Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2008 | 106 | 1044–1051 doi: 10.1111/j.1471-4159.2008.05460.x

The ketogenic diet increases mitochondrial glutathione levels

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Abstract

The ketogenic diet (KD) is a high-fat, low carbohydrate diet that is used as a therapy for intractable epilepsy. However, the mechanism(s) by which the KD achieves neuroprotection and/or seizure control are not yet known. We sought to determine whether the KD improves mitochondrial redox status. Adolescent Sprague–Dawley rats (P28) were fed a KD or control diet for 3 weeks and ketosis was confirmed by plasma levels of β-hydroxybutyrate (BHB). KD-fed rats showed a twofold increase in hippocampal mitochondrial GSH and GSH/GSSG ratios compared with control diet-fed rats. To determine whether elevated mitochondrial GSH was associated with increased *de novo* synthesis, the enzymatic activity of glutamate cysteine ligase (GCL) (the rate-limiting enzyme in GSH biosynthesis) and protein levels of the catalytic (GCLC) and modulatory (GCLM) subunits of GCL were analyzed. Increased GCL activity was observed in KD-fed rats, as well as up-regulated protein levels of GCL subunits. Reduced CoA (CoASH), an indicator of mitochondrial redox status, and lipoic acid, a thiol antioxidant, were also significantly increased in the hippocampus of KD-fed rats compared with controls. As GSH is a major mitochondrial antioxidant that protects mitochondrial DNA (mtDNA) against oxidative damage, we measured mitochondrial H_2O_2 production and H_2O_2-induced mtDNA damage. Isolated hippocampal mitochondria from KD-fed rats showed functional consequences consistent with the improvement of mitochondrial redox status i.e. decreased H_2O_2 production and mtDNA damage. Together, the results demonstrate that the KD up-regulates GSH biosynthesis, enhances mitochondrial antioxidant status, and protects mtDNA from oxidant-induced damage.

Keywords: DNA damage, epilepsy, glutathione, ketogenic diet, mitochondria, oxidative stress.

J. Neurochem. (2008) **106**, 1044–1051.

Metabolic factors are a recent addition to previously established mechanisms known to contribute to the control of epilepsies. Additionally, epilepsy is now among the diverse neurological disorders in which mitochondrial dysfunction is implicated (Sullivan *et al.* 2003, 2004; Ziegler *et al.* 2003; Patel 2004; Bough *et al.* 2006; Maalouf *et al.* 2007; Nazarewicz *et al.* 2007). Much of the support for metabolic control of epilepsy arises from the success of the ketogenic diet (KD). The KD is a high-fat, low carbohydrate diet that has been utilized as a treatment for epilepsy for over 80 years. The KD is particularly impressive in its broad anticonvulsant effects, as it is able to control seizures of many different types. In this way it is a more effective anticonvulsant therapy than any of the currently available antiepileptic drugs.

Despite a growing body of literature demonstrating the protective actions of the KD, the underlying mechanism(s) remain unknown. Recent work suggests that the KD inhibits oxidative damage and improves mitochondrial bioenergetics in the brain (Sullivan *et al.* 2003; Ziegler *et al.* 2003; Bough *et al.* 2006; Maalouf *et al.* 2007; Nazarewicz *et al.* 2007). One important function of mitochondria is the production of reactive oxygen species (ROS) and maintenance of cellular redox state. However, to date, it remains unclear to what extent the KD influences mitochondrial redox status and thus the oxidative burden of this subcellular organelle. Our laboratory has demonstrated that mitochondria contribute disproportionately to seizure-induced ROS production (Liang *et al.* 2000; Jarrett *et al.* 2008; Patel *et al.* 2008). An important acute consequence of seizures is the depletion of mitochondrial GSH. GSH, a non-protein thiol, and its

Received January 23, 2008; revised manuscript received April 17, 2008; accepted April 18, 2008.

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Abbreviations used: BHB, β-hydroxybutyrate; CoASH, reduced CoA; CoASSG, CoASH disulfide with GSH; GCL, glutamate cysteine ligase; GCLC, catalytic subunit of GCL; GCLM, modulatory subunit of GCL; GS, glutathione synthetase; KD, ketogenic diet; LA, lipoic acid; mtDNA, mitochondrial DNA; PUFAs, polyunsaturated fatty acids; QPCR, quantitative PCR; ROS, reactive oxygen species.

disulfide (GSSG) are the most widely used indicators of redox status (Liang and Patel 2006). Furthermore, a decrease in blood and brain GSH levels has been observed in epilepsy patients and is associated with low seizure threshold (Rothstein *et al.* 1994; Abe *et al.* 2000). Therefore, we sought to determine whether the KD influences mitochondrial redox status in the adolescent rat brain.

