Neuroscience 145 (2007) 256–264

KETONES INHIBIT MITOCHONDRIAL PRODUCTION OF REACTIVE OXYGEN SPECIES PRODUCTION FOLLOWING GLUTAMATE EXCITOTOXICITY BY INCREASING NADH OXIDATION

M. MAALOUF,[a] P. G. SULLIVAN,[b] L. DAVIS,[b] D. Y. KIM[a] AND J. M. RHO[a]*

[a]*Neurology Research, NRC 4th Floor, Barrow Neurological Institute and St. Joseph's Hospital and Medical Center, 350 West Thomas Road, Phoenix, AZ 85013, USA*

[b]*Spinal Cord and Brain Injury Research Center, Department of Anatomy and Neurobiology, University of Kentucky, 240 Health Sciences Research Building, Lexington, KY 40536, USA*

Abstract—Dietary protocols that increase serum levels of ketones, such as calorie restriction and the ketogenic diet, offer robust protection against a multitude of acute and chronic neurological diseases. The underlying mechanisms, however, remain unclear. Previous studies have suggested that the ketogenic diet may reduce free radical levels in the brain. Thus, one possibility is that ketones may mediate neuroprotection through antioxidant activity. In the present study, we examined the effects of the ketones β-hydroxybutyrate and acetoacetate on acutely dissociated rat neocortical neurons subjected to glutamate excitotoxicity using cellular electrophysiological and single-cell fluorescence imaging techniques. Further, we explored the effects of ketones on acutely isolated mitochondria exposed to high levels of calcium. A combination of β-hydroxybutyrate and acetoacetate (1 mM each) decreased neuronal death and prevented changes in neuronal membrane properties induced by 10 μM glutamate. Ketones also significantly decreased mitochondrial production of reactive oxygen species and the associated excitotoxic changes by increasing NADH oxidation in the mitochondrial respiratory chain, but did not affect levels of the endogenous antioxidant glutathione. In conclusion, we demonstrate that ketones reduce glutamate-induced free radical formation by increasing the NAD^+/NADH ratio and enhancing mitochondrial respiration in neocortical neurons. This mechanism may, in part, contribute to the neuroprotective activity of ketones by restoring normal bioenergetic function in the face of oxidative stress.

Key words: glutamate, neurotoxicity, diet, mitochondria, oxidation, stress.

Calorie restriction can decrease the risk of neurodegenerative disease and protect the brain against acute insults such as stroke (Mattson et al., 2002). Similarly, the ketogenic diet, a high-fat, low-carbohydrate diet created to mimic the effects of calorie restriction, is an extremely efficacious treatment for medically intractable epilepsy (Freeman et al., 1998; Vining et al., 1998). Several metabolic changes occur during calorie restriction and the ketogenic diet, notably an increase in serum concentrations of the ketones β-hydroxybutyrate (BHB) and acetoacetate (ACA) (Gilbert et al., 2000; Koubova and Guarente, 2003; Denny et al., 2006; Mahoney et al., 2006).

Recent work has shown that ketones exert a protective effect on brain. For example, BHB prevents the death of hippocampal neurons exposed to $A\beta_{1-42}$, protects cultured mesencephalic dopaminergic neurons from the toxic effects of 1-methyl-4-phenylpyridinium (MPP^+, an inhibitor of NADH dehydrogenase that increases oxygen radical formation) and reduces brain injury in rodents subjected to glycolysis inhibition and focal or generalized ischemia (Kashiwaya et al., 2000; Suzuki et al., 2001 and 2002). Furthermore, ACA protects hippocampal neurons against glycolysis inhibition *in vivo* and *in vitro* (Massieu et al., 2003). In parallel, clinical data suggest that seizure control in epileptic patients treated with the ketogenic diet correlates with the serum concentration of ketones (Gilbert et al., 2000). The mechanisms underlying the therapeutic effects of ketones remain, however, largely unknown.

Studies in cardiac tissue have suggested that ketones might reduce oxidative stress (Veech et al., 2001), a pathogenic process implicated in many disorders ranging from atherosclerosis and traumatic injuries to diseases more specific to the nervous system (Droge, 2002; Keller et al., 2005). To investigate the antioxidant activity of ketones in neurons, we used a glutamate excitotoxicity model because of its equally important relevance to several neurological diseases (including stroke, epilepsy, trauma and Alzheimer's disease) and because of the well-known fact that glutamate excitotoxicity is associated with higher cellular levels of reactive oxygen species (ROS) (Nicholls, 2004; Sullivan et al., 2005). The antioxidant effects of BHB and ACA were therefore assessed in acutely dissociated neocortical neurons subjected to glutamate excitotoxicity and in isolated neocortical mitochondria exposed to high concentrations of calcium. Our results show that excitotoxic injury is associated with increased mitochondrial production of ROS and that ketones inhibit these deleteri-

*Corresponding author. Tel: +1-602-406-3156; fax: +1-602-406-5779.
E-mail address: jong.rho@chw.edu (J. M. Rho).

