats compared with controls, evidenced by the reduced amounts of [3-¹³C]alanine and [3-¹³C]lactate. Additionally, neuronal oxidative metabolism of [1-¹³C]glucose was decreased in ketotic rats compared with controls, since amounts of [4-¹³C]glutamate and [4-¹³C]glutamine were lower than those of controls. Although the amount of glutamate from [1-¹³C]glucose was decreased, this was not the case for GABA, indicating that relatively more [4-¹³C]glutamate is converted to GABA. Astrocytic metabolism was increased in response to ketosis, shown by increased amounts of [4,5-¹³C]glutamine, [4,5-¹³C]glutamate, [1,2-¹³C]GABA and [3,4-¹³C]-/[1,2-¹³C]aspartate derived from [1,2-¹³C]acetate. The pyruvate carboxylation over dehydrogenation ratio for glutamine was increased in the ketotic animals compared to controls, giving further indication of increased astrocytic metabolism. Interestingly, pyruvate recycling was higher in glutamine than in glutamate in both groups of animals. An increase in this pathway was detected in glutamate in response to ketosis. The decreased glycolysis and oxidative metabolism of glucose as well as the increased astrocytic metabolism, may reflect adaptation of the brain to ketone bodies as major source of fuel.

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1. Introduction

In the 1920s, the ketogenic diet (KD) was developed to mimic the metabolic state of starvation as a treatment for childhood seizures (Schwartzkroin, 1999; Stafstrom, 1999). The KD is high in fat and low in protein and carbohydrates, and induces a metabolic state in which the brain metabolizes ketone bodies produced by the liver (Fig. 1). Fatty acid oxidation leads to the formation of acetyl CoA in the liver. Since the diet contains sparse amounts of carbohydrates, hepatic metabolism is shifted towards production of glucose via gluconeogenesis rather than breakdown of glucose via

glycolysis. Oxaloacetate and malate leave the tricarboxylic acid (TCA) cycle to be converted after several steps to glucose. As a consequence, less oxaloacetate is available for condensation with acetyl CoA, which will accumulate, stimulating the formation of ketone bodies (acetoacetate and β-hydroxybutyrate). These are transported to extra hepatic tissue where they serve as metabolic fuel. The use of ketone bodies requires that acetoacetate is reactivated to its CoA derivative, a reaction carried out in most tissues except the liver. Acetoacetyl CoA is subsequently converted to acetyl CoA, which in turn enters the TCA cycle for production of energy.

During the suckling period, infant rats develop a nutritional state of ketosis due to the high fat content in maternal milk (Dombrowski et al., 1989; Nehlig and Pereira de Vasconcelos, 1993). In that period, acetoacetate and β-hydroxybutyrate represent a high proportion of brain metabolic fuels whereas the adult brain depends on a continuous supply of glucose for its metabolic needs (Hawkins et al., 1971; Cremer, 1982).

* Corresponding author at: Department of Neuroscience, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), Olav Kyrres gate 3, N-7489 Trondheim, Norway. Tel.: +47 73 59 88 56; fax: +47 73 59 86 55.

E-mail address: torun.melo@ntnu.no (T.M. Melø).

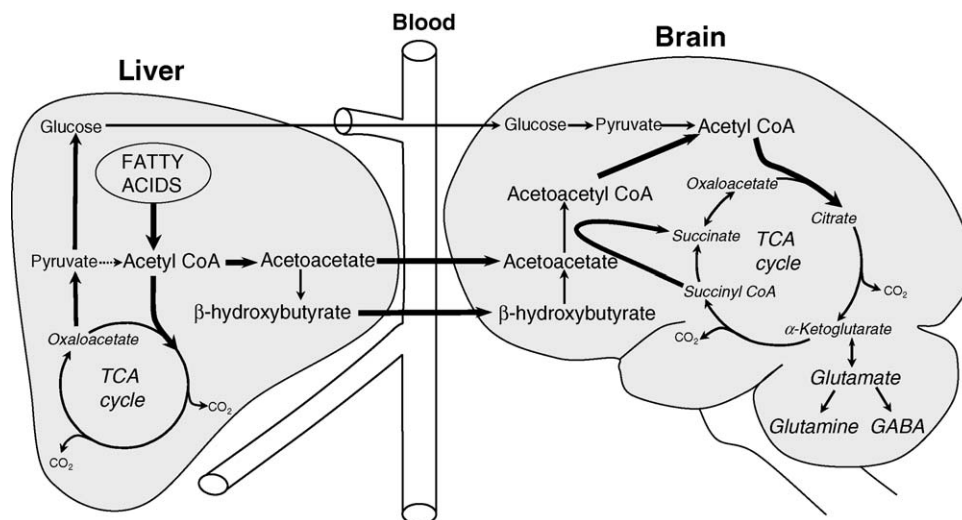


Fig. 1. Simplified illustration of hepatic and cerebral metabolism of relevant substrates in ketosis. Ketone bodies (acetoacetate and β -hydroxybutyrate) produced by the liver are fuel for the brain when glucose is in short supply. In the liver, increased fatty acid oxidation raises the level of acetyl CoA and NADH. Very little acetyl CoA enters the TCA cycle since the intermediates, oxaloacetate and malate are consumed for glucose production. Furthermore, the high amount of NADH allosterically inhibits the TCA cycle and the accumulating acetyl CoA will be converted to ketone bodies. The ketone bodies are transported to the brain via the blood. Acetoacetyl CoA is converted to acetyl CoA, which in turn enters the TCA cycle for production of energy and metabolic intermediates.

However, the use of ketone bodies can be reactivated in the adult brain during prolonged ketosis. Both neurons and astrocytes are able to take up and catabolize β -hydroxybutyrate and acetoacetate (Kunnecke et al., 1993; Martinez and Toledano, 1970; Sokoloff, 1973). Ketone bodies are used in mitochondria predominately for energy production and amino acid synthesis (DeVivo et al., 1975; Kunnecke et al., 1993). The mechanistic basis underlying the efficacy of the KD in epilepsy is still not clarified. It has been hypothesized that the KD could alter the nature of cerebral energy metabolism, and also affect neurotransmitter function and release, hence altering the inhibitory–excitatory balance leading to a decrease in excitability (Schwartzkroin, 1999). The main neurotransmitters involved in this balance are glutamate and GABA, whose synthesis is coupled to the TCA cycle via α -ketoglutarate.