Materials and methods

Animals and dietary regime

Animal housing was conducted in compliance with University of Colorado Denver procedures and protocols. Adolescent male rats (P28) were housed under a 12 h light dark cycle with water *ad lib*. All animals were fasted 8 h prior to initiation of the KD or control diet. KD rats received Bio-Serv F3666 (Frenchtown, NJ, USA) and control rats received Bio-Serv F3517. Diets were fed isocalorically, calorie restricted to 90% of the recommended daily requirement, and maintained for 3 weeks. Blood plasma was obtained via retro-orbital collection and immediately centrifuged at 13 000 *g* at 4°C for 5 min. BHB levels were assessed using the BHB Liquicolor kit (Stanbio 2440CE, Boerne, TX, USA) and blood glucose was measured using a Precision Xtra (Abbott Laboratories, Alameda, CA, USA) glucose monitor.

Mitochondrial isolation

For GSH and GSSG measurements, hippocampal mitochondria were isolated as previously described by Liang and Patel (Liang and Patel 2006). For all other analyses, mitochondria were isolated as described by Castello *et al.* (2007). Briefly, two hippocampi were pooled together and homogenized with a Dounce tissue grinder (Wheaton, Milville, NJ, USA) in isolation buffer (70 mmol/L sucrose, 210 mmol/L mannitol, 5 mmol/L Tris–HCl, 1 mmol/L EDTA; pH 7.4) and then diluted 1 : 1 in 24% Percoll. Homogenates were centrifuged at 30 700 *g* at 4°C for 10 min. The sediment was subjected to Percoll gradient (19% on 40%) centrifugation at 30 700 *g* at 4°C for 10 min. The material located at the interface of the lowest two layers was slowly diluted 1 : 4 with mitochondrial isolation buffer containing 1 mg/mL bovine serum albumin and centrifuged at 6700 *g* at 4°C for 10 min to obtain final pellets consisting of respiring mitochondria. The final pellet was either used immediately for H_2O_2 production or DNA damage studies or frozen in liquid nitrogen and stored at −80°C until needed.

Measurement of GSH and GSSG

GSH and GSSG were measured by HPLC equipped with electrochemical detection as previously described (Liang and Patel 2006). Analytes were detected using a CoulArray system (Model 5600; ESA, Waters Comp., Milford, MA, USA) on two coulometric array cell modules, each containing four electrochemical sensors attached in series. Electrochemical detector potentials were 120/355/480/560/680/800 mV (vs. Pd). Frozen mitochondrial fractions of hippocampus were sonicated in 0.2 N perchloric acid ($HClO_4$) immediately before thawing to prevent artificial formation of thiol disulfide during sample preparation. The homogenates were centrifuged at 13 000 *g* at 4°C for 15 min. Aliquots of the supernatant were injected into HPLC and separated on a 5 μm, 4.6 × 150 mm YMC ODS-A column (Waters Com.). The mobile phase was composed of 125 mmol/L sodium phosphate, 1% methanol, pH 2.8, and a flow rate of 0.7 mL/min was maintained. As the redox potentials of GSH and GSSG are ∼550 and ∼750 mV respectively, the highest potential for the quantitative measurement of both GSH and GSSG was set at 800 mV. GSH and GSSG peaks were verified by adding standard solutions of known concentrations to the samples. The concentration of mitochondrial protein was determined in each sample using the Bradford method, and levels of GSH and GSSG were normalized per mg of mitochondrial protein.

