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Higher ketogenic diet ratios confer protection from seizures without neurotoxicity

Kristopher J. Bough, Suellan G. Yao, Douglas A. Eagles *

Department of Biology, Georgetown University, Washington, DC 20057-1229, USA

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Abstract

The present study was designed to establish a dose-response relationship for the efficacy of the ketogenic diet (KD). Sprague–Dawley rats were fed ketogenic diets containing varying ratios of fats; (carbohydrates + proteins) whereas control animals were fed rodent chow. Unless otherwise indicated, all animals were fed calorie-restricted, isocaloric diets beginning at P37 and ketonemia, seizure threshold and neurotoxic effects were determined. Despite being provided isocaloric quantities, animals fed lower ketogenic ratios gained weight relative to those fed diets having greater proportions of fats. A significantly increased metabolic rate was noted for animals fed a high-fat diet, suggesting a basis for the weight differences. Results also showed that the animals fed calorie-restricted high-fat diets exhibited significant ketonemia and protection from pentylenetetrazole (PTZ)-induced seizures. There were no detectable neurotoxic effects for any diet group. For animals of the same age, there was no correlation between β-hydroxybutyrate (β-OHB) and seizure threshold. These findings suggest that β-OHB is not directly involved in the anticonvulsant mechanism of the diet. Also, data presented here show that the conventional 4:1 ketogenic diet does not confer the greatest level of seizure protection. We conclude that a 6:1 ketogenic diet, which shows no evidence of neurotoxicity, may be maximally efficacious in rats.

Keywords: Ketogenic diet; Pentylenetetrazole; Calorie-restriction, Neurotoxicity; β-hydroxybutyrate; Metabolism

1. Introduction

The ketogenic diet (KD) is a high fat, low carbohydrate, low protein diet that was originally described as a treatment for human epilepsy in the early 1920s (Wilder, 1921). Despite clinical reports of its success for over 75 years (Wilder, 1921; Peterman, 1925; Helmholz and Keith, 1930; Huttenlocher et al., 1971; Livingston, 1972; De Vivo et al., 1973; Schwartz et al., 1989) interest in the diet had waned until recently (Wheless, 1995).

The efficacy of the diet as a treatment for human epilepsy has been well established. It has been shown that more than two-thirds of those children that are maintained on the KD exhibit $>50\%$ improvement from seizures (Kinsman et al., 1992; Freeman et al., 1994; Swink et al.,

* Corresponding author. Tel.: +1-202-6875905; fax: +1-202-6875662.

E-mail address: eaglesd@gusun.acc.georgetown.edu (D.A. Eagles)

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1997). Moreover, the protection afforded by a KD does not appear to be limited to particular seizure types (Freeman et al., 1998).

Despite the clinical successes of this treatment, there have been remarkably few studies of the anticonvulsant mechanism of action and fewer still defining the parameters for treatment (Nordli and De Vivo, 1997). Of these, there have been reports of diminished seizure intensity (Millichap et al., 1964), an increased resistance to maximal seizures (Uhlemann and Neims, 1972) and indications of an elevated seizure resistance (Appleton and De Vivo, 1974; Nakazawa et al., 1983; Otani et al., 1984; Hori et al., 1997).

Animal studies, however, have employed a wide variety of diet compositions, diet treatments (i.e. calorie restriction vs. ad libitum), species and genera, age at onset, duration of diet treatment and method of seizure induction. Cross-comparisons have been difficult. While adding to the variety with the introduction of another rat model (Bough and Eagles, 1999), we have shown that age at diet onset, calorie-restriction and type of seizure test are important variables in a single species and dietary regimen (Bough et al., 1999).

One of the clinical hallmarks of success for the KD is a notable accumulation of ketone bodies in blood plasma, particularly β-hydroxybutyrate (β-OHB) (Freeman et al., 1994). Accordingly, there has been considerable speculation regarding the role of β-OHB as an anticonvulsant. Despite early evidence suggesting that the infusion of ketone bodies was anticonvulsant (Helmholz and Keith, 1930), such a role has not yet been clearly defined. Although correlation between plasma levels of β-OHB and seizure threshold has been demonstrated (Appleton and De Vivo, 1974; Bough and Eagles, 1999), these correlative data do not implicate β-OHB as the cause of seizure resistance. Further analysis of the relationship between β-OHB and seizure threshold has more closely associated seizure protection with age (at diet onset) rather than the level of ketosis (Bough et al., 1999). Therefore, the present study was intended to: (1) further examine the relationship between plasma levels of β-OHB and seizure threshold; (2) establish a dose–response relationship for the efficacy of the diet in seizure protection, and (3) behaviorally analyze potential neurotoxic effects of the high-fat diets.

