Mitochondrial Biogenesis in the Anticonvulsant Mechanism of the Ketogenic Diet

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Objective: The full anticonvulsant effect of the ketogenic diet (KD) can require weeks to develop in rats, suggesting that altered gene expression is involved. The KD typically is used in pediatric epilepsies, but is effective also in adolescents and adults. Our goal was to use microarray and complementary technologies in adolescent rats to understand its anticonvulsant effect.

Methods: Microarrays were used to define patterns of gene expression in the hippocampus of rats fed a KD or control diet for 3 weeks. Hippocampi from control- and KD-fed rats were also compared for the number of mitochondrial profiles in electron micrographs, the levels of selected energy metabolites and enzyme activities, and the effect of low glucose on synaptic transmission.

Results: Most striking was a coordinated upregulation of all (n = 34) differentially regulated transcripts encoding energy metabolism enzymes and 39 of 42 transcripts encoding mitochondrial proteins, which was accompanied by an increased number of mitochondrial profiles, a higher phosphocreatine/creatine ratio, elevated glutamate levels, and decreased glycogen levels. Consistent with increased energy reserves, synaptic transmission in hippocampal slices from KD-fed animals was resistant to low glucose.

Interpretation: These data show that a calorie-restricted KD enhances brain metabolism. We propose an anticonvulsant mechanism of the KD involving mitochondrial biogenesis leading to enhanced alternative energy stores.

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Epilepsy affects 1 to 2% of people worldwide and is defined symptomatically by the appearance of spontaneous, recurrent seizures. The ketogenic diet (KD) is a high-fat diet that has been used to treat various types of medically refractory epilepsy.[1] The KD might also slow the progression of the disorder.[2,3] During treatment, ketone bodies are thought to become a significant energy substrate for the brain, but how this metabolic adaptation confers the anticonvulsant effect is unknown.

Because the KD may require several days or longer to become maximally effective in rats,[4] we hypothesized that alterations in gene expression are involved in its anticonvulsant action. Microarrays were used to identify functional groups of genes induced or repressed in the hippocampus after KD. Although microarray analysis of brain regions is complicated by massive heterogeneity of cell types, global changes in gene expression have provided important insights into underlying mechanisms of neurological disease. For example, transcriptional profiling has yielded insights into epilepsy, schizophrenia, Alzheimer's disease, and multiple sclerosis.[5–8]

The hippocampus was chosen for microarray, electron micrographic, biochemical, and electrophysiological studies because of previous findings showing that KD affects several hippocampal processes associated with diminished neuronal excitability and altered epileptogenesis.[2,3,9–13] Normal animals were studied rather than "epileptic" animals because a KD-induced change in seizure frequency would itself change gene expression that would cloud interpretation. Age at diet

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onset has not been linked with anticonvulsant efficacy experimentally,[4,14] and only via anecdotal reports clinically[1,15,16]; indeed, in a prospective trial of 150 children and adolescents there was no age-related difference in outcome.[1] The KD is effective in infants,[17] adolescents,[18,19] and adults with generalized and partial epilepsy.[16] Experimental studies in adult rats support this notion.[4] Although the KD is used primarily for pediatric epilepsy, we selected adolescent rats (37–41 days old)[20] rather than very young rats to study to circumvent ontogenetic profiles that would be superimposed on diet-induced changes,[20] an age group that also has a documented KD-induced anticonvulsant effect.[18,19] We have previously identified the key variables associated with KD efficacy in rats.[14,21–24] This study provides novel insight into how the KD may act via cellular metabolism to increase the resistance to seizures and, possibly, limit neurodegeneration.

Materials and Methods

Animals and Diet Treatment

For all experiments, male Sprague–Dawley rats (Harlan, Indianapolis, IN) were housed individually and fed either a calorie-restricted KD or normal, ad libitum (control [CON]) diet beginning on postnatal days 37 to 41 (initial weights, 135–175gm). A detailed description of the constituents of the normal diet (Purina 5001, St. Louis, MO) and KD (#F-3666 Bio-Serv; Frenchtown, NJ) is published.[22] All experiments were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and was approved by Institutional Animal Care and Use Committees. Seizure threshold was measured every 3 to 4 days via timed venous infusion of pentylenetetrazole (PTZ).[25] In separate experiments, seizure threshold was measured only once, after 3 weeks on a control diet or KD, via timed exposure to PTZ (n = 25) or flurothyl (n = 20). A threshold dose of PTZ or flurothyl (measured in mg/kg) was calculated from the time when the rat first exhibited a bilateral forelimb clonic jerk.[24] Seizures were induced between 1:00 and 5:00 PM before feeding to minimize possible effects of circadian rhythms and postprandial hormonal fluctuations. Plasma levels of β-hydroxybutyrate (BHB) and glucose were measured using a Keto-Site (GDS Technologies, Elkhart, IN) and Precision Xtra (Abbott Labs, Alameda, CA) meters, respectively.

Microarray Analysis

Seizure-naive male rats (n = 24) were maintained on the diet for 22 days, lightly anesthetized with isoflurane, and decapitated. Their brains were removed rapidly and placed in ice-cold phosphate-buffered saline solution for 10 to 15 seconds, then hippocampi were dissected over ice and frozen. To minimize biological variability, we pooled together left hippocampi from either 2 KD or 2 control animals and stored at −80°C to produce 6 pooled samples from each treatment group of 12 rats. Pooled, right hippocampi (n = 12) were stored individually at −80°C for corroborative analyses. All tissue samples were sent to the National Institute of Neurological Disorders and Stroke-National Institute of Mental Health (NINDS-NIMH) Affymetrix Microarray Consortium (TGEN, Phoenix, AZ) for total RNA isolation, quality-control assessment, probe generation, hybridization to rat 230A GeneChips (Affymetrix, Santa Clara, CA), and GeneChip scanning. Total RNA was inadequately isolated from one of the ketogenic samples, thus only 11 total samples were used for array testing (5 KD, 6 CON). Image data from each chip were normalized to a mean target intensity value of 150.

The relative abundance of each probe set and an evaluation of whether a particular transcript was expressed above background were calculated using Microarray suite (MAS 5.0; Affymetrix). The assignment of each probe pair on the rat 230A GeneChip to a gene was originally based by Affymetrix on the sequences available in Unigene build #99, whereas the most recent build is #144. The probe pair assignments have not been updated by Affymetrix, and approximately 11% of the original accession numbers assigned to probe sets on the RAE 230A chip either match less than half of the probe pairs in the corresponding set or are retired from current databases.[26] Dai and colleages[26] created a custom CDF file (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp) that can be read by the MAS 5.0 program to assign signal intensities of each probe pair to genes. All probe pairs for a particular transcript are pooled into a single probe set, which eliminates duplicate or triplicate instances of genes on the chip. Moreover, probes hybridizing to the noncoding strand of a transcript are deleted from analysis, which greatly reduces the number of expressed sequence tags called. Discrimination scores of the signal intensities for each spot on an individual chip were determined to be significantly different from background (i.e., present, marginally present, or absent calls) using a one-sided Wilcoxon signed rank test. We selected genes for subsequent statistical analysis if signal intensities were significantly above background (i.e., called "present") in at least 5 of the 11 arrays tested.

