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REDUCED SEVERITY OF ISCHEMIC STROKE AND IMPROVEMENT OF MITOCHONDRIAL FUNCTION AFTER DIETARY TREATMENT WITH THE ANAPLEROTIC SUBSTANCE TRIHEPTANOIN

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Abstract—Triheptanoin, an oily substance, consists of glycerol bound to three molecules of heptanoic acid, a C7 odd-chain fatty acid. A triheptanoin-rich diet has anaplerotic effects because heptanoate metabolism yields succinate which delivers substrates to the Krebs cycle. While previous studies on the effects of triheptanoin focused on metabolic disorders and epilepsy, we investigated triheptanoin's effect on ischemic stroke. Mice were fed a triheptanoin-enriched diet for 14 days; controls received soybean oil. Only mice fed triheptanoin had measurable quantities of odd-numbered fatty acids in the plasma and brain. Transient ischemia was induced in the brain by occlusion of the middle cerebral artery (MCAO) for 60 min. One day later, mice were tested for neurological function (chimney, rotarod and corner tests) which was found to be better preserved in the triheptanoin group. Microdialysis demonstrated that the strong, neurotoxic increase of extracellular glutamate, which was observed in the mouse striatum during MCAO, was strongly reduced in triheptanoin-fed mice while glucose levels were not affected. Triheptanoin diet reduced the infarct area in stroked mice by about 40%. In *ex vivo*-experiments with isolated mitochondria, ischemia was found to cause a reduction of mitochondrial respiratory activity. This reduction was attenuated by triheptanoin diet in complex II and IV. In parallel measurements, ATP levels and mitochondrial membrane potential were reduced in control animals but were preserved in triheptanoin-fed mice. We conclude that triheptanoin-fed mice which sustained an experimental stroke had a significantly improved neurological outcome. This beneficial effect is apparently due to an improvement of mitochondrial function and preservation of the cellular energy state. Our findings identify triheptanoin as a promising new dietary agent for neuroprotection.

Key words: anaplerosis, brain ischemia, heptanoic acid, microdialysis, middle cerebral artery occlusion, mitochondrial respiration.

INTRODUCTION

Stroke is one of the leading causes of death worldwide and the most common cause for long-term care (Lopez et al., 2006; Strong et al., 2007). Although numerous pharmacological options have been investigated, there is a dire lack of neuroprotective agents (Dirnagl et al., 1999). As an alternative option, dietary approaches have been investigated, and the ketogenic diet – which is successfully used for the treatment of childhood epilepsy – has shown promising effects in experimental models of ischemia (Gibson et al., 2005; Gasior et al., 2006). The strict regimen of the ketogenic diet, however, is poorly accepted by adults. In the experiments described in this paper, we tested potential neuroprotective effects of a different (and more palatable) dietary approach, namely the feeding of an anaplerotic diet. In this approach, intermediates of the citric acid cycle (CAC) are produced from dietary compounds which may affect mitochondrial metabolism in the brain leading to a replenishment of the CAC during ischemia, especially when CAC intermediates such as alpha-ketoglutarate or succinate are removed for the synthesis of large amounts of glutamate or GABA (Haberg et al., 2009).

Triheptanoin, chemically glycerol triheptanoate, is an oil which has anaplerotic actions (Brunengraber and Roe, 2006; Borges and Sonnewald, 2012). It is hydrolyzed to three molecules of heptanoic acid and provides, after ß-oxidation, six molecules of acetyl-CoA and three molecules of propionyl-CoA. Acetyl-CoA is the main supplier for carbon atoms to the Krebs cycle. Propionyl-CoA can fuel the Krebs cycle via its metabolism to methylmalonyl-CoA, succinyl-CoA, and succinate (Brunengraber and Roe, 2006; Borges and Sonnewald, 2012). Succinyl-CoA can also directly be used for energy production by succinate dehydrogenase, which is in the complex II of the mitochondrial respiratory chain (Kinman et al., 2006; Gu et al., 2010). Triheptanoin was previously tested for therapeutic effects in inherited metabolic disorders such as pyruvate carboxylase deficiency, adult polyglucosan body disease or various fatty acid oxidation disorders (Mochel et al., 2005; Roe et al., 2010; Spiekerkoetter et al., 2010), as well as in Huntington's disease (Mochel et al., 2010). Borges and coworkers

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Abbreviations: CAC, citric acid cycle; CCA, common carotid artery; ECA, external carotid artery; ETS, electron transfer system; HBSS, Hanks balanced salts solution; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; MMP, mitochondrial membrane potential; TTC, 2,3,5-triphenyl-tetrazolium chloride.

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described beneficial effects of a triheptanoin-rich diet (35% of calories) in several epileptic models (Willis et al., 2010; Thomas et al., 2012; Kim et al., 2013). To our knowledge, this is the first investigation of the effects of triheptanoin in an experimental stroke model.

EXPERIMENTAL PROCEDURES

Animals and dietary treatment

Female CD-1 mice (18–22 month, 30–35 g, Charles River) were kept under standardized conditions: 12-h-light/dark circle, 22 °C temperature, 70% humidity, food and water available *ad libitum*. All animal procedures were carried out to minimize animal suffering in accordance with German and European law. The study was registered with the local authorities (Regierungspräsidium Darmstadt).