2. Methods

2.1. Animals

Two hundred and seventy male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) were housed five to a cage at a temperature of 21 ± 1°C, on an alternating 12:12 light: dark cycle with lights on at 07:00 h. All animals began diet treatment at P37 (mean weight $= 130 \pm 5$ g).

2.2. Diet treatment

Animals were divided into groups of 5–35 and fed high-fat (ketogenic) diets of different ratios (i.e. fats: [carbohydrates + proteins]). Table 1 shows ratios that are expressed in terms of metabolizable constituents (including carbohydrates in the vitamin mix). A detailed description of all ingredients of a KD has been reported previously (Bough and Eagles, 1999). All animals were fasted for 6 h prior to the initiation of experimental and control diets. Animals were fed individually once each day, beginning between 15:00 and 17:00 h, and allowed to feed for at least 2.5 h. Control animals were fed a normal (rodent chow) diet (Table 2). Except for those animals fed a normal (rodent chow) diet ad libitum (NAL), all diets were fed in quantities designed to make them both isocaloric and calorie-restricted to $\sim 90\%$ of the normal daily requirement (Appleton and De Vivo, 1974; Rogers, 1979). Water was provided for all animals ad libitum throughout each experiment.

2.3. Determination of weight-specific metabolic rate

Twenty-five of the animals were used for a study of metabolism and were not subjected to prior testing. They were divided into three diet groups: (1) a calorie-restricted KD (6.3:1) group ($n = 9$); (2) a calorie-restricted normal (rodent chow) diet group ($n = 9$) and (3) a NAL group ($n = 7$). As with the other studies, all animals

Table 1
Constituents of the various high-fat (ketogenic) diets (KD) expressed by percent weight[a]

Diet ingredients	1:1	3:1	4:1	5:1	6:1	6.3:1[b]	8:1	9:1
Fat	25.4	61.8	68.3	72	76.3	76.7	81.2	82.9
Protein	35.2	14.9	12.3	10.6	9.2	8.8	7.3	6.6
Fiber	12.7	7.3	6.1	5.5	4.6	4.6	3.8	3.4
Ash[c]	8.0	4.8	4.0	3.6	3.0	2.9	2.4	2.1
Moisture	9.0	5.6	4.6	4.5	3.4	3.6	2.5	2.4
Vitamin mix[d]	7.0	4.2	3.7	2.9	2.7	2.64	2.2	2
Carbohydrates	2.7	1.8	1.0	0.9	0.8	0.76	0.6	0.6

[a] Ratios (1:1, etc.) represent fats: [proteins + carbohydrates] and are calculated to within $\pm 2\%$.

[b] KD # F3666 (BioServ, Frenchtown, NJ) that we have used previously (Bough and Eagles, 1999).

[c] Formula AIN-76 Mineral Mix # F8505 contains the following (gm kg^{-1}): calcium phosphate; dibasic 500.0; potassium citrate 220.0; sucrose 117.53; sodium chloride 74.0; potassium sulfate 52.0; magnesium oxide 24.0; ferric citrate 6.0; manganese carbonate 3.5; zinc carbonate 1.6; chromium potassium sulfate 0.55; calcium phosphate; tribasic 0.5; cupric carbonate 0.3; potassium iodate 0.01 and sodium selenite 0.01.

[d] Formula AIN-76 Vitamin Mix # F8000 contains the following (gm kg^{-1}): sucrose 971.73; vitamin E acetate 20.0; niacin 3.0; calcium pantothenate 1.6; retinyl palmitate 1.0; pyridoxine 0.7; riboflavin 0.6; thiamine 0.6; vitamin D3; (400 000 IU gm^{-1}) 0.25; folic acid 0.2; menadione sodium bisulfite (33%) 0.2; vitamin B12 (1%); 0.1 and biotin 0.02.

began experimental diets at age P37. Metabolism was measured at 7-day intervals and two measurements were made before animals began experimental diets in order to obtain baseline metabolic rates.

