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A ketogenic diet increases succinic dehydrogenase (SDH) activity and recovers age-related decrease in numeric density of SDH-positive mitochondria in cerebellar Purkinje cells of late-adult rats

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ABSTRACT

Ketogenic diets (KDs) have been applied in the therapy of paediatric epilepsy for nearly a century. Recently, beneficial results have also been reported on metabolic disorders and neurodegeneration, designating aged individuals as possible recipients. However, KDs efficacy decrease after the suckling period, and very little is known about their impact on the aging brain. In the present study, the effect on the neuronal energetic supply of a KD containing 20% of medium chain triglycerides (MCT) was investigated in Purkinje cells of the cerebellar vermis of late-adult (19-month-old) rats. The animals were fed with the KD for 8 weeks, and succinic dehydrogenase (SDH) activity was cytochemically determined. The following parameters of SDH-positive mitochondria were evaluated by the use of a computer-assisted image analysis system connected to a transmission electron microscope: numeric density (Nv), average volume (*V*), volume density (Vv), and cytochemical precipitate area/mitochondrial area (*R*). Young, age-matched, and old animals fed with a standard chow were used as controls. We found significantly higher Nv in MCT-KD-fed rats vs. all the control groups, in young vs. late-adult and old controls, and in late-adult vs. old controls. *V* and Vv showed no significant differences among the groups. *R* was significantly higher in MCT-KD-fed rats vs. all the control animals, and in old vs. young and late-adult controls. Present data indicate that the ketogenic treatment counteracted age-related decrease in numeric density of SDH-positive mitochondria, and enhanced their metabolic efficiency. Given the central role of mitochondrial impairment in age-related physio-pathological changes of the brain, these findings may represent a starting point to examine novel potentialities for KDs.

1. Introduction

Classic ketogenic diets (KDs) are high-fat and low-carbohydrate alimentary regimens that derive their name from the capability of inducing systemic ketosis. They stimulate the hepatic 3-hydroxy-3-methylglutaryl-CoA pathway leading to the synthesis of ketone bodies (KB, i.e. aceto-acetate, β-hydroxybutyrate (βHB), and acetone), which are entirely released in the blood because the liver lacks the enzymes necessary to use them as energetic substrates. KDs have been applied in the therapy of several forms of paediatric epilepsy for nearly a century (Zupec-Kania and Spellman, 2008), and in the last decades positive results have also been obtained in hypoxia–ischemia (Puchowicz et al., 2008; Tai et al., 2008), defects of the brain glucose transporters (Klepper, 2008), traumatic brain injuries (Hu et al., 2009; Prins, 2008), brain cancer (Seyfried et al., 2008; Skinner et al., 2009; Zhou et al., 2007), as well as neurodegeneration (Gasior et al., 2006; Kashiwaya et al., 2000; Maalouf et al., 2009; Noh et al., 2003; Van der Auwera et al., 2005). The latter achievement designates aged individuals as possible recipients of KDs, but major concerns derive from age-dependent differences in their effectiveness.

Several studies have shown that the adult central nervous system (CNS) is less reactive to a ketogenic dietetic regimen than the young one (Appelberg et al., 2009; Bough et al., 1999; Prins

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et al., 2005; Prins and Houvada, 2009; Stafstrom, 1999). Besides, several factors influencing cerebral KB metabolism already start decreasing after the suckling period, including the amount of the proton-coupled monocarboxylic acid transporter protein 1 (MCT1) at the blood–brain barrier, and the activity of the three mitochondrial enzymes, which catalyze KB utilization (3-hydroxybutyrate dehydrogenase, succynil-CoA-3oxoacid-CoA transferase, and mitochondrial acetoacetyl-CoA thiolase). During aging, the plastic reactivity needed to face the metabolic challenge may be even more impaired, but only a few data are available on this matter. Recently, we have examined the consequences of two medium chain triglycerides (MCT)-KDs on the ultrastructural features of hippocampal and cerebellar synapses and synaptic mitochondria of late-adult rats, and we have provided the first evidence that the ketogenic approach offers promising potentialities along with not negligible risks: the effects may be even opposite in different brain regions, depending critically on neuronal vulnerability to age and on daily ketogenic intake (Balietti et al., 2008; Balietti et al., 2009b). Further investigation in aged animals is required to evaluate whether positive results found in younger organisms can be at least partly replicated, and whether potential side-effects associated with the therapeutic use can be reduced.

The CNS is characterized by a very intense metabolism, and energy impairments are known to play a key role in its age-related physio-pathological changes (Bertoni-Freddari et al., 2006; Calabrese et al., 2001; Sas et al., 2007). Since KB are able to improve the cellular metabolic efficiency (Cahill and Veech, 2003; Nylen et al., 2009; Sato et al., 1995; Veech et al., 2001), it is important to consider whether this property is maintained in aged individuals, in order to assess its possible application in the prevention and in the therapy of age-related brain diseases.