The Significance Analysis of Microarrays (SAM) program (http://www-stat.stanford.edu/~tibs/SAM)[27] was used to determine differences in patterns of gene expression between groups at a 1% false discovery rate. Functional categories were ascribed to differentially expressed genes using NetAffx Analysis Center (www.affymetrix.com) and explore Gene Ontology (eGOn; http://www.genetools.microarray.ntnu.no/common/intro.php). Because not all named genes were associated with a Gene Ontology biological process, functions for the remaining named genes were identified by manual searching through EntrezGene (National Institutes of Health), the Rat Genome Database, and GeneCards. To determine whether the ratio of induced-to-repressed genes in a particular category was significantly different from the ratio of all induced-to-repressed genes (see Fig 2C), we performed two-sided Fisher's exact tests for each of the eight categories.

Analysis of Cerebral Metabolites, Nucleotides, and Enzyme Activities

Cohorts of KD (n = 29) and CON (n = 28) animals were maintained on diet treatment for 20 to 28 days. For nucleotide measurements, rats (n = 9 KD and 8 CON) were

killed by high-energy microwave irradiation (6kW, 70% power, 1.3 seconds).[28] Hippocampi were rapidly dissected, fresh frozen over dry ice, weighed, and homogenized in 2% trichloroacetic acid. For metabolite (n = 10 KD and 10 CON) and enzyme (n = 10 KD and 10 CON) measurements, rats were lightly anesthetized with isoflurane and decapitated. The head was immediately cooled in ice-cold phosphate-buffered saline for 10 to 15 seconds to minimize the postmortem accumulation of GABA, and the brain was removed rapidly.[29] Hippocampi were dissected rapidly over ice, frozen on dry ice, and weighed. Metabolites and nucleotides were measured by high-performance liquid chromatography; enzyme activities were measured fluorometrically or spectrophotometrically (see Supplementary Methods section for details). All enzyme reactions were verified to be linear with time and concentration of tissue.

Electron Microscopy

Nine animals (KD = 4, CON = 5) were maintained on diet treatment as described earlier for at least 4 weeks. After deep anesthesia with halothane, animals were perfused transcardially with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Brains were postfixed in the same mixture for 8 to 12 hours at 4 to 8°C. Coronal sections (60μm) were cut with a vibratome, dehydrated, and embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). Regions of the dentate and hilus (approximately 2mm^2) were microdissected and mounted on blocks, and 60nm sections were collected onto Pioloform-coated slot grids, then counterstained with uranyl acetate and lead citrate.

Sections were examined using a Zeiss EM10C electron microscope (Zeiss, Thornwood, NY). Electron micrographs were taken randomly at 20,000× magnification through the dentate-hilus region. Data from 4 KD-fed (160 micrographs representing 2,680μm^2) and 5 control animals (184 micrographs representing 3,080μm^2) were examined for mitochondrial counts. Images were captured on a Leica DMRBE microscope with a Spot RT color digital camera (Diagnostic Instruments, Sterling Heights, MI), saved in a jpg-file format, and printed using a Kodak 8660 Thermal printer (Kodak, Rochester, NY). Identification of dendrites, terminals, spines, and axons was based on ultrastructural characteristics and size, as described previously.[30] Mitochondria were identified by their localization within identified processes, dual outer membranes, and presence of internal cristae.

Electrophysiology

Six pairs of animals were fed either a KD or control diet for 20 or more days as described earlier, anesthetized with halothane, and decapitated. Brains were removed rapidly and chilled in an ice-cold, carbogenated (ie, bubbled with 95% O_2/5% CO_2) cutting artificial cerebrospinal fluid containing the following (in mM): NaCl 130, KCl 3.5, Na_2HPO_4 1.25, $NaHCO_3$ 24, $MgSO_4$ 4, $CaCl_2$ 1, glucose 10; osmolarity 300 ± 5mOsm. For each experiment, transverse entorhinal-hippocampal slices (500–550μM thick) were cut using a vibratome from each pair of animals (one KD and one control) simultaneously. Slices were transferred to a carbogenated holding chamber in artificial cerebrospinal fluid containing 2mM each of $MgSO_4$ and $CaCl_2$, where they were maintained for approximately 30 minutes at 25°C until recordings were begun. Slice recordings from pairs of animals were conducted concurrently in a submerged chamber, where slices were perfused continuously with carbogenated bathing medium at a rate of 2 to 3ml/min. With our chamber dead volume of 2.2ml, this allowed for fluid exchange within a few minutes. All experiments were performed at 32 to 34°C.

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from the dentate molecular layer using glass micropipettes (5–10μ) filled with artificial cerebrospinal fluid and an aliquot (about 25μl) of India ink to visualize recording electrode placement. Slices from the middle hippocampus were used preferentially. Responses were evoked by stimulation of the medial perforant path (MPP, located in the middle third of the molecular layer of the dentate gyrus) using a Teflon-coated, Pt/Ir monopolar microelectrode (approximately 1MΩ impedance). Electrode placements in the medial perforant path were corroborated by observing paired-pulse depression (about 15%) at a 50-millisecond interpulse interval.[31] Responses that did not exhibit consistent paired-pulse depression were not used. Stimulus intensity (0.1-millisecond duration, ≤70μA) was adjusted to evoke fEPSPs of approximately 50% maximum amplitude. A solution containing low (2mM) glucose supplemented with 8mM mannitol to maintain osmolarity was bath applied for 7 minutes.

Results

Ketogenic Diet Treatment Induces Rapid Ketonemia but Delayed Seizure Protection

When effective, the KD produces sustained ketonemia and increased seizure resistance to a variety of proconvulsant challenges.[4,22,32] To determine how long the diet must be administered to achieve an anticonvulsant effect in adolescent rats, we monitored seizure threshold repeatedly in the same cohort of rats over 9 weeks (KD = 16, CON = 11). Despite a precipitous increase in plasma BHB levels and moderate reduction in blood glucose that developed within 1 day of diet initiation, PTZ seizure threshold was not consistently elevated above threshold in the control group until at least 13 days of diet treatment (Figs 1A–D). To determine whether the observed decline in seizure threshold in the control group was due to kindling by repeated administration of PTZ,[33] we fed separate groups of seizure-naive rats KD or control diets for 3 weeks and tested once for seizure threshold against either PTZ (n = 25) or flurothyl (n = 20). In both tests, seizure threshold was elevated compared with the control group after the same duration (20–21 days) of KD treatment (see Figs 1E, F). These findings suggest that the decline in PTZ seizure threshold over 3 weeks in control rats was not due to a chemical kindling effect, but instead may reflect a developmental change. We