Metabolic rate was measured by indirect calorimetry. Animals were placed within a plexiglass cylinder having a total volume of 1 l. Exhaled CO_2 was absorbed by Soda Lime, Indicating Type (4–8 mesh) (J.T. Baker, Phillipsburg, N.J.) and water vapor was absorbed by Indicating Drierite (anhydrous $CaSO_4$) (W.A. Hammond Drierite Company, Xenia, OH). Both absorbents were mechanically isolated from the animals. Tubing attached to the metabolic chambers permitted intermittent aeration, without opening the chamber, and a syringe was used to calibrate the gas volume in a chamber (chamber volume minus the volume of the absorbents and the rat) and calibration volumes were recorded with each experimental trial. Measurements were made at room temperature (20–22°C) for brief periods (from 1 to 5 min), interrupted by approximately equal intervals in which chambers were purged with fresh air. Two to five measurements were taken from each animal every 7 days. Chamber temperature never differed from room temperature by more than 2°C. Neither these temperature changes nor ambient barometric pressure were factored into volume calculations because each contributed a negligible error ($<$ 1.4%).

Pressures were measured using pressure transducers of either of two types (Model PNEU05 or Model BLPR) connected to a Transbridge transducer amplifier, all manufactured by World Precision Instruments (Sarasota, FL) and were calibrated for volume using a syringe as described above. Data were collected using CODAS software (DATAQ Instruments, Akron, OH).

Measurements of metabolism for all 25 animals were made between 13:00 and 17:00 h each week by a class of students previously trained in such measurements and were conducted blind with re-

Table 2
Constituents of the normal (rodent chow) diet[a]

	Normal diet[b]
1.5%	Saturated fat
8.5%	Unsaturated fat
23.4%	Protein
5.3%	Fiber
6.9%	Ash
49.0%	Carbohydrates

[a] Values shown in percent weight.

[b] Denotes Laboratory Rodent Chow, Purina # 5001.

spect to the identity of the animals, which were rotated among the groups making measurements.

2.4. Analysis of ketosis

Blood concentrations of β-OHB were measured spectrophotometrically using a StatSite Meter (GDS Technology, Elkhart, IN) and taken as an indication of ketosis. Blood samples (0.1 ml) were collected at the same time of day (13:00–16:00 h) from the tail vein of the rat after 19 days of diet treatment and immediately analyzed (25 μl).

2.5. Pentylenetetrazole seizure induction

All animals were maintained on their respective diet treatments for 20–21 days prior to seizure testing and were tested at age P57–P58. Pentylenetetrazole (PTZ) was dissolved in bacteriostatic saline (Abbot) to a final concentration of 10 mg ml^{-1} prior to each experiment. PTZ was infused into the tail vein via a 27-gauge needle at a rate of 1.0 ml min^{-1} using a syringe pump (Stoelting, Model 100) as described previously (Foote and Gale, 1984; Pollack and Shen, 1985). The threshold dose of PTZ (mg kg^{-1}) was derived from the time at which the rat first exhibited bilateral forelimb clonus. All animals were seizure-naïve when tested and were subjected to seizure testing only once. Seizures were always induced between 13:00 and 17:00 h to minimize possible complicating effects of circadian rhythms (Woolley and Timiras, 1962; Löscher and Fiedler, 1996).

2.6. Neurotoxic assessment

To assess the potential neurotoxic effects of treatment with high-fat KDs, behavioral tests were performed on Day −3 and Day 19 relative to the initiation of experimental diets. Six behavioral tests were performed: (1) positional sense; (2) righting reflex; (3) gait and stance; (4) muscle tone; (5) equilibrium and (6) rotorod (Swinyard et al., 1952). For the positional test, the hind leg was pulled over the edge of a table to observe if the rat could quickly pull it back into a normal position. For the righting-reflex test, the rat was placed in a supine position to determine its ability to return to an upright stance. The gait and stance test noted any alteration in gait, such as a zig-zag or circular motion. Palpation of the upper part of the hind leg was used to assess muscle tone. For a test of equilibrium, the rat was placed on the edge of a polycarbonate colony cage ($\sim$ 1 cm in width) and the ability of the rat to walk along this edge was noted. The rotorod test was completed to supplement the aforementioned tests in order to determine any loss of motor-reflex coordination. The rotorod was 2.5 cm in diameter and revolved at 5.5 r.p.m. The rats' inability to maintain equilibrium on the rod for at least 1 min (in three attempts) was taken as a failure (Swinyard et al., 1952; Dunham and Miya, 1957). Test scores were based on the following 3-point scale: 2 = strong/quick response; 1 = positive response; 0 = failure (Table 3). Failure in any one of the above-mentioned tests was considered an indication of a minimally neurotoxic dose (KD ratio) for that animal.

