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Leptin Contributes to Slower Weight Gain in Juvenile Rodents on a Ketogenic Diet

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ABSTRACT: The ketogenic diet (KD) is an efficacious therapy for medically refractory childhood epilepsy that also slows weight gain. We tested the hypothesis that the KD slows weight gain *via* neurohormones involved in energy homeostasis. We found that juvenile rodents fed a KD had slower weight gain than those fed a standard diet (SD). Rats fed a KD had higher serum leptin levels and lower insulin levels compared with those fed an SD. We investigated the increase in leptin further because this change was the only one consistent with slower weight gain. Although rats fed the SD experienced slower weight gain when calorie restricted, they had serum leptin levels similar to those fed the SD *ad libitum*. Furthermore, leptin deficient (*ob/ob*) and leptin receptor deficient (*db/db*) mice did not show slower weight gain on the KD. All animals on the KD had elevated serum β-hydroxybutyrate (βHB) levels. Thus, ketosis is insufficient and a functioning leptin signaling system appears necessary for the KD to slow weight gain. The increase in leptin may contribute to the anticonvulsant effects of the KD. **(*Pediatr Res* 60: 413–417, 2006)**

Few treatment options exist for the 20–30% of epileptic children whose seizures are refractory to medication. One option is the KD, a high-fat, low-carbohydrate, and adequate protein diet. The KD is remarkably effective in children with medically refractory epilepsy as 5–10% become seizure free and 30% have a >90% reduction in seizure frequency (1). Yet, its adverse effects and the parental effort involved in implementing and maintaining the diet limit its use. Thus, all epileptic children would benefit from a simpler method of attaining the diet's anticonvulsant effects, which requires elucidating its mechanism of action.

Here we focus on what the impaired weight gain associated with the KD might reveal about the diet's anticonvulsant mechanism. Several studies report that children on the KD drop about 10 percentiles in weight, although appropriate weight gain is the goal (2,3). Recognizing that low-carbohydrate diets, such as the Atkins diet, are ketogenic makes this impaired weight gain less surprising. Indeed, the Atkins diet and other low-carbohydrate KDs result in a 5% to 10% weight loss over 6 mo (4,5). Furthermore, rodents placed on a KD show slower weight gain (6,7).

We hypothesized that the anticonvulsant and weight effects of the KD share a common mechanism involving a change in the serum levels of leptin, insulin, ghrelin, or cortisol. These peripherally released hormones help determine body weight because they regulate energy homeostasis (8). Importantly, leptin and insulin also modulate neuronal excitability (9,10).

METHODS

Dietary protocols. Experimental protocols were approved by the Washington University Animal Studies Committee. Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), male *ob/ob* mice (Stock 000632, The Jackson Laboratory, Bar Harbor, ME), male C57BL/6J mice (Stock 000664, The Jackson Laboratory), male *db/db* mice (Stock 000642, The Jackson Laboratory), and male C57BLKS/J mice (Stock 000662, The Jackson Laboratory) were housed under a 12-h light/dark cycle. Experiments with rats began on postnatal day (PD) 21, the day of weaning, and those with mice began when *ob/ob* and *db/db* mice are phenotypically identifiable. Cages held four to six rats or two to three mice. All animals had *ad libitum* access to water. They were fed a SD (Rodent Diet 20, PicoLab, Richmond, IN), a KD (F3666; Bio-Serv, Frenchtown, NJ), or a calorie-restricted diet (CD). Animals on the SD received 12% of their calories from fat, 65% from carbohydrate, and 24% from protein. By weight, the SD was 20% protein, 5% fat, 5% fiber, 55% carbohydrate, and 6% ash. Animals on the KD received 92% of their calories from fat, 3% from carbohydrate, and 5% from protein. By weight, the KD was 8% to 9% protein, 75% fat (45% lard, 19% butter, and 10% corn oil), 4% fiber, 3% ash, 4% to 6% carbohydrate, <10% moisture, and 2% vitamin. The KD had a 6:1 ratio of fat to carbohydrate + protein by weight. Animals on the SD and the KD had *ad libitum* access to chow. Animals on the CD received about 40% of the calories that those on the SD received. Animals on the CD were fed at 11:00 a.m., and they consumed all allotted chow in two hours. Caloric intake was calculated by weighing the chow consumed by one cage daily and assuming all animals had the same caloric intake when normalized to body weight. Animals were killed on PD 26 to 50 between 1000 and 1400 h. Although leptin and insulin are at their nadir at this time, their diurnal variation is markedly diminished with fasting and that of leptin is markedly diminished with high-fat diets (11–13). Blood was collected by a right ventricular cardiac tap.