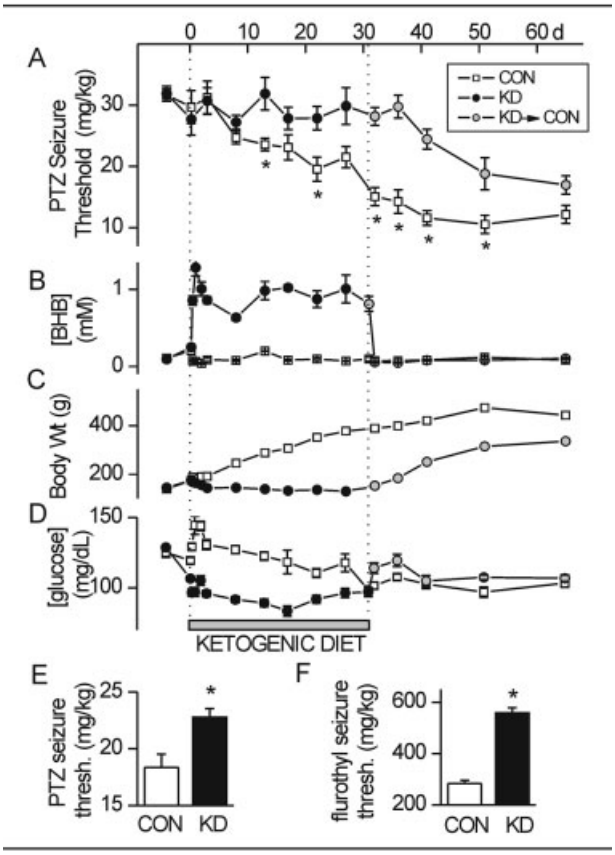

Fig 1. The anticonvulsant effect of the ketogenic diet (KD) develops slowly. (A) Pentylenetetrazole (PTZ) seizure threshold, (B) plasma levels of β-hydroxybutyrate (BHB), (C) body weight, and (D) plasma levels of glucose were measured repeatedly for 65 days before, during, and after administration of a KD to rats. The bar *(D) represents the time during which a KD was administered to the KD group* (solid circles). *Control (CON) animals were maintained on normal chow, ad libitum throughout the experiment* (open squares). *On day 31, KD-fed animals were reverted to a control diet* (gray circles). Points *represent the mean ± standard error of the mean (SEM) of each diet group: KD-fed (n = 16) and control animals (n = 11).* *p < *0.05, analysis of variance. Separate cohorts of seizure-naive animals exhibited similar elevations in seizure threshold as evaluated by either PTZ (E; n = 25) or flurothyl (F; n = 20) after 20 to 21 days of diet treatment. Bars represent group mean + SEM.* *p < *0.05, unpaired* t *test.*

conclude that chronic but not acute ketosis is associated with the anticonvulsant action of the KD.

Metabolic Genes Are Coordinately Upregulated after Ketogenic Diet

The Affymetrix GeneChip (Affymetrix) has been the most popular microarray platform, yet over the past few years, there has been a growing gulf between the historical assignment by Affymetrix of oligonucleotide sequences to genes and modern assignments. Dai and colleagues[26] resolved this problem by reassigning sequences to transcripts based on the most up-to-date Unigene build (see Materials and Methods). The updated CDF file[26] was used to assign probe pairs to accession numbers for the Affymetrix rat 230A GeneChip. The updated assignments resulted in a reduction of the number of transcripts available on this chip from 15,866 (reported by Affymetrix) to 10,179. Normalization and filtering of the signal intensities resulted in 5,518 genes that were expressed reliably (i.e., "present") in either or both treatment groups. Statistical analysis at a 1% false discovery rate yielded 658 differentially expressed genes in the hippocampus after KD, 7 of which are expected to be false-positives. Only 60% of these differentially expressed genes were identified using the original CDF file available through Affymetrix. A total of 384 transcripts were induced (297 with known functions), whereas 274 were repressed (199 identified genes) after KD (Fig 2A). A list of all differentially expressed transcripts is available in Supplementary Table 1. Differentially expressed genes occurred for transcripts exhibiting a 100-fold range of control expression levels (see Fig 2A). Low variability was generally observed across arrays (see Figs 2A, B) and resulted in the ability to identify transcripts with as little as a ±16% repression or induction. The mean coefficient of variation of the expression level was 9%.

Batch searches of the differentially regulated genes produced a Gene Ontology biological process for about half of the named genes. Many of the remaining genes could be assigned to functional categories after searches of EntrezGene, GeneCards, the Rat Genome Database, or PubMed. In all, 462 genes could be assigned to 1 of 8 functional categories (see Fig 2C). More than half of these genes were associated with 1 of 3 categories: metabolism (104 genes), signal transduction (101 genes), and transcription (77 genes).

Transcripts in one functional category (metabolism) were coordinately upregulated after KD, whereas those in the synaptic transmission category were downregulated (*$p < 0.02$, Fisher's exact tests; see Fig 2C). Within the metabolism category, 3 subcategories of transcripts involved in energy (34 genes), lipid (17 genes), and protein metabolism (34 genes encoding proteolytic enzymes or proteasomes) predominated; 19 others were not members of these 3 subcategories. Energy metabolism genes accounted for the coordinate induction of this transcript category after KD ($p < 0.0001$, Fisher's exact test with Bonferroni correction; Fig 3A). All 34 of the transcripts in this category were upregulated. Hierarchical clustering of expression values for differentially expressed energy metabolism genes showed that all transcripts associated with glycolysis (2), the tricarboxcylic acid (TCA) cycle (6), and oxidative phosphorylation (21), were upregulated after KD (see Fig 3B). The oxidative phosphorylation transcripts encoded protein subunits of complex I (five subunits of

nicotinamide adenine dinucleotide dehydrogenase), complex II (subunits A and D of succinate dehydrogenase), complex IV (cytochrome *c* oxidase subunit Via1), and five subunits of the F_0-F_1 adenosine triphosphate (ATP) synthase complex. Transcripts for creatine kinase, glycogen phosphorylase, glucose 6-phosphate dehydrogenase, and acetyl-coenzyme A synthase 2 were also upregulated.

If the modest but concerted upregulation of transcripts encoding metabolic proteins were focused in a subpopulation of principal cells rather than distributed throughout the hippocampus, differential expression of these transcripts should be much larger in cells harvested by laser-capture microscopy and assayed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Based on estimates of the relative amounts of RNA in each neuron population compared with whole hippocampus, and assuming all of the change was concentrated within one cell population, one would predict a $\log_2$ ratio for most of the differentially expressed transcripts greater than 3, well within the limit of detection for qRT-PCR. $\log_2$ ratios of more than 0.8 can be consistently detected in qRT-PCR.[34] If, however, the modest upregulations observed were distributed evenly over various cell types in hippocampus, qRT-PCR should show no differences. We tested this hypothesis for dentate granule cells and hippocampal CA1 pyramidal cells by assaying the levels of eight energy metabolism transcripts by qRT-PCR (see Supplementary Methods section). In this experiment, there was no measurable difference in energy transcript levels for neurons isolated from KD or control-fed rats. These findings suggest that the KD causes a widespread, coordinate upregulation of energy metabolism transcripts across hippocampal cell types.