^{13}C Nuclear magnetic resonance (NMR) spectroscopy can be used to study metabolic interactions between neurons and glia (Sonnewald and Kondziella, 2003). Acetate and glucose are both precursors for acetyl CoA which enters the TCA cycle. Acetate is selectively taken up by astrocytes that express a specialized transport system, which is absent or less active in neurons (Waniewski and Martin, 1998). Acetyl CoA derived from glucose is metabolized more extensively in the neuronal than in the astrocytic TCA cycle (Kunnecke et al., 1993; Qu et al., 2000). It is possible to differentiate between metabolic events taking place in neurons and glia in the same animal, by simultaneous injection of $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate (Taylor et al., 1996).

The present study was conducted to investigate how the KD influences metabolism and neuronal–glial interactions, with a specific focus on the GABA–glutamate–glutamine cycle. We chose to perform the study on a strain of spontaneously epileptic rats, the genetic absence epilepsy rat from Strasbourg (GAERS). These rats display spontaneous spike-and-wave

discharges in the cortex and thalamus concurrently with behavioral arrest (Danober et al., 1998). A recent study of neuronal–glial interactions in GAERS compared with a non epileptic control strain, showed that glutamate metabolism was mainly increased in the cortex, which may underlie the occurrence of spontaneous seizures in this model (Melø et al., 2006). However, the KD does not affect the expression of spike-and-wave discharges and has no effect on the behavior of GAERS (Nehlig, unpublished data). ^{13}C NMR spectroscopy and HPLC were used to study metabolism in astrocytes and neurons in brain extracts from cortex of 5-month-old GAERS receiving the KD for 3 weeks and controls on a standard pelleted rat chow after the injection of $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate.

2. Materials and methods

2.1. Animals

Ten adult male rats, 5-month-old (58th generation, genetic absence epilepsy rats from Strasbourg, GAERS) were kept on a KD for 3 weeks prior to the experiment. The KD consisted of 70% fat, 14% protein, no carbohydrate, and appropriate vitamins, minerals and fiber; 92% of energy provided was contributed by fat and 8% by protein (TD 96355, Harlan Tekland, Indianapolis, Indiana, USA). Eight GAERS were kept on a conventional diet. The animals were maintained at 22 °C room temperature in quiet, uncrowded animal facilities under a 12/12 h normal light/dark cycle (lights on at 7:00 a.m.) with food and water ad libitum. All animal experimentation was performed in accordance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC), and the French Department of Agriculture (License No. 67–97).

All rats were given an i.p. injection of sodium $[1,2-^{13}\text{C}]$ acetate (504 mg/kg, 0.6 M solution) plus $[1-^{13}\text{C}]$ glucose (543 mg/kg, 0.3 M solution; 99% ^{13}C enriched, Cambridge Isotope Laboratories, Woburn, Massachusetts, USA). The weight of the rats on the KD (330 ± 13 g) was statistically significantly higher ($p < 0.05$) than that of the control group (314 ± 16 g). Fifteen minutes after the injection, the brains of the animals were subjected to micro-wave fixation, 6.3 kW, 2 s (Sairem, Villeurbanne, France). Animals were decapitated

and blood was collected freely flowing from the carotid artery. Cerebral cortex (frontal, parietal and lateral cortices) was dissected, weighed and frozen (-80°C). Blood samples were centrifuged for 5 min at $3000 \times g$ and plasma was thereafter frozen at -80°C until extraction.

2.2. Tissue and plasma extraction

Each frozen tissue sample was homogenized in 1 ml ice-cold 7% perchloric acid and centrifuged for 10 min at 4°C and $4000 \times g$. The supernatants were transferred into new tubes and the precipitates were re-extracted with 0.5 ml 7% perchloric acid. The combined supernatants were centrifuged again for 10 min at 4°C and $4000 \times g$. The tubes were kept on ice at all times possible. Plasma samples (typically 300 μl) were extracted with 1 ml ice-cold 7% perchloric acid and centrifuged for 10 min at 4°C and $4000 \times g$. Each sample was neutralized with 2 M KOH, centrifuged, and lyophilized. The lyophilized samples were dissolved in 1 ml D_2O (70%, Cambridge Isotope Laboratories, Woburn, Massachusetts, USA) and 20 μl were taken out for HPLC analysis and the samples were again lyophilized and stored at -20°C .

2.3. ^{13}C and ^1H NMR spectroscopy

Lyophilized samples were dissolved in 99% D_2O and pH was adjusted to values between 6.8 and 7.0. Proton decoupled ^{13}C NMR spectra were accumulated on BRUKER DRX500 (BRUKER Analytik GmbH, Rheinstetten, Germany). The following acquisition parameters were applied; 30° pulse angle, acquisition time of 1.3 s and a relaxation delay of 0.5 s. The number of scans was typically 20,000. Some spectra were also broad band decoupled only during acquisition and accompanied by a relaxation delay of 20 s, to achieve fully relaxed spectra without nuclear Overhauser effects. From several sets of spectra, correction factors were obtained and applied to the integrals of the individual peaks.

^1H NMR spectra were acquired, on the same instruments, with the following acquisition parameters: 90° pulse angle, an acquisition time of 1.36 s and a relaxation delay of 10 s, 320 scans were accumulated for each sample. Water suppression was achieved by applying a low-power presaturation pulse at the water frequency. In both the brain and plasma extracts, the amounts of glucose, lactate, alanine and β -hydroxybutyrate were measured by ^1H NMR spectroscopy. Additionally, glutamate, glutamine, GABA and aspartate were measured in brain extracts.

2.4. HPLC analysis

Branched chain amino acids in the brain extracts were quantified by high performance liquid chromatography (HPLC) analysis on a Hewlett-Packard 1100 system (Agilent, CA, USA) with fluorescence detection, after derivatization with *o*-phthalaldehyde and were separated on a ZORBAX SB-C18 (4.6×250 mm, 5 mm) column from Agilent with 50 mM sodium acetate buffer (pH 7.0) and methanol as eluents (Geddes and Wood, 1984).

2.5. Metabolic fate of $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate

To interpret ^{13}C NMR spectra (Fig. 2) it is necessary to know the metabolic fate of $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate. Via glycolysis, $[1-^{13}\text{C}]$ glucose is metabolized to $[3-^{13}\text{C}]$ pyruvate that can be converted to $[3-^{13}\text{C}]$ lactate, $[3-^{13}\text{C}]$ alanine or enter the tricarboxylic acid (TCA) cycle as $[2-^{13}\text{C}]$ acetyl CoA (Fig. 3). $[4-^{13}\text{C}]$ Glutamate and subsequently $[4-^{13}\text{C}]$ glutamine or $[2-^{13}\text{C}]$ GABA may be formed in their respective cell types from $[4-^{13}\text{C}]$ α -ketoglutarate. Labeled oxaloacetate can be transaminated to give $[2-^{13}\text{C}]$ - or $[3-^{13}\text{C}]$ aspartate. In astrocytes, $[3-^{13}\text{C}]$ pyruvate can condense with CO_2 , via pyruvate carboxylase, leading to the formation of $[3-^{13}\text{C}]$ oxaloacetate, and further in the cycle give rise to $[2-^{13}\text{C}]$ glutamate or $[2-^{13}\text{C}]$ glutamine. If $[2-^{13}\text{C}]$ glutamine is transported from the astrocyte to a glutamatergic or a GABAergic neuron it can be converted to $[2-^{13}\text{C}]$ glutamate or to $[4-^{13}\text{C}]$ GABA, respectively (Fig. 3). Pyruvate carboxylase is an anaplerotic enzyme, supplying the TCA cycle with intermediates, when metabolites such as α -ketoglutarate leaves the cycle in the form of glutamate. Pyruvate carboxylase (PC) over pyruvate dehydrogenase (PDH) ratio for glutamate is calculated as follows:

$$\frac{\text{PC}}{\text{PDH}} = \frac{([2-^{13}\text{C}] \text{glutamate} - [3-^{13}\text{C}] \text{glutamate})}{[4-^{13}\text{C}] \text{glutamate}}$$

The same type of calculation is valid for glutamine. The ratio for GABA is the following:

$$\frac{\text{PC}}{\text{PDH}} = \frac{([4-^{13}\text{C}] \text{GABA} - [3-^{13}\text{C}] \text{GABA})}{[2-^{13}\text{C}] \text{GABA}}$$

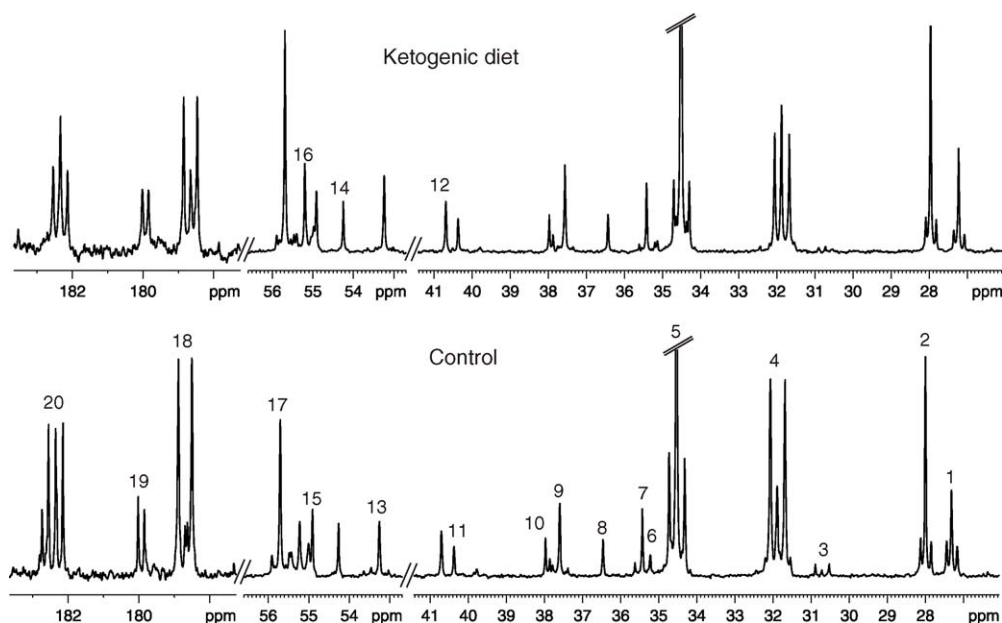


Fig. 2. Typical ^{13}C NMR spectrum of an extract from the cortex of a rat receiving the ketogenic diet (upper) and a spectrum of a control rat (lower). The animals were given an i.p. injection of $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate, and were sacrificed 15 min later. Monolabeled (singlet) metabolites are mainly derived from $[1-^{13}\text{C}]$ glucose whereas double-labeled (doublet) from $[1,2-^{13}\text{C}]$ acetate. Peak assignment: 1, glutamine C-3; 2, glutamate C-3; 3, oxoproline C-4; 4, glutamine C-4; 5, glutamate C-4; 6, succinate C-2/3; 7, GABA C-2; 8, taurine C-2; 9, aspartate C-3; 10, creatine C-2; 11, GABA C-4; 12, *N*-acetyl aspartate C-3; 13, aspartate C-2; 14, *N*-acetyl aspartate C-2; 15, creatine C-4; 16, glutamine C-2; 17, glutamate C-2; 18, glutamine C-5; 19, *N*-acetyl aspartate C-4 and -1; 20, glutamate C-5.