2.7. Statistics

Differences between the means were compared by Student Newman–Keuls tests as performed by SAS (Figs. 1–3). Regression analysis was performed using Sigma Stat for Fig. 4.

3. Results

3.1. Weight gain, metabolism and health

Animals fed a higher proportion of proteins and carbohydrates (i.e. lower ketogenic ratios) gained more weight than did animals fed isocaloric diets containing larger proportions of fat. The mean weights for each of the groups at the time of diet testing (20–21 days after diet treatment) are depicted in Fig. 1A. Animals fed higher ketogenic ratios exhibited significantly smaller weights ($P < 0.05$, Newman–Keuls).

Fig. 1B shows the weight-specific metabolism for animals fed a calorie-restricted KD, a calorie-restricted normal diet or a NAL. Animals fed a calorie-restricted KD exhibited a significantly in-

Table 3
Neurotoxicity tests for animals fed high-fat (ketogenic) or normal diets[a]

Behavioral test	N, ad lib ($n=5$)	N ($n=10$)	1:1 ($n=15$)	4:1 ($n=15$)	6.3:1[b] ($n=15$)	8:1 ($n=15$)	9:1 ($n=15$)
Positional	2	1.9	2	1.9	1.9	1.9	1.8
Righting	2	2	2	2	2	2	2
Gait and stance	2	2	2	2	2	2	2
Muscle tone	2	2	2	1.9	2	1.8	1.7
Equilibrium	1.6	1.9	2	1.9	2	2	2
Rotorod	1.6	1.7	1.1[c]	1.8	2	1.9	1.8
Weights	288[c]	127[e]	146[d]	126[e]	102	100	99
($\pm$SD)	($\pm$18.4)	($\pm$5.3)	($\pm$11.6)	($\pm$6.6)	($\pm$7.8)	($\pm$9.8)	($\pm$11.9)

[a] For high-fat diets, ratios represent the proportion of fats: (carbohydrates + proteins). Behavioral tests were completed as described previously (Swinyard et al., 1952) and were scored quantitatively on a 3-point scale (see Section 2). Mean scores are reported for each test. Behavioral tests were completed on 3 days prior to and 19 days after diet treatment. The number of animals from each group tested is indicated within each column.

[b] KD # F3666 (BioServ, Frenchtown, NJ) that we have used previously (Bough and Eagles, 1999).

[c–e] Mean weights ($\pm$SD) with different letters were determined to be significantly different ($P<0.05$).

creased metabolic rate 7 days after diet treatment began ($P<0.05$, Newman–Keuls). Moreover, this increased level of metabolism for ketogenic animals was maintained for all time points tested (7, 14, 21, 28 days) as compared to controls ($P<0.05$, Newman–Keuls). Animals fed calorie-restricted high-fat (ketogenic) diets did not show any ill effects, nor did calorie-restricted normal controls (Table 3). All animals remained well groomed, all diets were readily consumed and there were no animal deaths during these experiments.

3.2. Effects of diet ratio on ketosis

As depicted in Fig. 2, all animals fed calorie-restricted high-fat diets ($\geq$1:1) exhibited a significantly higher blood level of β-OHB compared to calorie-restricted controls. The mean blood level of β-OHB for animals fed a calorie-restricted normal diet was significantly less than that of the 1:1 group ($P<0.004$). The mean β-OHB level for animals fed a 1:1 diet was significantly less than that of animals in 3:1–8:1 groups ($P<0.05$ for all, Newman–Keuls). Comparatively, the β-OHB level for those animals fed a 9:1 KD was significantly greater than that of animals fed a 3:1–6.3:1 KD ($P<0.05$), but was statistically similar to that of animals fed an 8:1 KD. We have noted previously that the blood level of β-OHB for animals fed a NAL is statistically identical to that of animals fed a calorie-restricted normal diet and, therefore, those data are not represented here (Bough et al., 1999).

3.3. Effects of diet ratio on seizure threshold

The data represented in Fig. 3 are taken from the same group of animals represented in Fig. 2. In comparison to those animals fed a NAL (rodent chow), all animals fed high-fat diets in excess of 3:1 exhibited a markedly greater resistance to seizures ($P<0.05$, Newman–Keuls). The efficacy of the KD was significantly greater for animals fed diets exceeding a 6:1 ratio, compared to those fed 4:1 or 5:1 ratios ($P=0.009$ and 0.02, respectively). Although the differences in seizure threshold for calorie-restricted and ad libitum controls were marginally significant ($P=0.05$), we have previously shown that animals fed a calorie-restricted normal diet exhibited a markedly higher seizure resistance compared to animals fed the same diet ad libitum (Bough et al., 1999).