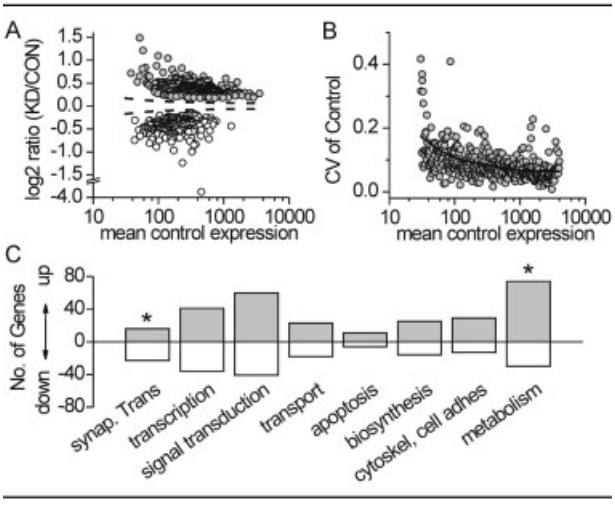

Fig 2. Changes in gene expression after ketogenic diet (KD). (A) Genes induced (gray circles) *or repressed* (white circles) *after KD (1% false discovery rate; n = 658; see Materials and Methods). Each symbol depicts one transcript. Variability* (dashed line) *is represented by the mean coefficient of variation (CV) determined as in B. (B) The relation between CV and control expression level was calculated from the best fit of the plot of the mean CV versus mean expression for all differentially expressed probe sets (n = 6 arrays). (C) The number of genes induced or repressed in each functional category after KD. Functional categories were assigned to 462 differentially expressed known genes. Each* bar *represents the total number of genes per category. Two categories were significantly upregulated or downregulated after KD (*$p < 0.02$, *Fisher's exact test).*

A coordinated upregulation of 19 proteasome-related transcripts occurred in hippocampus after the KD, compared with only 4 downregulated transcripts. A previous report[35] showed that proteasome inhibition was associated with reduction in the activity of complex I and II components of oxidative phosphorylation in hippocampal mitochondria; thus, upregulation of proteasome transcripts is consistent with enhanced oxidative phosphorylation.

A total of 39 genes directly associated with ion channels or synaptic transmission changed after KD. Downregulated transcripts (n = 23) predominated (see Fig 2C, $p < 0.01$), which included two voltage-dependent calcium-channel subunits (γ4 and α1D), the ClCN1 chloride channel, the KCNH3 and KCNE1-like potassium channels, P2X3 and P2X7 purinergic receptors, and synaptotagmins 6 and XI. Upregulated transcripts (n = 16) include the glutamate receptor subunits GluR2 and KA1, the KCNN2 potassium channel, the SCN1a type Iα sodium channel subunit, and the SLC1A1 glutamate transporter (EAAC1). No genes encoding $GABA_B$ receptors, metabotropic glutamate receptors, or subunits of *N*-methyl-D-aspartate receptors were induced or repressed after KD. Elucidation of the functional consequences of these changes requires further study.

No Differences in Activities of Selected Energy Metabolism Enzymes

In view of the microarray results, we determined whether a coordinate upregulation in energy transcripts in the hippocampus was paralleled by an increase in metabolic enzyme activities after KD. We found that activities of glycolytic enzymes (hexokinase, glucose 6-phosphate dehydrogenase, and lactate dehydrogenase) and enzymes involved in the TCA cycle (citrate synthase, α-ketoglutarate dehydrogenase, and malate dehydrogenase) were not significantly altered after KD (Table). Except for malate dehydrogenase (see Fig 3B), transcripts for the other enzymes were unchanged after KD or below detection level. These data suggest that the modest increases in transcript levels observed (<35%; see Fig 3B) were insufficient to cause significant increases in enzyme activities measured in vitro. At $\alpha = 0.05$, the power to detect a 25% change in enzyme activity was 91% or more for all of these en-

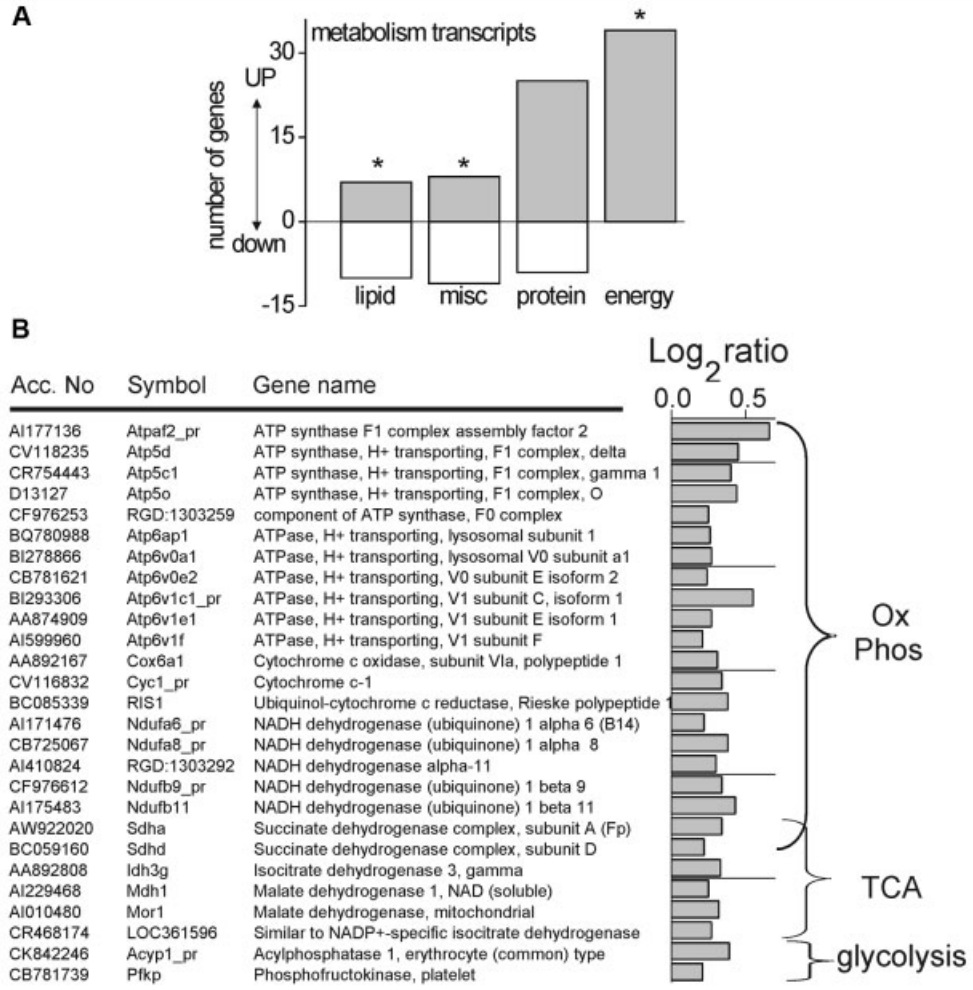

Fig 3. Metabolic genes changed after ketogenic diet (KD). (A) The 104 metabolism-related genes in Fig 2C could be further subcategorized into energy metabolism, lipid metabolism, protein (proteolysis and proteasome) metabolism, and a miscellaneous subgroup. Bars *indicate the total number of genes per subgroup. Two categories showed coordinate regulation of the genes in the category (*$p < 0.0001$ *for energy metabolism, and* $p < 0.02$ *for lipid metabolism, Fisher's exact test with Bonferroni correction). (B) Relative expression levels of 24 energy metabolism genes that were upregulated by the KD. Each row corresponds to one gene. Genes associated with glycolysis, the tricarboxylic acid (TCA) cycle, or oxidative phosphorylation (Ox Phos) are grouped.*