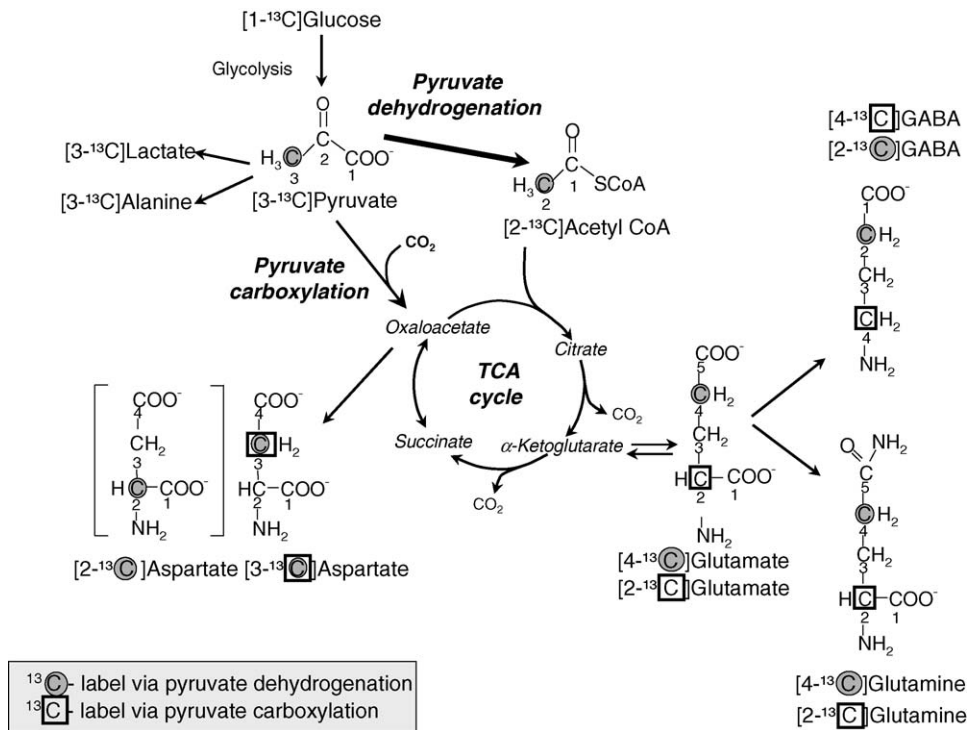


Fig. 3. Schematic representation of possible isotopomers arising from [1-¹³C]glucose from the first turn of the TCA cycle. Via glycolysis, [1-¹³C]glucose is converted to [3-¹³C]pyruvate, which can either be converted to [3-¹³C]lactate, [3-¹³C]alanine or [2-¹³C]acetyl CoA. If [2-¹³C]acetyl CoA condenses with unlabeled oxaloacetate it will after several steps give rise to [4-¹³C]glutamate and subsequently [4-¹³C]glutamine or [2-¹³C]GABA in their respective cell types. Alternatively, in astrocytes [3-¹³C]pyruvate is carboxylated to yield labeled oxaloacetate via pyruvate carboxylation leading to the formation of [2-¹³C]-/[3-¹³C]aspartate, [2-¹³C]glutamate or [2-¹³C]glutamine or [4-¹³C]GABA. A circle around a carbon atom symbolizes ¹³C which has been metabolized through pyruvate dehydrogenase, while a square symbolized ¹³C which has been metabolized via pyruvate carboxylase. Abbreviation: TCA, tricarboxylic acid.

Label in the C-2 position in glutamate and glutamine and in the C-4 position of GABA is not uniquely derived from PC activity. In the second turn of the TCA cycle equal amounts of label in the C-2 and -3 positions of glutamate and glutamine and C-3 and -4 positions of GABA, will be produced from [1-¹³C]glucose. Therefore, when calculating PC activity, label in the C-3 position of glutamate, glutamine and GABA has to be subtracted from the C-2 position for glutamine and glutamate and from the C-4 position for GABA.

An increase in PC over PDH could either be due to an increase in PC activity or a decrease in PDH activity.

[1,2-¹³C]Acetate is converted to [1,2-¹³C]acetyl CoA that enters the TCA cycle, leading to the formation of [4,5-¹³C]glutamate or [4,5-¹³C]glutamine or [1,2-¹³C]GABA (Fig. 4). Labeled oxaloacetate can be transaminated to [2,3-¹³C]-/[3,4-¹³C]aspartate.

Pyruvate recycling takes place when TCA cycle intermediates oxaloacetate or malate are decarboxylated to pyruvate and the resulting pyruvate re-enters the TCA cycle as acetyl CoA. Malic enzyme converts malate to pyruvate generating NAD(P)H and CO₂. The action of the two enzymes phosphoenolpyruvate carboxykinase and pyruvate kinase converts oxaloacetate into pyruvate. Pyruvate recycling from [1,2-¹³C]acetate can be detected if labeled oxaloacetate or malate are converted to [3-¹³C]pyruvate or [1,2-¹³C]pyruvate. If [1,2-¹³C]pyruvate is dehydrogenated to [1-¹³C]acetyl CoA, which re-enters the TCA cycle, [5-¹³C]glutamate or [5-¹³C]glutamine may be formed. Pyruvate recycling of [3-¹³C]pyruvate will give rise to [4-¹³C]glutamate and [4-¹³C]glutamine. However, it is not possible to differentiate pyruvate recycling which occurs from [3-¹³C]pyruvate from TCA metabolism of [3-¹³C]pyruvate, directly derived from [1-¹³C]glucose, because both give rise to [4-¹³C]glutamate and [4-¹³C]glutamine. Pyruvate recycling from [1-¹³C]glucose as a precursor will also lead to label in the C-5 position of glutamate and glutamine. Thus, by calculating the percent enrichment in the C-5 positions of glutamate and glutamine it is possible to detect pyruvate recycling obtained from [1-¹³C]glucose and [1,2-¹³C]acetate. The amount of GABA is too small to give quantifiable peaks for

measuring pyruvate recycling. Percent enrichment in glutamate C-5 was calculated from the following formula:

$$\% \text{ enrichment} = \frac{([5 - ^{13}\text{C}] \text{glutamate} - 1.1\%[\text{glutamate}]) \times 100}{[\text{glutamate}]}$$

where [glutamate] represents the total amount of glutamate measured by ¹H NMR spectroscopy. The amount of [5-¹³C]glutamate was determined by comparing the integral of [5-¹³C]glutamate with the integral of [4,5-¹³C]glutamate in the C-5 position since the amount of [4,5-¹³C]glutamate is the same in the C-5 as in the C-4 position and the latter is known. The same type of calculation is also valid for glutamine.

In this study, [1-¹³C]glucose and [1,2-¹³C]acetate are used to analyze neuronal and astrocytic metabolism, respectively. Therefore, the correct interpretation of these data depends on whether glucose (at the acetyl CoA level) is metabolized more in the neuronal TCA cycle than in the astrocytic one and whether acetate is selectively taken up by astrocytes and metabolized in the astrocytic TCA cycle as mentioned above. However, it is conceivable that during exposure to the ketogenic diet, specific uptake and use of metabolic substrates by the two cell types may be shifted. To ensure the correct interpretation of the data, we need to verify that [1-¹³C]glucose and [1,2-¹³C]acetate at the acetyl CoA level are tracers of neuronal and astrocytic TCA cycle metabolites, respectively. In order to evaluate this possibility, the cellular localization of glutamine (astrocytes), glutamate (glutamatergic neurons) and GABA (GABAergic neurons) can be used. By comparing the amounts of [4-¹³C]glutamate to [4-¹³C]glutamine and [2-¹³C]GABA to [4-¹³C]glutamine (all derived from [1-¹³C]glucose, as described above) it is possible to get an indication of the metabolism of [1-¹³C]glucose in neurons and astrocytes both in rats on the conventional diet and on the ketogenic diet. Furthermore, to investigate whether [1,2-¹³C]acetate may also enter neurons during the ketogenic diet, the amounts of [4,5-¹³C]glutamine to [4,5-¹³C]glutamate and [4,5-¹³C]glutamate to [1,2-¹³C]GABA, were compared.