Aim of the present study was to investigate the effect of a MCT-KD on parameters related to the energetic supply in the aging brain. Late-adult (19-month-old) rats were fed for 8 weeks with the ketogenic regimen, and succinic dehydrogenase (SDH) activity (an important marker of the mitochondrial capability to provide ATP, being part of both the Krebs cycle and the respiratory chain) was cytochemically revealed in Purkinje cells (PC) of cerebellar vermis using the copper ferrocyanide method. Young, age-matched, and old animals fed with a standard chow were used as controls. Since PC are large neurons that have very high metabolic needs and it is known that largest neurons are preferentially and more severely affected by aging (Rogers et al., 1984), they can be considered a good model to study the energetic potentialities of KDs during advancing aging.

2. Materials and methods

2.1. Animals

Eight late-adult (19-month-old) male Wistar rats from the breeding colony of INRCA (Italian National Research Centres on Aging, Ancona, Italy) were housed 1/cage, divided into two groups (average body weight pair-matched), and fed ad libitum with different dietary regimens for 8 weeks: MCT-KD-fed animals had a diet with a 20% content of MCT (Neobee 895, Stepan, Inc., Northfield, IL, USA), whereas control animals were fed with the standard commercial chow 25/18 from Charles River, Milano, Italy (25/18CR). The detailed composition of the two diets is shown in Table 1. Four young (5-month-old) and 4 old (26–27-month-old) rats fed with 25/18CR were used as further controls. The animals were treated in accordance with the European Community guidelines on animal care.

Table 1
Percent composition of 25/18CR and MCT-KD.

Calculated values (%)	25/18CR	MCT-KD
Carbohydrate	54.0	38.2
Protein	18.5	19.0
Fat	5.0	7.0
MCT	–	20.0
Fiber	4.5	5.0
Ash	6.0	2.8
Moisture	12.0	8.0

2.2. Seric βHB concentration

To verify the ketogenic efficacy of the MCT-KD, approximately 250 μl of blood were collected from the tail vein of late-adult MCT-KD-fed and control animals: (i) before the beginning of the treatment (week 0), (ii) once a week during the first 4 weeks, and (iii) every 2 weeks in the last period to reduce the vessel trauma. All the drawings were done at the same time of the day (between 9.00 and 10.00 a.m.). The blood samples were maintained 1 h at 37 °C, then centrifuged at 17,900 × *g* for 10 min at 4 °C to separate the serum. Seric βHB levels were measured using a colorimetric commercial kit (β-hydroxybutyrate Liqui Color, Stanbio Laboratory).

2.3. Cytochemical analysis

The animals were anaesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (200 mg/kg body weight) and sacrificed through decapitation. The cerebellar vermis was excised, immediately sectioned into thin slices, and treated following the copper ferrocyanide method (Kerpel-Fronius and Hajos, 1968). Fresh tissue pieces were incubated for 45 min at 37 °C in the reaction medium as previously described (Fattoretti et al., 1998; Bertoni-Freddari et al., 2001), then washed, post-fixed in 1% osmium tetraoxide, dehydrated, and embedded in Durcupan resin according to conventional electron microscopic procedures. The ultrathin sections (60 nm thickness) were contrasted by lead citrate and uranyl acetate (Fig. 1). The method is based on the reduction of ferricyanide to ferrocyanide by SDH activity and on the coupled capture of ferrocyanide by copper. The granular reaction product (copper ferrocyanide) is highly electron opaque and is confined exclusively to the mitochondrial membranes. The use of a chelating agent in the incubating medium prevents the diffusion of the dark spots and guarantees their precise localization at the site of SDH activity. According to the 'equal opportunity rule' (Coggeshall and Lekan, 1996), a systematic random sampling of SDH-positive mitochondria was carried out in the perikaryon of PC, for an overall area of 667.10 μm^2/animal. The operators were blind to the experimental groups. The parameters of SDH-positive mitochondria taken into account were: the numeric density (Nv: number of mitochondria/μm^3 of tissue), the average volume (*V*), and the volume density (Vv: overall volume of mitochondria/μm^3 of tissue).

The following morphometric formulas were applied to calculate the ultrastructural indexes: Nv = ((number of mitochondria/sampled area)^3/(total mitochondrial area/sampled area))$^{0.5}$ × 0.6369; Vv = total mitochondrial area/sampled area; $V = Vv/Nv_m$ (Bertoni-Freddari et al., 1993). Moreover, the ratio (*R*) between the overall area of the cytochemical precipitate and the mitochondrial area was calculated: the parameter estimates the fraction of the internal mitochondrial membrane involved in SDH activity (Bertoni-Freddari et al., 2001). Finally, a quartile analysis of *R* was performed by ordering the mitochondrial areas by increasing value, matching the corresponding *R*, and dividing the data obtained in the four groups.