zyme assays. Small, individually unremarkable increases in the activity of several sequential enzymes in a metabolic pathway can, however, cause a substantial increase in total flux through the pathway.[36] Indeed, the increased glutamate level (see the Table) probably reflects a higher flux through the TCA cycle. The integration of glutamate, glutamine, and alanine levels, all of which are energy metabolites, also shows a larger combined level in the hippocampus of KD (17.7 ± 0.4nmol/mg wet weight) rats than control rats (16.3 ± 0.2; $p = 0.02$).

Increased Number of Mitochondrial Profiles in the Hippocampus after Ketogenic Diet

Ketosis and fasting result in loss of oxidative phosphorylation and mitochondrial function and numbers in muscle.[37,38] We next determined whether the concerted increase in expression of energy metabolism genes in hippocampus (see Fig 3) was accompanied by mitochondrial biogenesis. By visually scoring electron micrographs taken from the dentate-hilar region of the hippocampus, we found significantly more mitochondrial profiles in animals fed a KD compared with control animals (Fig 4). Data were remarkably consistent across animals (coefficient of variation = 3% for both KD and control animals), indicating a 46% increase in mitochondrial profiles for KD-fed animals versus control animals (see Fig 4B, left panel). In the same micrographs, there was no difference in the number of dendritic profiles (see Fig 4B, right panel). Most mitochondria counted in both control and KD tissue appeared to be located in neuronal processes (dendrites or axon terminals). The finding of increased numbers of mitochondrial profiles was reinforced by our observa-

tion that 39 of 42 differentially regulated transcripts encoding mitochondrial proteins were upregulated after KD (see Fig 4C). In addition to 23 energy metabolism genes, upregulated mitochondrial transcripts included two mitochondrial ribosome subunits (L20 and L53), the ANT3 and ANT4 nucleotide transporters, ornithine aminotransferase, acyl-coenzyme A synthase 3, and hydroxyacyl-coenzyme A dehydrogenase.

Energy Reserves Are Increased after Ketogenic Diet

We next determined whether the coordinate induction of energy metabolism genes and mitochondrial biogenesis led to increased production of energy metabolites in hippocampus after KD. There was a significantly larger phosphocreatine/creatine (PCr:Cr) ratio (see the Table; $p < 0.05$, t test). ATP, adenosine diphosphate, and adenosine monophosphate levels were not significantly increased in hippocampus after KD, although adenosine diphosphate level showed a twofold trend toward an increase. The BHB level was significantly elevated ($p < 0.001$). Glycogen was significantly ($p = 0.01$) decreased after KD as expected from the moderate hypoglycemia (see Fig 1), whereas glutamate and glutamine were elevated (see the Table). Because most of the tissue glutamate in the brain is used as an energy source rather than neurotransmitter[39]; these results are consistent with the notion that energy reserves are elevated in hippocampus after KD. The magnitude and direction of change of metabolites measured here in hippocampal tissue after the KD are similar to those that DeVivo and colleagues[40] reported for whole brain, although absolute levels in the hippocampus (see the Table) were about 6-fold higher for BHB, 3-fold lower for ATP, and 10-fold higher for adenosine monophosphate.

Synaptic Transmission Is More Resistant to Low Glucose after Ketogenic Diet

Synaptic transmission is highly dependent on ATP availability.[41] In view of our findings of a diet-induced modest enhancement in brain energy reserves, we asked whether synaptic transmission in hippocampal slices

Table. Hippocampal Metabolites and Enzymes Measured in Animals Fed Control or Ketogenic Diet 20 to 28 Days after Diet Treatment

Enzyme or Metabolite	CON (mean ± SD)	n	KD (mean ± SD)	n	*p*
Enzymes					
Hexokinase	7.20 ± 1.46	10	7.19 ± 0.71	10	0.98
Glucose 6-phosphate dehydrogenase	1.13 ± 0.09	9	1.21 ± 0.21	9	0.296
Lactate dehydrogenase	26.0 ± 1.32	6	23.9 ± 2.31	6	0.087
β-Hydroxybutyrate dehydrogenase	1.22 ± 0.09	10	1.28 ± 0.16	10	0.32
Citrate synthase	24.3 ± 2.33	10	26.2 ± 4.89	10	0.28
α-Ketoglutarate dehydrogenase	1.54 ± 0.17	10	1.54 ± 0.24	10	0.97
Malate dehydrogenase[a]	98.6 ± 10.3	10	90.8 ± 14.2	10	0.17
Metabolites					
Glutamate	11.5 ± 0.43	9	12.2 ± 0.48	9	0.03
Glutamine	4.04 ± 0.24	9	4.53 ± 0.41	9	0.01
Aspartate	1.98 ± 0.17	9	1.99 ± 0.18	9	0.84
Glycine	2.83 ± 0.15	9	3.11 ± 0.32	9	0.03
Taurine	5.79 ± 0.28	9	4.63 ± 0.37	9	<0.001
Alanine	0.74 ± 0.03	9	0.81 ± 0.07	9	0.01
Citrate	0.0923 ± 0.011	9	0.0856 ± 0.0127	10	0.24
Glycogen	3.68 ± 0.22	8	2.88 ± 0.19	9	0.01
β-hydroxybutyrate	0.0997 ± 0.034	9	0.386 ± 0.106	9	<0.001
AMP	0.692 ± 0.149	7	0.73 ± 0.11	9	0.884
ADP	0.290 ± 0.098	6	0.626 ± 0.15	9	0.115
ATP	0.708 ± 0.155	7	0.811 ± 0.20	9	0.67
PCr	5.90 ± 0.163	7	5.99 ± 0.20	9	0.396
Cr	4.52 ± 0.085	7	4.08 ± 0.18	9	0.054
Protein/wet weight[b]	0.119 ± 0.004	10	0.119 ± 0.007	10	0.88
PCr:Cr ratio	1.32 ± 0.038	7	1.48 ± 0.045	9	0.017

Glycogen, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), phosphocreatine (PCr), and creatine (Cr) measurements were performed on rats killed by head-focused, high-energy microwave irradiation to preserve in vivo nucleotide levels. The other analyses were from fresh-frozen tissue. Data are expressed as nmol/mg wet weight (metabolites) or nmol/mg wet weight/min (enzymes). An unpaired Student's t test was used to compare statistical differences of the mean between groups.