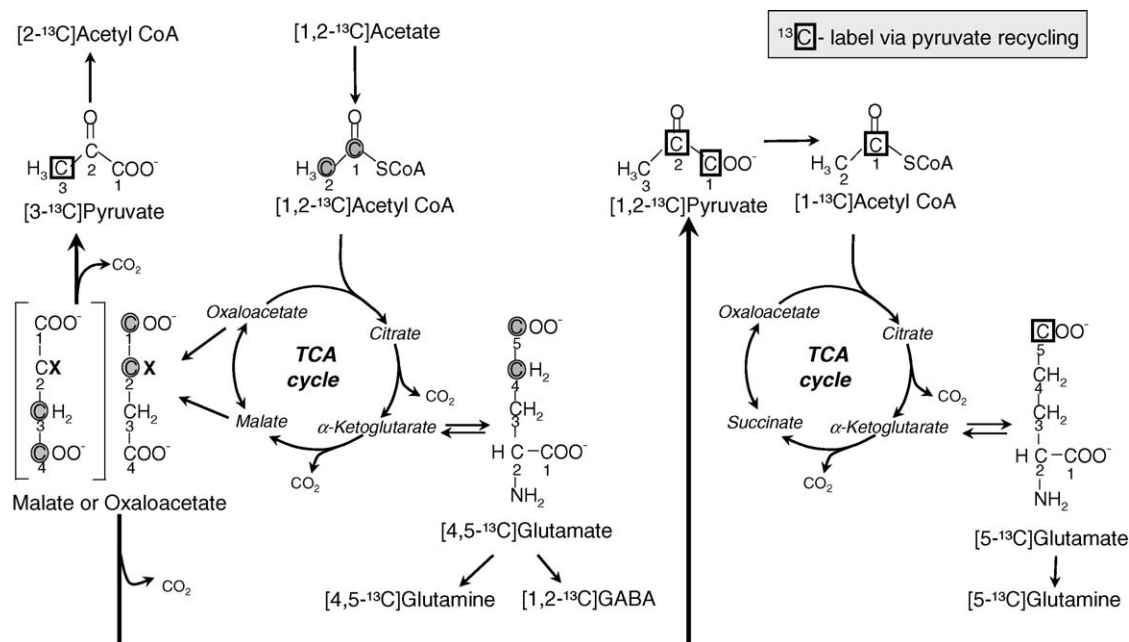


Fig. 4. Schematic representation of possible isotopomers arising from [1,2-¹³C]acetate via the first turn of the TCA cycle as well as isotopomers arising from pyruvate recycling. [1,2-¹³C]Acetyl CoA condenses with unlabeled oxaloacetate resulting in formation of [4,5-¹³C]glutamate, [4,5-¹³C]glutamine or [1,2-¹³C]GABA. Labeled oxaloacetate can be transaminated to [3,4-¹³C]-/[1,2-¹³C]aspartate. Pyruvate recycling from [1,2-¹³C]acetate can be detected if labeled oxaloacetate or malate are converted to [3-¹³C]pyruvate or [1,2-¹³C]pyruvate. If [1,2-¹³C]pyruvate is dehydrogenated to [1-¹³C]acetyl CoA, which re-enters the TCA cycle, [5-¹³C]glutamate or [5-¹³C]glutamine may be formed. One cannot differentiate pyruvate recycling which occurs from [3-¹³C]pyruvate from TCA cycle metabolism of [3-¹³C]pyruvate, directly derived from [1-¹³C]glucose, because both give rise to [4-¹³C]glutamate or [4-¹³C]glutamine. Pyruvate recycling with [1-¹³C]glucose as a precursor will also lead to label in the C-5 position of glutamate and glutamine. The marked carbon atoms symbolize ¹³C. More specifically a circle around a carbon atom symbolizes ¹³C which has been metabolized through the TCA cycle, while the squared marked carbon atom symbolized ¹³C which has been metabolized via pyruvate recycling. Abbreviation: TCA, tricarboxylic acid.

2.6. Data and statistical analysis

Relevant peaks in the ¹³C and ¹H NMR spectra were identified and integrated using XWINNMR software. The amounts of ¹³C were quantified from the integrals of the peak areas, using ethylene glycol as internal standard. Relevant peaks in the ¹H spectra were also integrated using ethylene glycol as an internal standard. All results are expressed as mean ± S.D. of 10 rats on the KD or eight rats on a conventional diet. All concentrations were corrected for tissue weight. Statistical differences between the two groups were analyzed using unpaired two-tailed Student's *t*-test. The level of significance was set at *p* < 0.05.

Table 1
Metabolite concentration in extracts from cortex (μmol/g tissue) and plasma (μmol/ml)

	Cerebral cortex (μmol/g tissue)		Plasma (μmol/ml)	
	Control	KD	Control	KD
β-Hydroxybutyrate ^a	ND	ND	0.12 ± 0.09	0.38 ± 0.17*
Glucose ^a	1.23 ± 0.19	1.54 ± 0.21*	2.10 ± 1.01	4.57 ± 1.66*
[1- ¹³ C]Glucose ^b	0.57 ± 0.06	0.92 ± 0.13*	1.22 ± 1.07	3.06 ± 1.39*
Lactate ^a	1.44 ± 0.19	1.33 ± 0.23	5.95 ± 2.36	5.72 ± 1.90
[3- ¹³ C]Lactate ^b	0.16 ± 0.02	0.12 ± 0.01*	0.28 ± 0.11	0.18 ± 0.08
Alanine ^a	0.31 ± 0.03	0.23 ± 0.03*	0.32 ± 0.09	0.21 ± 0.10
[3- ¹³ C]Alanine ^b	0.03 ± 0.005	0.02 ± 0.004*		
Glutamate ^a	7.00 ± 0.66	6.41 ± 0.61*		
Glutamine ^a	2.80 ± 0.45	2.86 ± 0.50		
GABA ^a	0.88 ± 0.09	0.97 ± 0.17		
Aspartate ^a	1.61 ± 0.12	1.54 ± 0.14		
[2- ¹³ C]Aspartate ^b	0.11 ± 0.01	0.09 ± 0.02*		
[3,4- ¹³ C]Aspartate ^b	ND	0.02 ± 0.002*		

Results are presented as mean ± S.D., *n* = 8–10. The metabolites were measured using either.

^a ¹H NMR spectroscopy.

^b ¹³C NMR spectroscopy.

* *p* < 0.05, Statistically significant difference between KD and control rats.

3. Results

3.1. Metabolites in plasma

The amount of β-hydroxybutyrate was significantly higher in plasma of ketotic compared to control rats (Table 1). Both the levels of unlabeled and [1-¹³C]glucose were increased, whereas the amounts of unlabeled and [3-¹³C]lactate and alanine were unchanged in ketotic rats compared with controls (Table 1).

3.2. Metabolites in cortex

Typical ^{13}C NMR spectra from cortical extracts of both a ketotic and a control rat are presented in Fig. 2. It is clear that the ratio of doublet to singlet in the separate peaks is higher in the rat on the KD compared with the control rat. Labeling of metabolites derived from $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate is presented in Figs. 3 and 4. Metabolic pathways are described in Section 2.