Measurement of CoASH and CoASSG

Reduced CoA (CoASH) and its GSH disulfide (CoASSG) were measured by HPLC equipped with UV detection as previously described (Liang and Patel 2006). Briefly, frozen hippocampus was placed in 0.1 mol/L sodium phosphate and 28.5 mmol/L *N*-ethylmaleimide buffer (pH 7.4) and sonicated immediately prior to thawing. The tissue was acidified with equal volumes of 4% $HClO_4$ and centrifuged at 12 000 *g* 4°C for 20 min. Aliquots of supernatant were separated on a Zorbax SB-C18 column using the following mobile phases: (A) 10% methanol and 10 mmol/L tetrabutylammonium hydrogen sulfate (pH 5.0), (B) 85% methanol and 10 mmol/L tetrabutylammonium hydrogen sulfate (pH 5.0). The gradient was 5 min at 100% A, 0% B, followed by a 30 min linear gradient to 40% A and 60% B. Peak height was quantified by UV detection at 254 nm. The CoASSG standard was prepared from a mixture of GSH (100 nmol) and CoASH (100 nmol) which was dissolved in 0.1 mol/L sodium phosphate buffer, pH 7.4. Diamide (100 nmol) was added to a final volume of 1 mL followed by the addition of *N*-ethylmaleimide (1000 nmol) and 10 μL H_3PO_4. The mixture was analyzed by HPLC using the individual standard solutions of known concentrations to analyze the samples.

Measurement of GCLM and GCLC

Hippocampal tissue (50 μg of protein) isolated from KD- and control diet-fed rats was used for immunoblotting. Protein levels of the glutamate cysteine ligase (GCL) subunits (GCLM and GCLC) were determined using selective antibodies (Applied Biosystems, Foster City, CA, USA). Membrane blots were incubated with 1 μg/mL antibodies, the primary antisera used at a dilution of 1 : 5000, and the secondary antibodies used at a dilution of 1 : 20 000. The bands were scanned on a Storm Optical Scanner (Molecular Dynamics Inc., Sunnyvale, CA, USA) and quantitative analysis of each band was performed by ImageQuant software (Amersham Biosciences, Buckinghamshire, UK). The densities of GCLM and GCLC were normalized to β-actin.

Measurement of GCL and glutathione synthetase

Hippocampal tissue isolated from KD- and control-diet fed rats was used for GCL and glutathione synthetase (GS) activity assays. The activities of GCL and GS were determined spectrophotometrically by the oxidation of NADH as previously described (Drechsel *et al.* 2007).

Measurement of lipoic acid

The levels of lipoic acid (LA) in the brain were quantified by HPLC equipped with electrochemical detection as previously described (Han *et al.* 1995b; Sen *et al.* 1999) with minor modifications.

Briefly, the tissue was weighed and sonicated in 40% methanol, 20% acetonitrile, and 20% water. Homogenates were incubated at 4°C for 10 min to facilitate extraction of LA from the tissue. An equivalent volume of 0.2 N $HClO_4$ was added to precipitate protein. Samples were centrifuged at 15 000 *g* at 4°C for 15 min and aliquots of the supernatant were injected into the HPLC. LA was analyzed using a CoulArray system with an EC detector set at 100/300/400/460/520/580/640/700 mV and a 5 µm, 150 × 4.6 mm C-18 ODS2 column (Waters Comp.). The mobile phase was composed of 50 mmol/L NaH_2PO_4, 35% acetonitrile at pH 3.5 and the flow rate was set to 1.2 mL/min. The retention time of LA was 12.40 min.

Measurement of H_2O_2 production

Hydrogen peroxide (H_2O_2) formation in mitochondrial fractions (10 µg samples) was measured by fluorometric detection using the horseradish peroxidase-linked Amplex Ultra Red assay (Molecular Probes, Eugene, OR, USA) as previously described (Castello *et al.* 2007). Briefly, cellular fractions (10 µg) were added to a 96-well plate containing 100 µL of reaction buffer containing 0.1 units/mL horseradish peroxidase, 50 µmol/L Amplex UltraRed, and 10 mmol/L succinate. Resorufin fluorescence was detected on a microplate reader equipped for excitation in the range of 530–560 nm and emission detection at 590 nm.