[a]Mitochondrial + cytosolic.

[b]Milligram protein/mg hippocampal fresh tissue, reported as a measure of potential protein catabolism or of hydration changes that might have affected analyses.

CON = control; SD = standard deviation; KD = ketogenic diet; PCr = phosphocreatine; Cr = creatine.

from KD-fed animals is significantly more resistant to mild metabolic stress compared with control animals (Fig 5). Paired stimuli (50-millisecond delay) were delivered to the medial perforant path, and fEPSPs were recorded from the dentate granule cell layer (see Fig 5A). Stimulus intensities required to evoke fEPSPs (30–70μA) did not differ across experiments or treatment groups. Reducing the bath glucose concentration from 10 to 2mM for 7 to 10 minutes rapidly and reversibly depressed the slope of the fEPSP by 53 ± 9% in control animals, but only by 27 ± 8% in KD-fed animals (see Fig 5B, top panel; $p < 0.001$ difference between control and KD, analysis of variance). Paired-pulse ratios increased in response to low glucose in slices taken from both control and KD rats (see Fig 5B, bottom panel; $p < 0.001$, analysis of variance), which supports the notion that diminished energy supply leads to an increased failure of transmitter release. The latency to onset of the effect induced by low glucose varied substantially from slice to slice in control rats (see Fig 5C), but for each pair of slices studied simultaneously (i.e., one from a KD-fed animal and one from a control animal), the onset of fEPSP reduction after low glucose always occurred more rapidly in con-

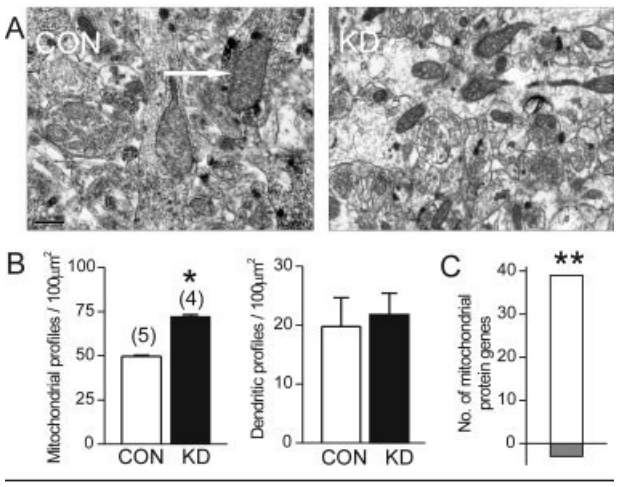

Fig 4. Mitochondrial biogenesis in the hippocampus of rats fed a ketogenic diet (KD). (A) Representative electron micrographs taken from control- (CON) or KD-fed animals (original magnification, ×20,000; calibration bar = 0.5μm). Arrow *indicates a mitochondrial profile. The number of mitochondrial profiles in each micrograph was counted. (B) There were significantly more mitochondrial profiles in the dentate gyrus of KD-fed animals than in control animals. Mitochondrial counts were taken from 4 KD-fed animals representing a total of 2,680μm² and 5 control rats representing 3,080μm². Data are presented as mean of averaged counts + standard error of the mean per 100μm² (n = 4 or 5).* *p < 0.001, t *test. By comparison, there was no difference in the density of dendritic profiles in the same micrographs, which guards against differential shrinkage of the tissue in KD versus control brains. (C) A total of 39 transcripts, assayed by microarray, encoding mitochondrial proteins were upregulated after KD* (open bar), *whereas only one transcript was downregulated* (gray bar). **p < 0.0001, Fisher's exact test.*

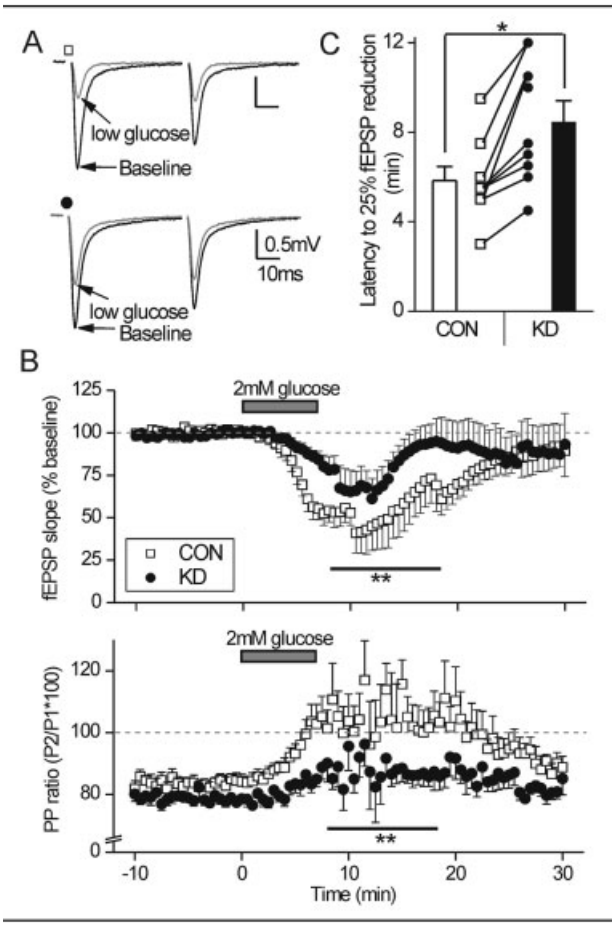

Fig 5. Synaptic transmission is more resistant to metabolic challenge in slices taken from ketogenic diet (KD)–fed animals than those from control (CON)-fed animals. (A) Representative paired-pulse evoked responses from control (top set of traces) and KD-fed (bottom set of traces) animals before (baseline) and 10 minutes after perfusion with low glucose (2mM). Responses are the average of five traces. Symbols denote stimulus artifacts. (B) Average response of hippocampal slices prepared from control (open squares; *n = 5 rats, 9 slices) and KD tissue* (filled circles; *n = 5 rats, 9 slices) to challenge with low (2mM) glucose. (Top) Symbols represent the mean slope of the field excitatory postsynaptic potential (fEPSP) slope ± standard error of the mean (SEM). The peak reduction in fEPSP slope (averaged over measurements during the period shown by the* bar*) after challenge with low glucose was significantly greater in control animals (**p < 0.001, analysis of variance [ANOVA]). (Bottom) Paired-pulse responses during low glucose (**p < 0.001 compared with control period, ANOVA). (C) Failure of synaptic transmission during low glucose was delayed in slices taken from KD-fed animals compared with control slices. Mean latencies to 25% reduction in synaptic efficacy are shown for experimentally matched slices prepared from control* (open squares) *and KD* (filled circles) *rats.* Bars *represent the mean latency per group + SEM.* *p < 0.002, paired t *test.*

trol tissue than in KD tissue (see Fig 5C; n = nine slice pairs from five pairs of rats). In slices taken from KD-fed animals, the latency to 25% inhibition of the fEPSP was 46 ± 10% longer than that of control slices (see Fig 5C; $p < 0.002$, paired t test). This difference, however, waned after slices were incubated in normal glucose-containing artificial cerebrospinal fluid at room temperature for greater than 3.5 hours (data not shown), as expected if brain metabolism could revert rapidly when excess glucose is supplied. Collectively, these findings support the notion that KD-induced enhancement in energy reserves can maintain synaptic transmission in the dentate gyrus for longer periods of time under metabolic stress.