Dual energy x-ray absorptiometry (DEXA) scans. The percentage body fat was obtained by scanning the torso and limbs of rats using the PIXImus

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Abbreviations: βHB, β-hydroxybutyrate; **CD,** calorie restricted diet; ***db/db*,** homozygous leptin receptor deficient mice; **DEXA**, dual energy x-ray absorptiometry; **KD,** ketogenic diet; ***ob/ob*,** homozygous leptin deficient mice; **PD,** postnatal day; **SD,** standard diet

densitometer and software (GE Lunar, Madison, WI) under ketamine (40 mg/kg) and xylazine (10 mg/kg) anesthesia.

Assays. Serum glucose concentrations were measured using a glucometer. Serum βHB concentrations were determined enzymatically except as shown in Figure 3 in which the KetoSite Test Kit (Stanbio Laboratory, Boerne, TX) and STAT-Site analyzer (GDS Diagnostics, Elkhart, IN) were used. Enzymatically determined values typically were higher. Serum cortisol, ghrelin, insulin, and leptin levels were determined by radioimmunoassay (DiaSorin S.p.A., Stillwater, MN or Linco Research, Inc., St. Charles, MO).

Data analysis. Plots of body weight or normalized caloric intake *versus* PD were compared using a repeated-measures analysis of variance (ANOVA) (SPSS, Chicago, IL). Means were compared by a *t* test or ANOVA with Tukey's *post hoc* comparison of means (OriginLab, Northampton, MA). Linear fits were obtained by linear regression. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

RESULTS

Juvenile Sprague-Dawley rats gained weight more slowly on a KD. We fed juvenile Sprague-Dawley rat littermates a KD or SD for 2 wk. We chose juvenile rats because younger animals are better adapted for ketosis (14). The KD chow was the same chow used in several other rodent studies (6,7,15). Rats tolerated the KD without difficulty. They appeared as healthy and active as their littermates fed the SD.

The littermates on the KD gained weight significantly more slowly than their siblings fed the SD as noted previously (6,7) (Fig. 1*A*). Heart, liver, and kidney wet weights were 33% to 48% lower while brain mass was preserved after 2 wk on the KD (Fig. 1, *B–E*). Aside from differences in mass, organs from rats fed the KD were not grossly different from those fed the SD except for the liver being fatty. To determine whether the effect of the KD on body weight was reversible, we placed rats on the SD from PD 21 to PD 25, the KD diet from PD 26 to PD 30, and the SD from PD 31 to PD 35. These animals had a slower growth rate while on the KD that reversed on return to the SD (Fig. 1*A*). Interestingly, rats on the KD had higher normalized caloric intakes than those on the SD (Fig. 1*F*). Thus, the KD reversibly slowed weight gain including that of lean body mass in juvenile rats while maintaining brain growth.