Triheptanoin, originally produced by Sasol (Brunsbüttel, Germany), was obtained from B. Braun (Melsungen, Germany). For the experiments, mice were fed over 14 days either a control diet (containing soybean oil) or the triheptanoin-enriched diet. Both chows were prepared by Ssniff Co. (Soest, Germany). The test diet contained 14% triheptanoin (w/w), which represents about 35% of total caloric intake (crude fat: 16.4%; starch 24.7%). The control chow consisted of the same caloric amount of soybean oil (crude fat: 5.4%; starch 35.7%). The amounts of (crude) protein (18%), fiber (4%), ash (5.5%) and sugar (11%) were identical in both diets, as were the food additives which included vitamins A, C, D_3, E, K_3, and copper.

Measurement of odd-numbered fatty acids

Samples were extracted using the Folch procedure (Folch et al., 1957). The aqueous phase was dried under a stream of nitrogen, and the residue was derivatized to the corresponding trimethylsilyl-ether or ester groups (Kombu et al., 2011) and measured on a GC–MS (Agilent 5973 series). The resulting total ion chromatogram was compared to the N.I.S.T database for qualitative analysis. Quantification was performed with internal and external standards.

Middle cerebral artery occlusion (MCAO)

Transient *in vivo*-ischemia in mouse brain was induced by MCAO as previously described (Schwarzkopf et al., 2013). Briefly, mice were anesthetized with isoflurane (2% in synthetic air) and kept at 37 °C using a thermostatic blanket coupled to a rectal thermometer (Harvard/Hugo Sachs, March-Hugstetten, Germany). Through a cervical incision, the left bifurcation of the common carotid artery (CCA) was dissected and all three branches (CCA, external carotid artery (ECA) and internal carotid artery (ICA)) were ligated. A 20-mm monofilament (Doccol, Redlands, California; size 6–0) was inserted into the ECA and gently advanced through the ICA into the brain until its tip occluded the origin of the left middle cerebral artery (MCA). Local cerebral blood flow was measured by laser Doppler flowmetry (Moor Instruments, Devon, UK; AP −0.5, L +3.5 from bregma) and dropped to 10–15% of basal flow during occlusion. After 60 min of occlusion, the filament was removed to allow reperfusion (defined as >50% of basal flow). The skin incision was closed with surgical clips, treated with local anesthetics, and mice were allowed to recover in their home cages. As analgesic agent, mice received buprenorphine (0.1 mg s.c.) immediately after wound closure and again eight hours later.

Behavioral testing

Neurological deficits were determined by behavioral testing in the morning before surgery and 24 h after MCAO. The ‘‘Chimney test’’ (modified from Heinecke, 1987) was performed for each mouse three times before and after surgery. A mouse was placed head in front at the entry of a tube (200 mm long and 20-mm diameter). When the mouse reached the bottom of the tube, the tube was raised to an angle of 45 degree. All mice responded by walking backward. The time needed to climb out of the tube was measured for a maximum of 120 s. In the ‘‘Rotarod test’’ (Jones and Roberts, 1968), all mice were forced to walk on an accelerating rod (starting at 4 rpm, increase of 4 rpm every 15 s). The time the mouse was able to walk on the rod before falling was measured (maximum value 120 s). The occurrence of two consecutive passive rotations without walking but clinging to the rod was considered as a fall. Each mouse was tested three times a day. The ‘‘Corner test’’ was used as described (Zhang et al., 2002). Mice were placed in a corner (30 degree angle) and the chosen sides to leave the corner were counted. Each mouse was tested for one trial (maximum time 120 s) before and after surgery. The laterality index (LI) was calculated: (left turns − right turns)/total number of turns (Bouet et al., 2007).

Microdialysis

For microdialysis experiments, a self-made dialysis probe was implanted into the left mouse striatum as described before (Schwarzkopf et al., 2013) one day prior to MCAO. Mice were anesthetized with isoflurane (2% in synthetic air) and placed in a stereotaxic frame. The probe (exchange length: 2.5 mm, cut-off 10 kDa) was implanted using the following coordinates from the bregma: AP +0.5 mm; L +2.2 mm; DV −3.8 mm (Franklin and Paxinos, 1997) and fixed at this position with Multilink Automix (Ivoclar Vivadent AG, Schaan, Liechtenstein). The wound was treated with local anesthetic (bupivacaine), and mice were allowed to recover over night in their home cages. Perfusion of the microdialysis probe was started 1 h before MCAO, was sustained while the MCAO surgery was performed and was continued for 2 h after MCAO. The perfusion fluid was artificial cerebrospinal fluid (aCSF: 147 mM NaCl; 4 mM KCl; 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$), the perfusion rate was 2 µl/min, and samples were collected in 10-min intervals. 24 h after MCAO, mice were deeply anesthetized with isoflurane and euthanized by decapitation. Brains were quickly removed, sectioned coronally into 1-mm slices and stained with 2,3,5-triphenyl-tetrazolium chloride (TTC). Images were acquired by a DinoLite camera