Mitochondrial DNA susceptibility to H_2O_2

The susceptibility of the mitochondrial genome to H_2O_2 (50 µmol/L)-induced oxidative stress was assessed using freshly isolated hippocampal mitochondria from KD and control rats re-suspended in 50 µmol/L H_2O_2 dissolved in mitochondrial isolation buffer (70 mmol/L sucrose, 210 mmol/L mannitol, 5 mmol/L Tris–HCl, 1 mmol/L EDTA; pH 7.4) for a period of 15 or 30 min. After the indicated exposure period, mitochondria were washed with fresh isolation buffer. DNA was extracted and quantitative PCR (QPCR) performed as previously described (Jarrett *et al.* 2008). The primer sequences were as follows for the rat mitochondrial genome: 5′-GGCAATTAAGAGTGGGATGGAGCGAA-3′ and 5′-AAAATC CCCGCAAACAATGACCACCC-3′. QPCR was carried out on a DNA Engine thermal cycler with all reactions being a total volume of 100 µL containing 15 ng of total genomic DNA, one unit of XL rTth polymerase, 3.3X XL PCR buffer II (containing potassium acetate, glycerol and dimethylsulfoxide) and final concentrations of 200 µmol/L deoxyribonucletoide triphosphate mix, 1.2 mmol/L $Mg(OAc)_2$, and 0.1 µmol/L primers.

The gene fragments were amplified using the following thermo-cycling profile: The PCR was initiated with the addition of one unit of XL rTth polymerase when samples had reached a temperature of 75°C. This was followed by an initial denaturation for 1 min at 94°C, cycles of denaturation at 94°C for 30 s and primer extension at 60°C for 13 min. After the PCR cycles had been completed a final extension at 72°C for 10 min was performed. After completion of QPCR, gene products were resolved on a 1% agarose gel and digitally photographed on a UV transilluminator (UVi Tec, Cambridge, UK). The intensity of the PCR product bands was quantified with Scion Image analysis software (Scion Corporation, Frederick, MD, USA, Version Beta 4.0.2). The DNA lesion frequency (λ) was calculated as the amplification of damaged DNA samples (A_d) relative to the amplification of control (A_0) (no damage).

Statistical analysis

For comparison between three or more experimental groups, one-way ANOVA with the Tukey test for multiple comparisons was used. A two-tailed *t*-test was used for comparison between two treatments. Values of $p \leq 0.05$ or less were considered statistically significant.

Results

The KD causes chronic ketosis and hypoglycemia

Rats were started on their respective diets on P28, following an 8-h fast and were maintained on the diets for 3 weeks. All rats remained healthy for the duration of the study. Ketosis was assessed weekly and serum BHB concentrations were significantly higher in KD- versus control diet-fed rats after only 1 week on the KD and reached steady state levels by 2 weeks (Fig. 1a). In addition, as expected of the KD, glucose levels in the KD-fed rats were significantly lower after 3 weeks on the diet compared with controls (Fig. 1b). These studies confirm that a 3-week period on the KD results in both hypoglycemia and ketosis in adolescent rats.

The KD improves hippocampal redox status

Redox status was assessed by measurement of GSH and GSSG levels in hippocampal mitochondria by HPLC-EC.

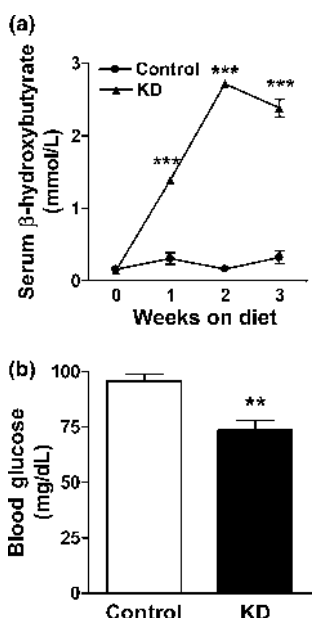

Fig. 1 Levels of β-hydroxybutyrate (BHB) and glucose of rats fed either ketogenic diet (KD) or control diet. Adolescent rats (P28) were housed under a 12 h light dark cycle with water *ad lib.* All animals were fasted 8 h prior to initiation of a control diet or KD and fed a calorie-restricted diet for 3 weeks as described in Materials and methods. (a) Ketone body levels were determined by measuring BHB once per week (control $n = 4$, Ketogenic $n = 7$). (b) Glucose levels were measured using a Precision Xtra glucose monitor ($n = 7$ rats per group). Data were presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.0001$ compared with control.