3.4. Ketosis as a predictive index for seizure threshold

Fig. 4 further examines the relationship between β-OHB and seizure threshold for animals of the same age at diet onset and shows no predic-

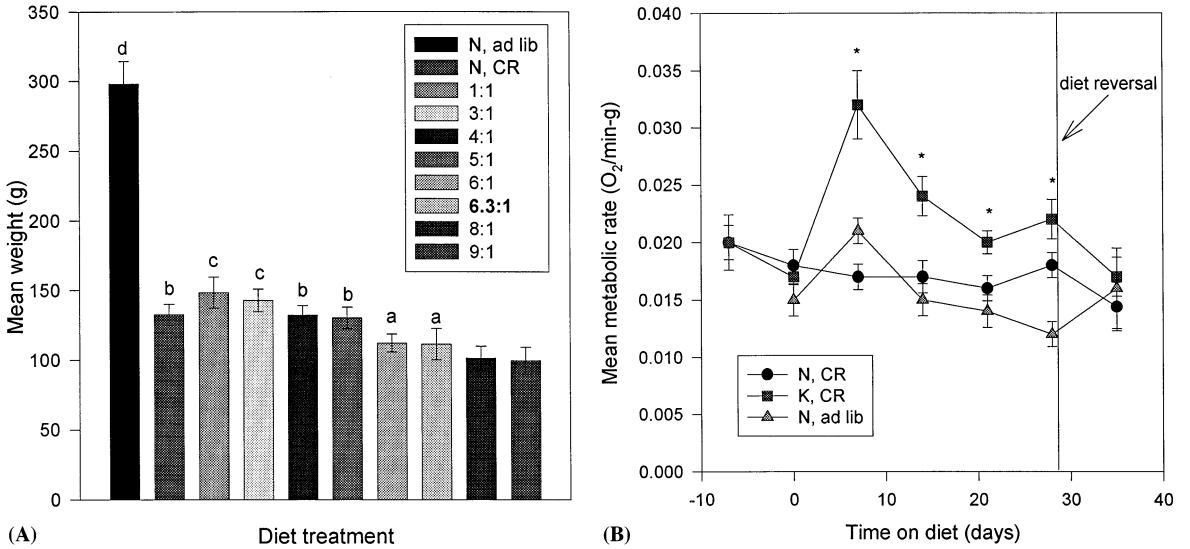

Fig. 1. A. Mean weights for animals fed each of the respective diet treatments, either a ketogenic or normal diet. Animal weights were taken at the time of seizure testing, after 20 days of diet treatment (age P57). Except for those animals fed a normal diet ad libitum, all diets were calorie-restricted and isocaloric (see Section 2). N, ad lib; normal diet, ad libitum; N, CR normal diet, calorie-restricted; pairs of numerals represent ketogenic diet (KD) ratios. The 6.3:1 KD is highlighted since it is the diet we have used previously (Bough and Eagles, 1999). Each bar represents the mean weight ± SD for each diet group. B. Weight-specific metabolic rate for animals fed a calorie-restricted ketogenic diet (K, CR) (6.3:1), a calorie-restricted normal diet or a normal diet ad libitum (NAL). Measurements were made prior to (days −7, 0) and after diet treatment began (days 7, 14, 21, 28) where * denotes $P < 0.05$, Newman–Keuls. After 28 days of diet treatment, animals fed calorie-restricted ketogenic and calorie-restricted normal diets were returned to normal chow ad libitum (diet reversal).

tive relationship. The slope of this regression line was not significant ($P = 0.126$) showing no positive correlation between β-OHB and seizure threshold ($r^2 = 0.04$).

3.5. Neurotoxic effects of high-fat diets

Prior to diet treatment (day −3), all animals exhibited normal responses to all neurobehavioral tests. After 19 days of diet treatment, all but three animals (87/90) passed all the neurobehavioral assessment tests. Of these three animals that failed the rotorod test, two were from the 1:1 KD group and one was fed a calorie-restricted normal diet.

The results of neurotoxicity tests were semi-quantitatively scored (see Section 2) and are presented in Table 3. As scored, these results show that the animals fed a 1:1 KD did significantly worse on the rotorod as compared to other animals fed either calorie-restricted KDs, a calorie-restricted normal diet or a NAL ($P < 0.05$). Analysis of the same data using the ± scale of Swinyard et al. (1952), however, shows that this disparity is insignificant as 13/15 of the 1:1 ketogenic animals completed all neurobehavioral testing.