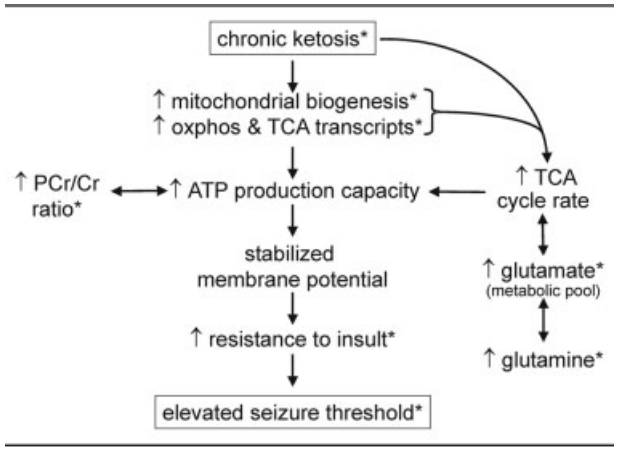

Fig 6. Hypothesis for anticonvulsant effect of the ketogenic diet (KD). Chronic ketosis in the brain is proposed to trigger mitochondrial biogenesis and associated induction of transcripts encoding proteins in the oxidative phosphorylation and tricarboxylic acid (TCA) pathways. Mitochondrial biogenesis increases adenosine triphosphate (ATP) production capacity, but under resting conditions, excess ATP is converted to phosphocreatine (PCr), leading to increased PCr/creatine (Cr) ratio. Ketones also serve as a substrate for glutamate synthesis via the TCA cycle, and some glutamate is reversibly converted to glutamine. Both glutamate and PCr act as energy buffers that can be drawn on to synthesize ATP when needed to fuel Na/K-ATPase and other pumps, which serve to stabilize neuronal membrane potential. The resulting enhanced resistance of hippocampal tissue to metabolic stresses accompanying hyperexcitability results in an elevated seizure threshold. Asterisk *indicates results observed in this study.*

Discussion

The molecular mechanisms underlying regulation of energy metabolism in the brain are not as well defined as those described for muscle. Indeed, chronic ketosis with caloric restriction appears to affect metabolism quite differently in skeletal muscle and brain. In muscle, ketonemia and/or fasting is accompanied by downregulation of oxidative phosphorylation, fewer mitochondria, less efficient mitochondrial respiration, and a decrease in glutamate and glutamine levels.[37,38,42,43] By comparison, in the hippocampus, chronic ketosis with caloric restriction results in upregulation of transcripts encoding oxidative phosphorylation and other mitochondrial proteins, mitochondrial biogenesis, elevated PCr:Cr ratio, and elevated glutamate and glutamine levels. Some of the energy transcripts were reported in a previous microarray study of KD,[44] but their use of pooled KD vs. pooled control samples on only one array makes interpretation difficult. In muscle, PGC1α, γ, and δ are powerful regulators of mitochondrial biogenesis and are downregulated during high-fat diets.[38,45] However, transcript levels for these proteins in both control and KD rats were below detection level in whole hippocampus and also in dentate granule cells and CA1 and CA3 pyramidal cells harvested by laser-capture microscopy (Borges, Shaw, Greene, and Dingledine, unpublished data). Our results suggest significant differences in how brain and muscle respond to high-fat diets.

The KD is a high-fat, calorie-restricted diet used to treat childhood epilepsies that do not respond to available drugs. Despite its clinical use for nearly 100 years, how the KD controls seizures remains unknown. Transcriptional profiling of rat hippocampus after KD showed a concerted upregulation of numerous transcripts encoding energy metabolism proteins and mitochondrial proteins. The most striking finding of this study was a 46% increase in the density of mitochondrial profiles in the dentate gyrus of KD-fed rats (see Fig 4), most of which were in neuronal processes. The increased PCr:Cr ratio and increased level of amino acid alternative energy sources, together with the improved resistance to low glucose (see Fig 5), are consistent with an increased capacity to sustain ATP production in hippocampus in the face of increased physiological need. These findings collectively point to an enhanced energy production capacity in the hippocampus of rats fed a KD.

An Energy Preservation Hypothesis for the Anticonvulsant Effect of the Ketogenic Diet

Several mechanisms for the anticonvulsant action of the KD have been proposed, including acidosis, enhanced GABA production, change in electrolyte balance or energy metabolism, activation by free-fatty acids of potassium or other channels, or dehydration.[46] One of the oldest theories stems from the idea that an increased production of ATP should enhance neuronal stability by stabilizing the resting membrane potential, perhaps via enhanced operation of the Na/K-ATPase.[40]

Taken together, our findings suggest a modification of the energetic hypothesis for the anticonvulsant effect of the KD (Fig 6, asterisk denotes findings in this study). We propose that chronic but not acute ketosis activates a genetic program that leads to mitochondrial

biogenesis in the hippocampus, which results in enhanced energy stores. The half-life of liver mitochondria is 3 to 4 days.[47] If that of brain mitochondria were similar, five half-lives (15–20 days) would be approximately the time required to achieve an anticonvulsant action of the KD (see Fig 1), consistent with the model presented in Figure 6. We suggest that mitochondrial biogenesis increases ATP production capacity, with excess high-energy phosphates stored as PCr. Glutamate and glutamine formed from the ketone-boosted TCA cycle provide an important second energy store that, in concert with PCr, can be drawn on to sustain ATP levels in times of need (ie, during hyperexcitability leading to a seizure). The ability to sustain ATP levels during metabolic or physiological stress should allow neurons to fuel Na/K-ATPase and other transporters that stabilize membrane potential in neurons, and thus maintain ionic homeostasis for longer periods of time. Under low glucose conditions, transmitter release from perforant path terminals could be maintained about 60% longer in hippocampal slices taken from KD compared with control-fed rats (see Fig 5), a likely consequence of enhanced energy stores.