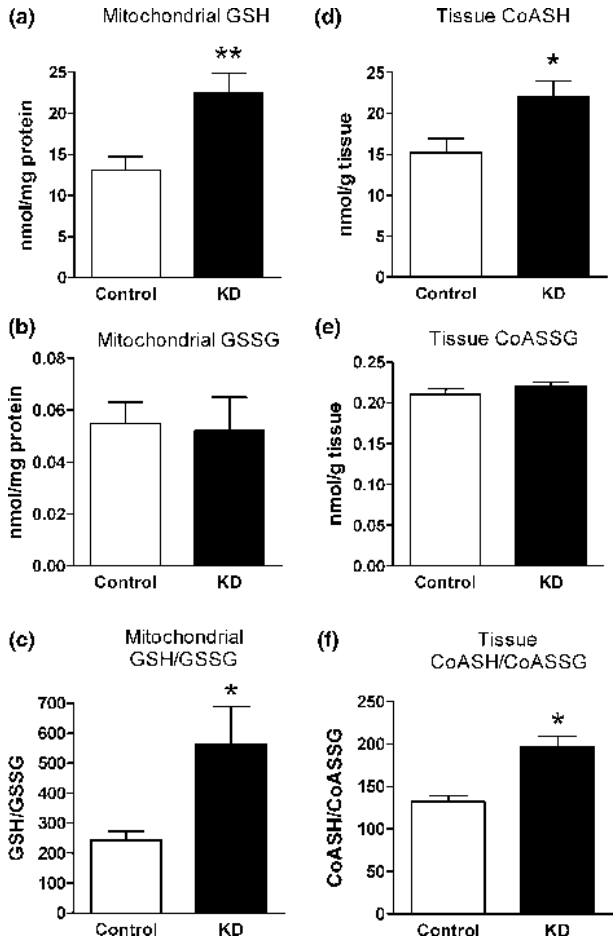

Fig. 2 Mitochondrial redox status of control and ketogenic diet (KD) rats. (a–c) Mitochondria were isolated from hippocampal homogenates and subjected to HPLC with electrochemical detection for analysis of GSH and GSSG levels ($n = 7$ per group). (d–f) Hippocampal homogenate was subjected to HPLC with UV detection for measurement of CoASH and CoASSG levels. Data were presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared with control.

Mitochondria from KD-fed rats showed a significant twofold increase in GSH levels, but not GSSG levels, compared with control rats (Fig. 2a and b). Furthermore, the ratio of GSH/GSSG, an index of mitochondrial redox status, was significantly increased in KD-fed rats compared with control diet-fed rats (Fig. 2c). The estimated GSH/GSSG redox potentials from this data using the Nernst equation revealed that hippocampal mitochondria from KD-fed rats exhibited significantly more reduced E_h values for the GSH/GSSG redox couple than controls (−230.0 ± 2.998 mV in control rats vs. −246.6 ± 3.703 mV in KD-fed rats, values represent mean ± SEM, $n = 7$ rats per group, two-tailed *t*-test revealed $p \leq 0.05$). In contrast, whole hippocampal tissue from KD-fed rats showed no significant changes in E_h values for the GSH/GSSG redox couple compared with hippocampal tissue from control rats (data not shown).

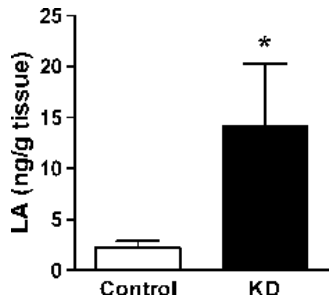

Fig. 3 Lipoic acid levels (LA) in the hippocampus of control and ketogenic diet (KD) rats. LA levels in hippocampus were assessed using HPLC equipped with UV detection ($n = 6$ per group). Data were presented as mean ± SEM. *$p \leq 0.05$ compared with control.

As the measurement of mitochondrial GSH and GSSG can be complicated by *in situ* oxidation of GSH and disulfide exchange reactions occurring during the isolation procedure, we measured a second redox couple (CoASH, CoASSG) that specifically resides in the mitochondrial compartment, allowing its measurement in intact tissue (Wong *et al.* 2001; Jenniskens *et al.* 2002; O'Donovan *et al.* 2002; Liang and Patel 2006). The levels of CoASH were significantly increased by the KD (Fig. 2d). While no significant change in CoASSG was observed (Fig. 2e), the ratio of CoASH/CoASSG increased significantly with the KD in comparison with control (Fig. 2f). We examined CoASH/CoASSG levels in the frontal cortex, as well. In contrast to the striking increase observed in the hippocampus, we found no significant changes in either CoASH or CoASSG in frontal cortex of KD-fed rats compared with controls (data not shown).