4. Discussion

Prior work has suggested that the classical (LCT) KD [formulated by Wilder (1921)] must be rigidly adhered to and exceed a ratio of 1.5:1 (fats: [carbohydrates + proteins]) in order to produce significant ketonuria/ketonemia (Withrow, 1980). Clinically, the KD is most commonly implemented in a 4:1 ratio (Freeman et al., 1994). Whereas the efficacies of high-fat diets have been compared qualitatively (i.e. MCT vs. LCT, Huttenlocher et al., 1971), we know of no studies that have evaluated quantitative differences. The present study characterizes the efficacy of KDs

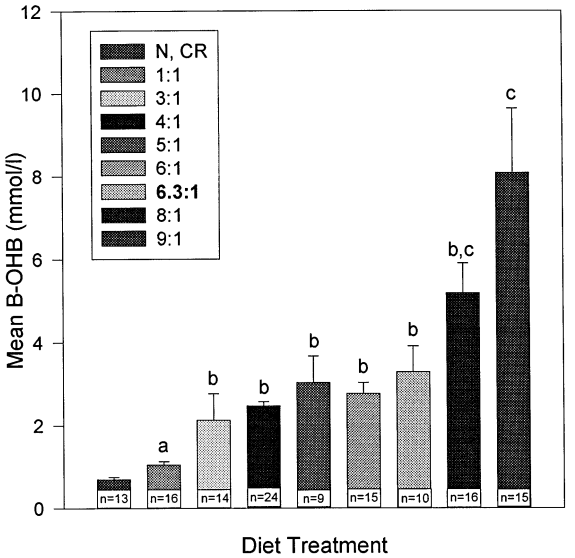

Fig. 2. Levels of β-hydroxybutyrate (β-OHB) for animals maintained on high-fat (ketogenic) diets of different ratios (fats: carbohydrates + proteins). N, CR animals were fed a calorie-restricted rodent chow diet. The different diet ratios tested are displayed in the legend. Blood samples were collected after 19–20 days and analyzed spectrophotometrically. Each bar represents the mean of the numbers of animals indicated within. Bars marked with different letters represent values that are significantly different from each other ($P < 0.05$). The 6.3:1 KD is highlighted since it is the diet we have used previously (Bough and Eagles, 1999). Error bars are ± SEM.

and shows that both ketonemia (Fig. 2) and seizure threshold (Fig. 3) are highly dependent upon the ratio of fats: [carbohydrate + proteins] in the diet but that seizure threshold in rats depends weakly, if at all, upon the level of ketonemia (as β-OHB).

4.1. Metabolic rate and weight gain

Surprisingly, there were significant differences in mean weights for animals fed KDs despite all diets being calorie-restricted and isocaloric (Fig. 1A). For those animals fed a greater percentage of proteins and carbohydrates (i.e. lower ketogenic ratios), there were marked increases in body weight. Although it is possible that imprecise measurements of daily amounts of each of the KDs resulted in significant differences in weight, this seems improbable as results show a consistent stair-step pattern of mean body weight (Fig. 1A) and all diets were prepared independently. Rather, it appears that increased proportions of dietary fat decreased weight gain. Intracerebroventricular infusion of β-OHB has been shown to limit weight gain without decreasing food intake, suggesting that heightened levels of β-OHB result in an increased metabolic activity or heat production (Davis et al., 1981). This conclusion is supported by data showing that long-term fasting stimulates a sympathetic, thermogenic response (Landsberg and Young, 1978; Sakaguchi et al., 1988) and data presented here showing that animals fed a KD exhibited a significantly elevated metabolic rate concomitant with minimal weight gain (Fig. 1).