Abbreviations: ACA, acetoacetate; BHB, β-hydroxybutyrate; DHE, dihydroethidium; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide p-[trifluoromethoxy]-phenylhydrazone; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; KCN, potassium cyanide; MCB, monochlorobimane; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogenase; NADP(H), nicotinamide adenine dinucleotide phosphate hydrogenase; PI, propidium iodide; R_a, access resistance; R_m, membrane resistance; ROS, reactive oxygen species; V_m, membrane potential.

0306-4522/07$30.00+0.00 © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2006.11.065

ous effects by enhancing NADH oxidation in the mitochondrial respiratory chain.

EXPERIMENTAL PROCEDURES

All protocols were approved by the Barrow Neurological Institute and the University of Kentucky Institutional Animal Care and Use Committees. Extreme care was taken to minimize the number of animals used and their suffering. Chemical products were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Fluorescent indicators were purchased from Molecular Probes (Eugene, OR, USA). Of note, the physiological isomer of BHB—i.e. R(−)BHB or D(−)BHB—was used in all experiments.

Tissue preparation

One to 3 week-old Wistar rats (Charles River Laboratories; Wilmington, MA, USA), deeply anesthetized with halothane, were killed and their brains quickly transferred to ice-cold, oxygenated PIPES buffer (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 20 mM PIPES, pH adjusted to 7.0 with Trizma base, 320–330 mOsm). Slices (500 μm) from somatosensory cortex were cut on a vibratome (The Vibratome Company, St. Louis, MO, USA), treated with Pronase (protease, *Streptomyces griseus*; Calbiochem; San Diego, CA, USA) 0.61 PUK/ml for 20 min at 37 °C and then allowed to recover in oxygenated PIPES buffer at room temperature for 1 h. Small areas (1 mm in diameter) were dissected and mechanically dissociated with fire-polished Pasteur pipettes of progressively decreasing diameter in 35 mm Petri dishes (Becton Dickinson; Franklin Lanes, NJ, USA) containing Hepes buffer (145 mM NaCl, 4.0 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH adjusted to 7.4 with Trizma base, 320–330 mOsm).

Electrophysiology

Borosilicate microelectrodes (1.5 mm external diameter, 0.75 mm internal diameter; WPI; Sarasota, FL, USA) were prepared with a Narishige PP-830 puller (Narishige International USA; East Meadow, NY, USA) and filled with an internal solution (150 mM K-gluconate, 8 mM $MgCl_2$, 10 mM Hepes, pH adjusted to 7.2 with Trizma base) containing 240 μg/ml amphotericin B (from streptomyces, ≈80%; Rae et al., 1991). Electrode impedances ranged from 5 to 9 MΩ. Acutely dissociated neurons in Petri dishes were placed under a Zeiss Axiovert 200 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). Electrodes were attached to a Sutter MP-225 micromanipulator (Sutter Instrument Company, Novato, CA, USA) and connected to an Axon Multiclamp 700B controlled by Multiclamp Commander (Axon Instruments; Union City, CA, USA). Electrical signals were digitized with an Axon Digidata 1322A and pClamp 9.2 (Axon Instruments).

Once a gigaohm seal was achieved, membrane (R_m) and access (R_a) resistances were continuously monitored using the Membrane Test function of pClamp for 10–40 min until the access resistance fell below 100 MΩ. Membrane potential (V_m) was recorded for 5 min and if the range did not fluctuate by more than 10 mV, the neuron was selected for further study with glutamate (L-glutamic acid monosodium salt, 99–100%), BHB (R-3-hydroxybutyric acid, ≥98%), ACA (lithium ACA, 90–95%) or catalase (from mouse liver). R_m was monitored again at the end of each protocol. In some experiments, cells were imaged under phase contrast to look for morphological changes. Pharmacological substances were applied with an ALA-VM8 pressure-controlled pump (ALA Scientific Instruments; Westbury, NY, USA). Electrophysiological data were analyzed with Clampfit 9.2 (Axon Instruments).