Finally we assessed the levels of LA, another thiol antioxidant. We assessed levels of LA in the hippocampus and frontal cortex of rats fed a control diet or KD. In a manner similar to GSH and CoASH levels, the levels of LA were significantly increased in the hippocampus but not frontal cortex of rats fed the KD (Fig. 3 for hippocampus, data not shown for frontal cortex).

The KD increases GCL enzymatic activity and protein levels of both GCL subunits in the hippocampus

As mitochondrial GSH is primarily dependent on the cytosolic pool, which ultimately arises because of *de novo* synthesis (Ha *et al.* 2006; St-Pierre *et al.* 2006), we measured the activities of the two GSH biosynthetic enzymes, GCL and GS. KD-fed rats showed a 1.3-fold increase in GCL activity, but no change in GS activity compared with control rats (Fig. 4a and b). GCL is a heterodimer comprised of a catalytic subunit (GCLC) and a modulatory subunit (GCLM) (Fujimori *et al.* 2004). By western blot analysis, we found that KD-fed rats exhibited increased protein levels of both GCLM (1.6-fold) and GCLC (1.9-fold) compared with control diet-fed rats (Fig. 4c and d).

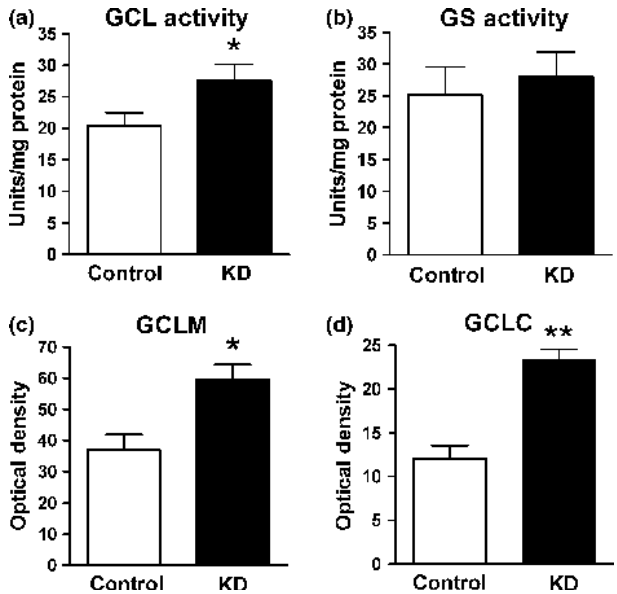

Fig. 4 A comparison of glutamate-cysteine ligase (GCL) and glutathione synthetase (GS) enzymatic activities and protein levels of GCL subunits (GCLM and GCLC) in control and ketogenic diet (KD) rats. After 3 weeks on either control diet or KD, hippocampi were homogenized for enzymatic activity assays and immunoblot analysis. (a and b) Enzymatic activity of GCL and GS in hippocampus of rats fed either a control or KD ($n = 6$ per group). (c and d) Protein levels of the modulatory and catalytic subunits of GCL (GCLM and GCLC, respectively) in control or KD rats ($n = 3$ per group). Data presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

The KD decreases H_2O_2 production in isolated hippocampal mitochondria

To determine whether improved redox status was associated with an improvement of mitochondrial function, we assessed H_2O_2 production in whole homogenates and purified respiring mitochondria from KD- and control-diet fed rat hippocampi. In contrast with whole hippocampal homogenates, isolated mitochondria from KD-fed rats exhibited decreased substrate-driven H_2O_2 production compared with control rats, which is consistent with a specific enhancement of mitochondrial antioxidant status (Fig. 5).

The KD protects mitochondrial DNA against oxidative stress

Finally, we asked whether an improvement of mitochondrial antioxidant status protected mitochondrial DNA (mtDNA), a sensitive and proximal target of ROS, from oxidative damage. Isolated hippocampal mitochondria obtained from control diet-fed rats exposed to exogenous H_2O_2 (50 μmol/L) for 15 and 30 min demonstrated a significant time-dependent increase in mtDNA damage compared with basal levels. In contrast, the KD-fed rats did not demonstrate any measurable mtDNA damage; the oxidative lesion frequency was significantly lower in KD-fed rats compared with the control rats at all time points studied (Fig. 6).