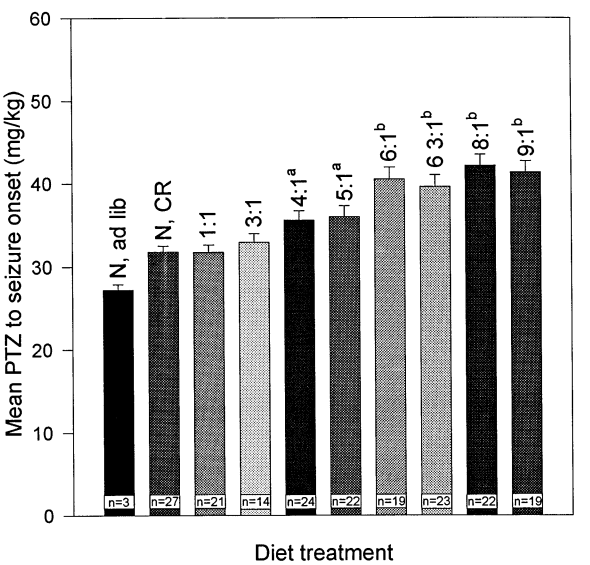

Fig. 3. Dose of pentylenetetrazole (PTZ) required to produce first clonus in animals maintained on high-fat (ketogenic) diets of different ratios (fats: carbohydrates + proteins) and normal (rodent chow) diets (either ad libitum or calorie-restricted). The different diet ratios tested are shown above each bar. Animals were tested after 20–21 days of diet treatment and were seizure-naïve when tested. Each bar represents the mean of the numbers of animals indicated within. Bars marked with different letters represent differences that are significant ($P < 0.05$). N, ad lib, normal diet ad libitum (NAL); N, CR, normal diet, calorie-restricted. The 6.3:1 KD is the diet we have used previously (Bough and Eagles, 1999). Error bars represent ± SEM.

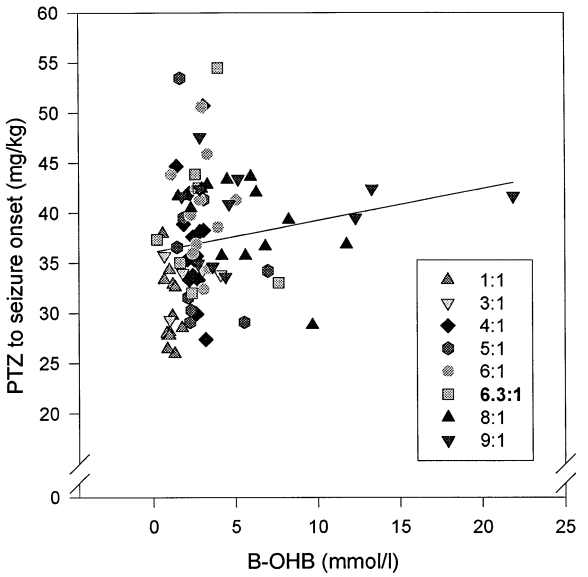

Fig. 4. Relationship between plasma levels of β-hydroxybutyrate (β-OHB) and seizure threshold for high-fat (ketogenic) diets (fats: carbohydrates + proteins). Seizure threshold and blood levels of β-OHB were determined as described in Figs. 1 and 2. Different symbols (legend) indicate the values for individual animals. The 6.3:1 KD is highlighted since it is the diet we have used previously (Bough and Eagles, 1999). Regression analysis of these data (SAS) gave an $r^2 = 0.04$.

4.2. The importance of calorie restriction

It is apparent from Fig. 2 that the level of ketonemia is highly correlated to the ketogenic ratio of the diet. Despite a previous review suggesting that, for humans, only KDs of greater than 1.5:1 would produce significant elevations in ketonuria and ketonemia (Withrow, 1980), rats fed a calorie-restricted 1:1 high-fat diet exhibited significant ketonemia (Fig. 2). Although animals fed either a calorie-restricted or a NAL do not exhibit ketonemia (data not shown), it seems likely that caloric restriction augments ketonemia in animals fed KDs, perhaps by limiting the potential exogenous precursors for gluconeogenesis (Owen et al., 1967; Cahill, 1970).

For animals fed high-fat diets, changes in seizure threshold parallel changes in ketonemia. As shown in Fig. 3, the mean level of seizure resistance significantly increased for animals fed diets containing a larger percentage of fat. Moreover, animals fed a calorie restricted normal diet had a minimally significant ($P = 0.05$) elevation in seizure resistance in comparison to animals fed the same diet ad libitum. These results (Fig. 3), in addition to data from previous work (Bough et al., 1999), suggest that calorie restriction serves to further increase the resistance to seizures, independent of ketonemia.