Fluorescence imaging

Neurons were dissociated in Hepes buffer containing either 50 μg/ml propidium iodide (PI), 1 μM dihydroethidium (DHE), 50 μM monochlorobimane (MCB) or 5 μM Rhod-2 (Macklis and Madison, 1990; Keelan et al., 2001; Vergun et al., 2001). Intracellular labeling was observed at 64× under the Zeiss Axiovert 200 equipped with an EXFO X-Cite 120 fluorescence system (Photonics Solutions Inc., Mississauga, ON, Canada) and Zeiss filter set 15 (excitation 546 nm, emission 590 nm; Zeiss) for PI and Rhod-2, Zeiss filter set 10 (excitation 450–490 nm, emission 515–565 nm; Zeiss) for DHE or Zeiss filter set 49 (excitation 365 nm, emission 545–550 nm; Zeiss) for MCB. Images were acquired and analyzed with Axiovision 4.3 (Zeiss). Neurons exposed to PI and DHE were studied immediately following dissociation whereas those treated with MCB and Rhod-2 were incubated with the dye for 30 and 60 min, respectively, prior to recording. For NAD(P)H fluorescence, neurons were dissociated in customized Petri dishes. The bottoms of the dishes were cut out and replaced with thin cover slides to allow imaging with a 40× oil-immersion objective (Plan-NEOFLUAR 40×/1.3 oil DIC; Zeiss). Neuronal fluorescence in all protocols was normalized to background (i.e. cell-free area) fluorescence.

Mitochondrial isolation

The following procedures were modified from previously described protocols (Sullivan et al., 2000, 2003; Brown et al., 2004). Adult male Sprague–Dawley rats (~250 g) were anesthetized with carbon dioxide in a sealed chamber and subsequently decapitated. The brains were quickly removed and the cortices dissected off. Cortical tissue was placed in cold isolation buffer with 1 mM EGTA (75 mM sucrose, 215 mM mannitol, 0.1% BSA, 1 mM EGTA, 20 mM Hepes with a pH adjusted to 7.2 using KOH) and all tissues were kept on ice at all times throughout the isolation.

Dissected cortical tissue was put into an all-glass dounce homogenizer with 3 ml of isolation buffer with EGTA, homogenized, split into four 2 ml tubes, topped off with isolation buffer with EGTA, and spun at 1300×*g* for 5 min at 4 °C. The supernatant was taken off and saved in separate tubes. The pellet was resuspended in isolation buffer with EGTA and spun at 1300×*g* for 5 min at 4 °C. The supernatant was again taken off and saved in separate tubes. The saved supernatant was topped off with isolation buffer with EGTA and spun at 13,000×*g* for 10 min at 4 °C. Supernatant was then discarded (by slinging off) and the pellets were resuspended and combined in 500 μl of isolation buffer with EGTA. The sample was placed in a cold nitrogen cell disruptor for 10 min at 1000 p.s.i. to burst synaptoneurosomes formed by homogenization (Brown et al., 2004).

After nitrogen disruption of synaptoneurosomes, the sample was spun on a Percoll gradient, with fresh stocks of 30%, 24%, and 40% Percoll made prior to spin with isolation buffer with EGTA. The gradient was made by first adding 3.5 ml of the 24% stock to the ultracentrifuge tube and then, using a 5 ml syringe, injecting 3.5 ml of the 40% stock to the bottom of the tube. Using equal amounts of 30% Percoll stock and sample a 15% Percoll solution was made and 3 mL was added to the top of the gradient. The gradient was then spun in a high-speed Sorval centrifuge for 10 min at 30,400×*g*. The third fraction containing the purified mitochondria was removed, put into clean tubes, and topped off with isolation buffer without EGTA. The samples were spun in the same centrifuge at 16,700×*g* for 15 min. The supernatant was removed and the pellet was resuspended in isolation buffer without EGTA and spun at 13,000×*g* in the high-speed Sorval centrifuge for 10 min. After the supernatant was removed and discarded the pellet was resuspended in 1 mM isolation buffer without EGTA and transferred to a microcentrifuge tube, which was then spun at 10,000×*g* for 10 min. The supernatant was removed and the pellet was resuspended in enough isolation buffer without EGTA to obtain a concentration of 10–15 μg/μl. A BCA protein assay kit was used to determine protein concentration by measuring absorbance at 560 nm with a BioTek Synergy HT plate reader (BioTek Instruments, Winooskin, VT, USA).