The amounts of $[4-^{13}\text{C}]$ glutamate and $[4-^{13}\text{C}]$ glutamine were decreased in cortex of KD compared with the control rats, whereas that of $[2-^{13}\text{C}]$ GABA was unchanged (Fig. 5A). The concentrations of $[2-^{13}\text{C}]$ - and $[3-^{13}\text{C}]$ aspartate, were lower in ketotic rats than in controls. Since equal amounts of $[2-^{13}\text{C}]$ - and $[3-^{13}\text{C}]$ aspartate are formed, due to the symmetrical succinate step, only $[2-^{13}\text{C}]$ aspartate is presented in Table 1. Levels of $[4,5-^{13}\text{C}]$ glutamate, $[4,5-^{13}\text{C}]$ glutamine, $[1,2-^{13}\text{C}]$ GABA, as well as $[3,4-^{13}\text{C}]$ aspartate from $[1,2-^{13}\text{C}]$ acetate were significantly increased in the cortex of the ketotic animals compared with the control group (Fig. 5B and Table 1).

PC/PDH ratios for glutamate, glutamine and GABA are shown in Fig. 6A (for calculations see Section 2). An increase in this ratio was seen for glutamine in ketotic compared with

control rats ($p = 0.002$). Pyruvate recycling (Fig. 4) was detected in glutamate and glutamine in both groups of rats, but this pathway was only increased for glutamate in rats on the KD compared with controls ($p = 0.04$) (Fig. 6B). Pyruvate recycling was higher in glutamine than in glutamate for both groups of animals.

The $[4-^{13}\text{C}]$ glutamate/ $[4-^{13}\text{C}]$ glutamine ratio was significantly ($p = 0.006$) higher in ketotic (4.3 ± 0.4) than in control animals (3.9 ± 0.1), as was the $[2-^{13}\text{C}]$ GABA/ $[4-^{13}\text{C}]$ glutamine ratio ($p = 0.001$) in ketotic (0.44 ± 0.05) and in control rats (0.36 ± 0.01). However, the $[4,5-^{13}\text{C}]$ glutamate/ $[4,5-^{13}\text{C}]$ glutamine ratio was similar in ketotic (1.93 ± 0.16) and control animals (1.79 ± 0.12). The same was true for the $[4,5-^{13}\text{C}]$ glutamine/ $[1,2-^{13}\text{C}]$ GABA ratio, which was 20.58 ± 3.91 in ketotic rats and 19.51 ± 3.67 in controls.

The amounts of unlabeled and $[1-^{13}\text{C}]$ glucose were increased, whereas the amounts of unlabeled and $[3-^{13}\text{C}]$ alanine were decreased in KD rats compared with controls (Table 1). The amount of unlabeled lactate was unchanged while that of $[3-^{13}\text{C}]$ lactate was decreased in ketotic animals compared with the control group. The level of glutamate was decreased in cortex of ketotic rats compared with controls, whereas those of glutamine, GABA and aspartate were similar.

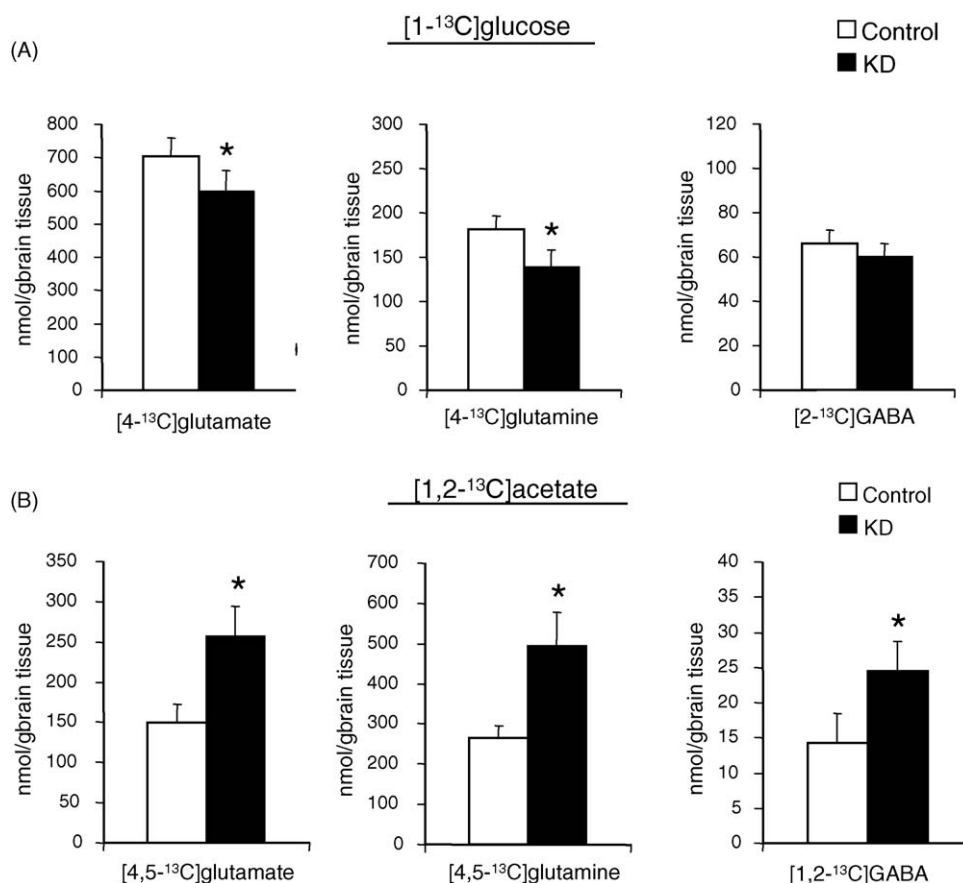


Fig. 5. Concentration of ^{13}C -labeled metabolites derived from $[1-^{13}\text{C}]$ glucose and $[1,2-^{13}\text{C}]$ acetate in the cerebral cortex in brain extracts from rats on the ketogenic diet (KD) and controls. ^{13}C -Labeled metabolites from $[1-^{13}\text{C}]$ glucose derived from α -ketoglutarate from the first turn of the TCA cycle are given in (A), while products of $[1,2-^{13}\text{C}]$ acetate metabolism from the first turn of the TCA cycle are given in (B). The amounts of ^{13}C (nmol/g tissue) were analyzed with ^{13}C NMR spectroscopy and were not corrected for naturally abundant ^{13}C (1.1%). Data represent mean \pm S.D. of 10 KD rats and eight control rats. * $p < 0.05$, statistically significant difference between KD rats and control rats.