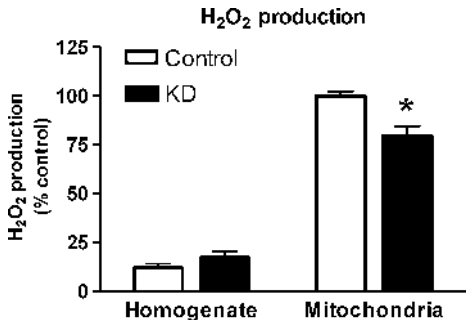

Fig. 5 Comparison of subcellular H_2O_2 production in control and ketogenic diet (KD) rats. Substrate (10 mmol/L succinate) driven H_2O_2 production was measured in whole hippocampal homogenates or isolated hippocampal mitochondria by a fluorometric assay ($n = 6$–8 per group). Data presented as mean ± SEM. $^{*}p < 0.05$ compared with control.

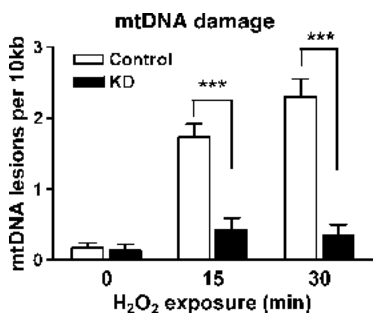

Fig. 6 Mitochondrial DNA (mtDNA) damage in control and ketogenic diet (KD) rat hippocampi following H_2O_2 exposure. Hippocampal mitochondria were freshly isolated and incubated with H_2O_2 (50 μmol/L) for 0, 15, or 30 min. After H_2O_2 exposure, the DNA was isolated and mtDNA lesion frequency was determined via quantitative PCR (QPCR) ($n = 3$–4 per group). Graph represents mtDNA lesions per 10 kb in control and KD rats. Data presented as mean ± SEM. $^{***}p < 0.0001$ compared with control.

Discussion

In this study, we demonstrate that the KD specifically increases mitochondrial GSH levels, stimulates *de novo* GSH biosynthesis, and improves mitochondrial redox status in the hippocampus, resulting in decreased mitochondrial ROS production and protection of mtDNA. Thus increased mitochondrial GSH may represent a possible candidate mechanism underlying the protection afforded by the KD.

The role of GSH in the protection against oxidative stress in neuronal disorders is well established; however, its role in neuroprotection by clinically utilized dietary therapies remains unexplored. This study provides evidence that GSH synthesis is up-regulated in KD-fed rats, as suggested by increased GCL activity and up-regulation of the GCL heterodimer subunits (GCLC and GCLM). It should be emphasized that dietary enhancement of mitochondrial

antioxidant capacity may have important implications for a variety of neurodegenerative diseases. Recent reports have suggested that the KD may provide symptomatic and disease-modifying activity in a broad range of neurological disorders in which oxidative stress is strongly implicated e.g. Alzheimer's disease, Parkinson's disease, traumatic brain injury, and stroke (Veech 2004; Zhao *et al.* 2006). Therefore, the KD-induced increase in mitochondrial GSH observed in this study may improve the ability of the brain to resist metabolic changes and oxidative stress, which, in part, underlie these neuronal disorders. In comparison with the frontal cortex, the hippocampus showed more pronounced changes in mitochondrial thiol status which is consistent with a report in which the KD was shown to selectively increase hippocampal activity of glutathione peroxidase (Ziegler *et al.* 2003).

In addition to the increased levels of GSH and CoASH, we found that the KD increased the levels of LA, another important thiol antioxidant with neuroprotective actions. This is not unexpected, as the immediate precursor of LA is acetyl-CoA, which is abundantly produced from the breakdown of ketone bodies in order to fuel the tricarboxylic acid (TCA) cycle in the relative absence of glucose that occurs in the KD. Importantly, LA has been shown to exhibit potent protective effects in models of hypoxia (Prehn *et al.* 1992; Cao and Phillis 1995), aging (Stoll *et al.* 1994; Palaniappan and Dai 2007), Parkinson's disease (Gotz *et al.* 1994; Bilska *et al.* 2007), diabetic neuropathy (Nagamatsu *et al.* 1995), as well as in human studies of inborn errors of metabolism (Matalon *et al.* 1984) and Alzheimer's disease (Hager *et al.* 2007; Moreira *et al.* 2007). Thus LA may be important for the protective effects seen with a KD.