The enhanced resistance to metabolic stress could thus elevate seizure threshold, if seizure initiation results from a crescendo barrage of neuronal impulses that eventually causes ATP levels to decline, leading to sustained membrane depolarization and runaway neuronal firing. The Na/K-ATPase inhibitor, ouabain, lowers seizure threshold to kainate,[48] as expected if oxidative metabolism opposes seizure initiation. Notably, the KD increases seizure threshold, but it cannot terminate a breakthrough seizure and may actually provide a greater energy supply that exacerbates spread of seizures once initiated,[23] which is consistent with our model. Although the degree of glutamate and PCr elevation is modest in KD-fed rat hippocampi (see the Table), a similar elevation in PCr:Cr ratio has been observed previously in total brain after KD.[40] Both glutamate and PCr are present in approximately 10-fold higher concentrations than ATP, befitting their role as energy buffers. Thus, small absolute adjustments should be effective. PCr opposes an acute activity-dependent reduction in ATP levels by donating its phosphoryl group to ATP.[49]

There is an established role for diminished ATP-production capacity in both patients with epilepsy[50, 51] and pilocarpine-treated rats.[52] In a recent study of human epileptic tissue, the rate of recovery of the resting membrane potential after an evoked stimulus train was positively correlated with the PCr/ATP ratio, but inversely correlated with granule cell bursting.[53] It is interesting to speculate whether GABAergic inhibitory interneurons, which tend to have a greater energetic requirement to maintain high-frequency modes of firing,[41] are particularly advantaged by improved energy levels associated with the KD. Electrophysiological evidence collected in vivo showed that KD decreases hippocampal network excitability in part by enhancing GABAergic inhibition.[3] Enhanced cellular metabolism might be expected to prolong activation of inhibitory interneurons, diminish network excitability, and thus improve seizure control. A critical test of this hypothesis will require the development of a method for selectively interrupting mitochondrial biogenesis in the brain.

Whereas glycogen was elevated in total brain,[40] we observed reduced levels of glycogen in hippocampus (see the Table). It is doubtful, however, that reduced glycogen level plays a particularly important role in seizure resistance because hypoglycemia can rapidly reduce levels of brain glycogen,[54,55] and KD-induced hypoglycemia occurred well before the anticonvulsant effect appeared (see Fig 1). It is thus unlikely that glycogen, the largest energy store in brain, is regulating seizure threshold directly.

Relevance to Childhood Epilepsies

We studied the effects of a KD in the hippocampus of nonepileptic, adolescent rats. Because this dietary treatment is often used in children with epilepsy originating outside the hippocampus, the validity of this model should be considered carefully and several points are relevant. First, the KD is effective in adolescents and adults, if tolerated.[18,19] Second, the KD has efficacy in a variety of epilepsies including temporal lobe epilepsy, which involves the hippocampus.[1] Third, the rat model faithfully reproduces at least four key aspects of KD treatment observed clinically: maintained ketonemia, maintained hypoglycemia, reduced weight gain, and increased resistance to seizures. Fourth, hippocampal sclerosis may coexist with nontemporal focal epilepsy[56] or cortical dysplasia, a more common cause of childhood epilepsy.[57] Moreover, Lundberg and colleagues[58] reported a high frequency of hippocampal asymmetry or sclerosis in benign childhood epilepsy with centrotemporal spikes, a common cause of childhood epilepsy. Therefore, although not involved in all childhood epilepsies, the hippocampus is a relevant structure. Finally, previous in vivo electrophysiological studies showed a robust anticonvulsant effect of the KD within the dentate gyrus of the hippocampus in a rat model.[3] KD-fed (and calorie-restricted) animals required greater stimulus intensities to evoke the same network excitability compared with ad libitum–fed control animals. Enhanced paired-pulse inhibition was observed, consistent with enhanced functional fast GABAergic inhibition, and electrographic seizure threshold was elevated in a kindling-like protocol. For these reasons, we decided to focus on a well-defined region of the brain, known to exhibit these KD-

induced effects and known to be involved importantly in the development and maintenance of epileptic phenotypes.

We (see Fig 1) and others[4] found that an elevation in seizure threshold required 2 or more weeks of the KD to fully develop in rats; this effect waned slowly following reversion to control (see Fig 1) or even high carbohydrate diet.[4] By contrast, Freeman and colleagues[59] reported that in 5 children with Lennox–Gastaut syndrome who were experiencing more than 20 myoclonic or atonic seizures per day, seizure frequency was drastically reduced 1 or 2 days after initiation of the KD. Aside from this report, there are only anecdotal reports of rapid loss of seizure control when plasma glucose level increases suddenly, or of rapid onset of KD effectiveness in epileptic patients, although typical practice is to assess efficacy after several weeks on the diet. The speed with which the anticonvulsant effects of the KD develop, and the mechanisms underlying its anticonvulsant effects, might well depend on age, physiological status (normal vs epileptic brain), and how the effect is measured (i.e., seizure threshold vs seizure frequency).

Implications for Neuroprotection

Mitochondrial dysfunction contributes to reperfusion injury, congestive heart disease, type 2 diabetes, and neurodegenerative disorders. Adaptive responses that induce mitochondrial biogenesis and enhance oxidative phosphorylation could therefore limit the progression of these disorders. The notion that mitochondrial biogenesis plays a role in neuronal survival in epilepsy is supported by the observation that surviving dentate-hilar neurons in humans with epilepsy contain more mitochondria than normal.[60] Several studies have shown that the KD can be neuroprotective. In mice treated with the KD for several weeks, Noh and colleagues[12] reported that kainate-induced status epilepticus caused less hippocampal cell death and less caspase-3 activation than control-fed mice. Whereas KD-fed mice exhibited a delay in seizure onset (increased seizure resistance), seizure severity was comparable with control animals. In rats, the KD is neuroprotective in models of controlled cortical injury[61] or hypoglycemia,[62] but unlike in mice, not status epilepticus produced by either kainate[9] or lithium-pilocarpine.[63]

Proteasome inhibition leading to mitochondrial dysfunction appears to contribute to several neurodegenerative disorders.[35,64] Mitochondrial biogenesis (see Fig 4), a coordinate increase in 19 proteasome transcripts, increased production of the UCP2 uncoupling protein, reduced reactive oxygen species generation, enhanced respiration rate of isolated mitochondria,[13,65] and enhanced alternative energy stores (see the Table), all of which point to a myriad of potential neuroprotective mechanisms induced after KD. The enhanced ability to maintain normal ATP levels via an enhancement in the PCr:Cr ratio (see the Table) should improve calcium homeostasis and limit synaptic dysfunction after metabolic challenges (see Fig 5; see also Yamada and colleagues[62]). The strong trend toward higher adenosine diphosphate levels may additionally protect against seizure-induced neuron death by inhibiting opening of the mitochondrial transition pore[66] and/or facilitating opening of K_{ATP} channels, which should reduce neuronal excitability.[67]

We conclude that diet can dramatically affect neuronal function within hippocampus. In response to a high-fat, calorie-restricted diet, the hippocampus responds by inducing mitochondrial biogenesis, enhancing metabolic gene expression, and increasing energy reserves. Our findings support an energy preservation hypothesis for the anticonvulsant effects of the KD, which might be particularly important for more metabolically active GABAergic interneurons. Because the enhanced ability of neurons to manage metabolic challenges (e.g., see Fig 5) after KD likely improves neuronal survival, as well as function under stressful conditions, the benefits of dietary therapies such as KD might also be extended to the treatment of other neurodegenerative disorders such as Alzheimer's or Parkinson's diseases.

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