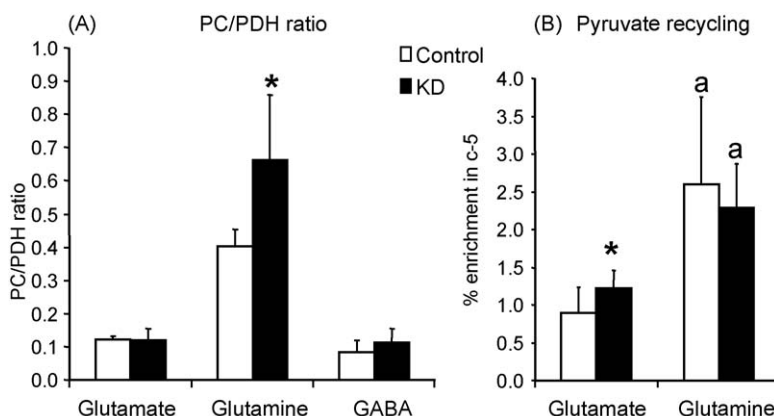


Fig. 6. (A) Pyruvate carboxylation over pyruvate dehydrogenation (PC/PDH) ratios for glutamate, glutamine and GABA in the cortex of rats receiving the ketogenic diet (KD) compared with control rats. (B) Pyruvate recycling in glutamate and glutamine detected as % enrichment in the C-5 position of glutamate and glutamine. The PC/PDH ratios and pyruvate recycling were calculated as described in Section 2. Data represent mean \pm S.D. of 10 KD rats and eight control rats. * $p < 0.05$, statistically significant difference between KD and control rats. ^a statistically significant different from glutamate in the same group of animals.

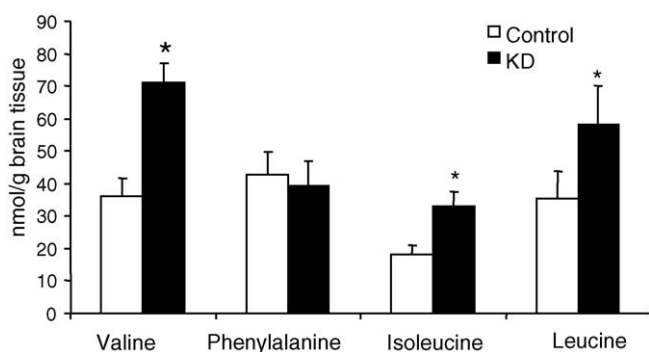


Fig. 7. Branched chain amino acid concentrations (nmol/g brain tissue) in cortex of rats receiving the ketogenic diet (KD) compared with control rats, measured by HPLC. Data represent means \pm S.D. of 10 KD rats and eight control rats. * $p < 0.05$, statistically significant difference between the two groups.

The amounts of the branched chain amino acids, valine, isoleucine and leucine were increased in ketotic rats compared with the control group (Fig. 7).

4. Discussion

As glycolysis and oxidative glucose metabolism both decrease during ketosis, alternative metabolic substrates increase their contribution to cerebral metabolism. Adaptive changes allow the switch from glucose to ketone bodies as the major brain substrates. In the present study, astrocytic metabolism was increased in ketosis, as evidenced both by increased use of [1,2- ^{13}C]acetate and increased pyruvate carboxylation. Additionally, decreased glutamate content was found in cortex.

4.1. Plasma levels of glucose and β -hydroxybutyrate

To ensure that the rats were in a ketotic state, the amount of β -hydroxybutyrate in blood was measured and was found to be significantly higher in rats on the KD compared with the controls. This is in agreement with many other reports (Al-Mudallal et al., 1996; Leino et al., 2001). Glucose levels,

on the other hand, have been reported to be decreased (DeVivo et al., 1978; Yudkoff et al., 2005) but mostly unchanged in plasma of adult rodents subjected to the KD (Al-Mudallal et al., 1996; Leino et al., 2001). In the present study, where a bolus injection of 0.3 M glucose (1 ml/kg) was given, labeled and unlabeled plasma glucose concentration was increased. The increase in unlabeled glucose must be due to altered metabolism in body tissues where ketone bodies are taken up and metabolized to a much higher degree than glucose. Thus, [1- ^{13}C]glucose extraction from the blood is most likely lowered, leading to the observed increased plasma [1- ^{13}C]glucose concentration. It is unlikely that the 5% increase in body weight in the ketotic rats causing a 5% increase in [1- ^{13}C]glucose injection could be the cause of more than doubling the blood [1- ^{13}C]glucose concentration.

4.2. Neuronal glucose metabolism was decreased

The amount of unlabeled and [1- ^{13}C]glucose was increased in the brain of rats receiving the KD. This might be caused by a slight increase in the number of glucose transporters which has been reported on brain endothelial cells in ketosis (Leino et al., 2001). However, in our study the blood-to-brain ratio for glucose was similar in rats receiving the KD and controls, confirming the regulating role of glucose concentration in the blood as a control for glucose entry into the brain (Paulson, 2002).

In addition, the increased glucose concentration could reflect reduced glycolysis. Even though the direct product of glycolysis, pyruvate, could not be measured, decreased glycolysis was evidenced by the reduced amounts of [3- ^{13}C]alanine and [3- ^{13}C]lactate from [1- ^{13}C]glucose. The amount of unlabeled alanine was decreased, whereas that of lactate was unchanged in the present study. These data are in line with the report of reduced glucose metabolism in brain of immature rats in a state of natural ketosis (Nehlig and Pereira de Vasconcelos, 1993). However, they are in contrast with data from Yudkoff et al. (2001) who found increased brain lactate concentration in mice on the KD. The discrepancy between the latter study and

the present one could be due to the difference in the period of exposure to the KD. Yudkoff et al. (2001) kept the animals on the KD for 3 days only, whereas we subjected GAERS to the KD for 21 days. It may not be certain that, after 3 days of exposure, all the mechanisms that underlie the switch from predominantly glucose to predominantly ketone body metabolism are fully operative, while this might be the case by 21 days. Indeed, when considering the temporal evolution of blood glucose concentration in animals on the KD, there is an initial decrease followed by a return to normal levels by 21 days (Leino et al., 2001). The same temporal evolution in blood glucose levels was found in GAERS exposed to the KD (Nehlig, unpublished data).

Oxidative metabolism of glucose (via the TCA cycle) was decreased in ketotic rats compared with controls. It is well known that high amounts of acetyl CoA, which in ketosis is derived largely from ketone bodies, inhibits pyruvate dehydrogenase and thus glucose access to the TCA cycle. This was evidenced by decreased amounts of [4-¹³C]glutamate and [4-¹³C]glutamine derived from [1-¹³C]glucose. Reduced oxidative metabolism of [1-¹³C]glucose in ketosis has also been reported by Yudkoff et al. (2005).