Although the KD has previously been shown to modulate mitochondrial function and decrease ROS production (Ziegler *et al.* 2003; Sullivan *et al.* 2004; Bough *et al.* 2006; Nazarewicz *et al.* 2007), to the best of our knowledge, the present study reveals for the first time that the KD affords protection to the mitochondrial genome against oxidative insults. This is particularly significant given the known association between mitochondrial dysfunction and epilepsy (Kunz 2002; Patel 2004). Epilepsy is a prominent feature of clinical syndromes associated with mtDNA mutations and genomic instability, such as myoclonic epilepsy with ragged-red fiber disease (Baron *et al.* 2007). Additionally, recent work in our laboratory suggests a strong association between mitochondrial oxidative stress and mtDNA damage in chemoconvulsant-induced epileptogenesis (Jarrett *et al.* 2008). Increased mitochondrial GSH could interact directly with ROS or indirectly as a cofactor for glutathione peroxidase thus preventing damage to mtDNA, proteins and lipids. Decreased ROS production in isolated mitochondria from KD-fed rats observed here is suggestive of increased mitochondrial antioxidant capacity because of elevated pools of reduced thiols.

The precise mechanism by which the KD specifically increases mitochondrial GSH remains to be determined. The mitochondrial pool of GSH represents a minor fraction of the total tissue pool (~10%) but plays an important role in the maintenance of mitochondrial redox balance (Griffith and Meister 1985; Lash *et al.* 1998). GSH biosynthesis primarily occurs in the cytoplasm, and transport systems are critical in maintaining mitochondrial glutathione. Despite the quantitative differences in the cytosolic and mitochondrial GSH pools, the mitochondrial pool is thought to play a far more important role in maintaining cell viability following toxic insults compared with the cytosolic pool (Meredith and Reed 1982). It is intriguing to note that the present study demonstrated differences in the mitochondrial and tissue pools of GSH in response to the KD. The inability of the KD to elevate tissue GSH levels, which predominantly represent the cytosolic pool, despite increased GCL subunit expression and activity may be indicative of increased GSH turnover coupled with enhanced transport into the mitochondria. Consistent with this, KD selectively decreased ROS production in the mitochondrial fractions, but not tissue homogenates which largely represent the cytosolic fraction. Additionally, the KD resulted in an increase in hippocampal levels of LA. Exogenously administered LA has been shown to elevate intracellular GSH (Han *et al.* 1995a; Hultberg and Hultberg 2006) and prevent the loss of GSH because of oxidative stress (Arivazhagan *et al.* 2002; Selvakumar *et al.* 2005; Winiarska *et al.* 2008). However, whether endogenously generated LA is sufficient to increase mitochondrial GSH levels needs to be further examined. Finally, although the KD has been shown to moderately increase mitochondrial numbers in the rat hippocampus (Bough *et al.* 2006), it is unlikely that this underlies the elevated GSH levels because we observe increases in GSH biosynthetic enzymes and have accounted for changes in mitochondrial numbers by expressing GSH values per mg of mitochondrial protein.

The combined up-regulation of GCLM and GCLC proteins is reminiscent of cellular stress as a causative factor. The KD contains high concentrations of polyunsaturated fatty acids (PUFAs), and these lipids may contribute to the increase in mitochondrial GSH observed in this study. First, PUFAs may act to regulate the expression of specific genes via transcription factors. Two possible candidates are the peroxisome proliferator-activated receptors-α or -γ and the nuclear factor erythroid-derived 2-related factor 2, as these pathways are critical for the antioxidant adaptive response to toxic stimuli and are also modulated by PUFAs (Ha *et al.* 2006; Qin *et al.* 2006). Secondly, the high concentrations of lipids in the KD are highly susceptible to oxidation by virtue of the unsaturated double bond. It is possible that these oxidized species, such as 4-hydroxynonenal, initiate an adaptive response thereby increasing mitochondrial antioxidant capacity. The adaptive response is a biological phenomenon which involves cells reacting at a molecular

level to acquire greater resistance against a wide range of physiological stresses, including ROS (Davies 2000).

In summary, this study demonstrates that the KD specifically enhances the antioxidant capacity of brain mitochondria. We propose that increased GSH levels may in part result from increased GCL (the rate-limiting enzyme in GSH biosynthesis). Improved mitochondrial redox status may further afford protection to the mitochondrial genome against oxidative stress. A greater understanding of the KD-induced enhancement of mitochondrial antioxidant capacity may be a useful target in developing future neuroprotective and anticonvulsant strategies.

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