4.3. Is the conventional 4:1 ketogenic diet maximally efficacious?

Interestingly, KDs with higher ratios of fats: [carbohydrates and proteins] produce a significantly greater seizure threshold (Fig. 3) while showing no signs of neurobehavioral deficit (Table 3). In light of this finding, and recent studies showing that rats fed a similar KD do not exhibit any deficits in learning and memory (Hori et al., 1997), we suggest that the conventional 4:1 KD may not be maximally efficacious in this species. Moreover, rats tolerate diets of high ketogenic ratio for extended periods of time, as we could not discern any deleterious effects in rats maintained on a 6.3:1 KD for 268 days. As shown in Fig. 3, animals fed a 6:1 KD showed a marked increase in seizure resistance over those fed 4 or 5:1 KDs, yet there was no significant increase in seizure protection for animals fed a KD greater than 6:1. Clearly, several other issues must be considered clinically (e.g. protein RDA's, hypercholesterolemia, growth/weight gain, dietary compliance, etc.) that might argue against use of such high ketogenic ratios for human diets.

4.4. β-hydroxybutyrate and seizure threshold are not correlated

Fig. 4 shows the relationship between β-OHB and seizure threshold. When all animals fed KDs are considered (and age is controlled), the range of β-OHB (mean values from 2.1 ± 0.6 to 8.1 ± 1.6) bears no relationship to the level of PTZ required to produce a first clonus in seizure-naïve rats. The poor correlation between the level of ketonemia (over a range of greater than 20-fold within the experimental population) and seizure threshold strongly suggests that these two vari-

ables (β-OHB and seizure threshold) are independent consequences of the consumption of KDs. It seems likely that there is either a threshold level of ketonemia involved in seizure protection (beyond which no additional protection is noted) or that ketonemia, per se, has no role in seizure resistance. Age is an important variable, as an earlier study (Bough et al., 1999) showed that both β-OHB levels seizure threshold (to PTZ infusion) were inversely correlated with age at diet onset.

4.5. Fasting versus high-fat diets — distinct mechanisms of protection?

KDs were originally developed to mimic fasting and they have been extremely successful in producing seizure protection for >75 years (Withrow, 1980; Prasad et al., 1996; Nordli and De Vivo, 1997). Because both starvation (De Vivo et al., 1975) and KDs protect against seizures (Freeman et al., 1994, 1998; Prasad et al., 1996; Nordli and De Vivo, 1997), there seems to be a tacit assumption that high-fat diets and fasting confer seizure protection by (a) common mechanism(s). The present study, however, found no correlation between β-OHB and seizure threshold (Fig. 4). This result is consistent with other findings. First, animals fed a calorie-restricted KD showed a marked elevation in β-OHB within 5 days (Bough and Eagles, 1999), while seizure protection was not noted until after 10–12 days of diet treatment (Appleton and De Vivo, 1974; Bough and Eagles, 1999). Second, seizure threshold was significantly elevated in animals fed calorie-restricted normal diets despite minimal levels of β-OHB (Bough et al., 1999). Third, we were unable to produce protection from PTZ-induced seizures in animals pre-treated (-30 to -45 min) with β-OHB (either i.v. or i.p.) (data not shown). Fourth, in experiments with cultured neocortical neurons, neither L- nor D-isomers of β-OHB altered $GABA_A$ receptor, AMPA receptor, NMDA receptor, sodium or calcium voltage-dependent channel activity (Rho J., personal communication). Even though there are physiological responses common to both fasting and treatment with a KD (e.g. transient acidosis, elevation of acetoacetic and β-OHB acids, lowered respiratory quotient), these observations suggest there may be differences in the mechanisms by which KDs and fasting confer seizure protection.

In summary, rats fed diets of high ketogenic ratio exhibit elevated levels of ketonemia and elevated metabolic rates, but show reduced rates of weight gain. Furthermore, there was no evidence of neurotoxicity associated with consumption of highly KDs. How well these animal data might be applied to the clinic is not clear. Adverse effects have been described in $\sim 10\%$ of children maintained on a KD (Herzberg et al., 1990; Ballaban-Gil et al., 1998; Tallian et al., 1998; Chesney et al., 1999) and so the nature of the diet is an important clinical consideration.

When age (at diet onset) as a variable was held constant, there was no correlation between the plasma level of β-OHB and seizure threshold. This and other observations suggest that the mechanism(s) of anticonvulsant action are not likely to be a result of direct actions of β-OHB. Rather, it seems more likely that protection from seizures by fasting or by high-fat diets is multifactorial, perhaps including changes in GABA or glutamate metabolism in response to elevated levels of ketone bodies (i.e. acetoacetate and β-OHB), as has been shown recently in in vitro studies by Erecińska et al. (1996) and by Yudkoff et al. (1997).

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