In addition to glutamate labeling, the amount of glutamate was also decreased in ketosis. This might directly reflect decreased glucose utilization. In cell cultures, it has been shown that only glucose, but not lactate, could maintain glutamate levels (Waagepetersen et al., 1998). In contrast, the amount and labeling of GABA from [1-¹³C]glucose were unchanged indicating that relatively more glutamate is converted to GABA, possibly as a result of increased glutamate release from the synaptic area and uptake into GABAergic neurons. This hypothesis is supported by the observation that β -hydroxybutyrate increases synaptosomal glutamate and GABA concentration (Erecinska et al., 1996). However, in GAERS fed a KD, we did not record any change in GABA concentration.

Glucose, after conversion to acetyl CoA, is metabolized more in the neuronal than the astrocytic TCA cycle (Qu et al., 2000) and is therefore mostly a neuronal marker. However, prolonged ketosis might alter cell type preferences, and changes in neuronal and glial properties, as hypothesized by Schwartzkroin (1999). In order to evaluate this possibility, labeling ratios can be used. [1-¹³C]Glucose labels [4-¹³C]glutamate mostly in neurons which can give rise to [4-¹³C]glutamine in astrocytes. Additionally, [4-¹³C]glutamine can be labeled from [1-¹³C]glucose in astrocytes. By comparing the amounts of [4-¹³C]glutamate to [4-¹³C]glutamine and [2-¹³C]GABA to [4-¹³C]glutamine it is possible to get an indication of the metabolism of [1-¹³C]glucose in neurons and astrocytes. The glutamate/glutamine ratio was significantly higher in ketotic than in control animals. This indicates that the [4-¹³C]glutamate level is relatively higher than the [4-¹³C]glutamine level and thus, that acetyl CoA from glucose is mainly metabolized in neurons also in ketosis. The same was true for GABAergic neurons, where the [2-¹³C]GABA to [4-¹³C]glutamine ratio was significantly higher in ketotic than in control rats.

4.3. Increased astrocytic metabolism

Some [1-¹³C]glucose is also metabolized in astrocytes as evidenced by labeling via pyruvate carboxylase (Fig. 3), which is a glial-specific anaplerotic enzyme, providing oxaloacetate for the TCA cycle (Shank et al., 1985). The PC/PDH ratio for glutamine was increased in ketotic rats. Since PDH activity is down regulated, the increased ratio indicates that pyruvate carboxylase activity is either increased or unchanged. Thus, relatively more label from [1-¹³C]glucose goes through pyruvate carboxylation in ketotic animals compared with controls. This can be explained by increased activation of PC by larger amounts of acetyl CoA from breakdown of ketone bodies. PC is activated allosterically by acetyl CoA. Pyruvate carboxylation appears to be critically involved in brain metabolism during ketosis. Indeed, KD was reported to have induced coma in a patient with pyruvate carboxylase deficiency (DeVivo et al., 1977). Pyruvate carboxylase is important for the modulation of the fractional distribution of intracellular acetyl CoA between the TCA cycle, the β -hydroxy- β -methyl-glutaryl CoA (linked to the synthesis of ketone bodies and cholesterol), and fatty acid synthesis (DeVivo et al., 1977). The present study confirms the activation of pyruvate carboxylation in GAERS fed the KD.

In astrocytes, [1,2-¹³C]acetate is converted to acetyl CoA. In ketotic animals, acetate metabolism was more active than in controls, as reflected by increased amounts of [4,5-¹³C]glutamate, [4,5-¹³C]glutamine, [1,2-¹³C]GABA and [3,4-¹³C]aspartate. Similar results were obtained by Yudkoff et al. (2004) who proposed that acetate uptake into the brain and intracellular processing was increased. It can be hypothesized that in the ketotic state, [1,2-¹³C]acetate may also enter neurons. By comparing the amounts of [4,5-¹³C]glutamine to [4,5-¹³C]glutamate and [4,5-¹³C]glutamine to [1,2-¹³C]GABA, it is possible to get information about the cellular specificity of [1,2-¹³C]acetate metabolism. The ratios were not altered in ketosis, implying that [1,2-¹³C]acetate only entered astrocytes and can therefore be used as a marker of astrocytic metabolism in ketosis.

4.4. Increased pyruvate recycling

Pyruvate recycling is the opposite of pyruvate carboxylation in the sense that one part of pyruvate recycling is decarboxylation of oxaloacetate or malate to form pyruvate (Fig. 4).

This pyruvate can be used for gluconeogenesis, lactate or alanine production, or re-enter into the TCA cycle after conversion to acetyl CoA. Hassel and Sonnewald (1995) have shown lactate formation via this pathway. In the present study, labeling of alanine or lactate from malate or oxaloacetate was not observed. However, re-entry of pyruvate via acetyl CoA into the TCA cycle was detected in [5-¹³C]glutamate and [5-¹³C]glutamine in all animals (for details see Section 2 and Fig. 4). Pyruvate recycling was first demonstrated in glutamate in brain by Kunnecke et al. (1993) and later by others (Aureli

et al., 1997; Haberg et al., 1998; Waagepetersen et al., 2002). In our study more recycling was detected in glutamine than in glutamate in both groups of animals, and increased pyruvate recycling was observed in glutamate in ketotic animals. This could be a consequence of the increase in the anaplerotic pathway, pyruvate carboxylation since pyruvate recycling allows the elimination of the excess oxaloacetate formed during carboxylation.

4.5. Functional implications for the use of the KD in epilepsy

The mechanism of action of the KD in controlling epilepsy is still not clarified. In the present study, total brain concentration of glutamate was decreased as a response to the KD. This may be part of the efficacy of the KD against seizures. Increased amounts of the branched chain amino acids valine, leucine and isoleucine were found in the brain of rats receiving the KD. This can be due to increased transport from the blood, since the concentration of these amino acids has been shown to be elevated in human plasma in ketosis (Sherwin, 1981). Leucine enters the brain via the L neutral amino acid transport system which primarily acts as an exchange system counter-transporting cerebral glutamine back to the blood (Boado et al., 1999). As pointed out by Yudkoff et al. (2004), the extraction of glutamine to the blood corresponds to a release of glutamate from the brain since glutamine is formed from glutamate in astrocytes. This decrease in glutamate concentration, together with an increase of GABA in some models (Cheng et al., 2004; Yudkoff et al., 2004), but not in the present study, would be in favor of a better control of brain excitability, and hence of epileptic seizures. However, as mentioned before, a 21 days exposure to the KD was unable to affect the expression of spike-and-wave discharges in GAERS (Nehlig, unpublished data). The reason why the KD is not effective in this model of nonconvulsive absence epilepsy is unclear.

In summary, the results of the present study show the adaptations of brain metabolism during ketosis at the level of both neurons and glial cells. As expected, both glycolysis and neuronal oxidative glucose metabolism is decreased while astrocytic acetate metabolism is increased. In addition pyruvate recycling and astrocytic pyruvate carboxylation is increased in animals fed a KD.

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