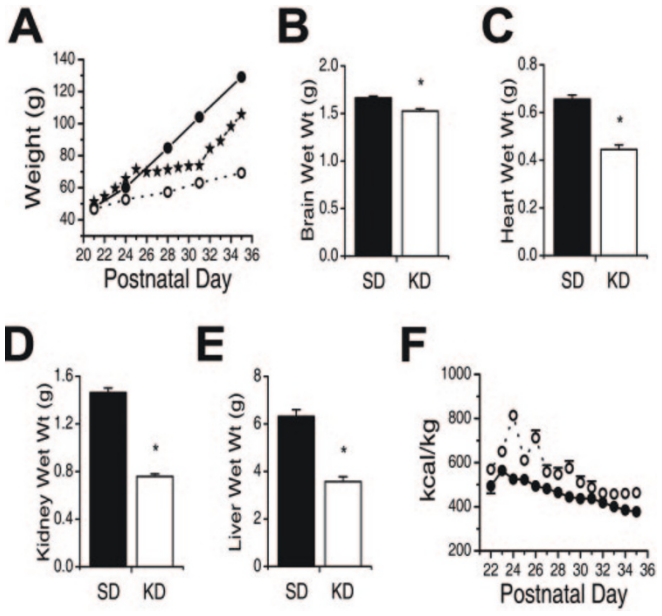

Figure 1. Rats fed a KD show slower weight gain. (*A*) Weight curves for rats on the SD (*filled circles*, $n = 25$) from PD 21 to PD 35, the KD (*open circles*, $n = 26$) from PD 21 to PD 35, or the KD from PD 26 to PD 30 (*stars*, $n = 12$). Symbols show mean body weight. Weight curves for rats on the SD and KD from PD 21 to PD 35 differ ($p < 0.05$). Normally growing rats on the SD show slower weight gain when placed on the KD and resume normal weight gain upon return to the SD (*stars*). (*B–E*) Mean organ wet weights at PD 35 from rats fed the SD (*filled columns*, $n = 25$) or the KD (*open columns*, $n = 26$) since PD 21. *$p < 0.05$ *vs* SD. (*F*) Curves for caloric intake on the SD ($n = 7$) and KD ($n = 11$) differ ($p < 0.05$). Symbols show mean caloric intake normalized to body weight and correspond to diets in *A*. Data in *A–E* are from a different experiment than the data in *F*. Error bars show SEM and are shown in *A* and *F* if larger than the symbol.

Increased serum leptin in juvenile Sprague-Dawley rats fed a KD. One mechanism by which the KD could slow weight gain is by altering serum leptin, insulin, ghrelin, and cortisol levels, which are hormones involved in regulating energy homeostasis (8). We hypothesized that the KD slows weight gain by increasing leptin, increasing insulin, decreasing ghrelin, or decreasing cortisol levels.

Rats on the KD for 2 wk had 10-fold higher midday serum levels of βHB and 30% lower glucose levels than rats on the SD (Fig. 2*A* and *B*). The elevated βHB levels indicated that the KD produced ketosis. The lower glucose was expected since low carbohydrate diets can lower blood glucose in humans (5,16). Rats on the KD also had 140% higher leptin levels, 72% lower insulin levels, and slightly increased cortisol levels but similar ghrelin levels at midday compared with rats on the SD (Fig. 2*C–F*). Five days on the KD produced smaller increases in serum βHB and leptin than 2 wk on the KD, with the changes being significant and reversing upon return to the SD (Fig. 2*A* and *F*). Although low-protein diets also slow weight gain, decrease insulin levels, and can increase leptin levels (17,18), they increase ghrelin levels (19), suggesting that our results do not arise solely from the low-protein content of the KD. Of the hormonal changes observed, only the increase in leptin could slow weight gain.

We examined how serum βHB and leptin varied with weight because of the known relationship between these parameters. Obese humans fed a low-carbohydrate diet experience greater weight loss with higher βHB levels (4). For both the SD and the KD, the rats with the highest βHB levels on the day of sacrifice weighed the least (Fig. 2*G*). In rodents, serum leptin levels correlate with body mass index (20). Although weight and leptin levels did not correlate in rats on the SD, rats having higher leptin levels on the KD weighed more (Fig. 2*H*). For both the SD and the KD, neither insulin nor the leptin:insulin ratio correlated with weight.

Serum leptin does not increase in juvenile Sprague-Dawley rats fed a CD. In another experiment, we compared the weight growth curves and serum leptin levels in rats fed a KD, CD, or SD. We fed juvenile rat littermates a KD, CD, or SD for 2 wk. Rats on the KD and CD gained weight more slowly than their siblings fed the SD (Fig. 3*A*). The fat mass of rats on the KD as a percentage of total mass was two to three times that of the rats on the SD or CD (Fig. 3*B*). Rats on the KD had sixfold higher βHB than rats fed either the SD or CD (Fig. 3*C*). Relative to the SD, the changes induced by the KD and CD in leptin levels correlated with the changes in fat mass (Fig. 3*D*). Rats on the CD had access to chow just before

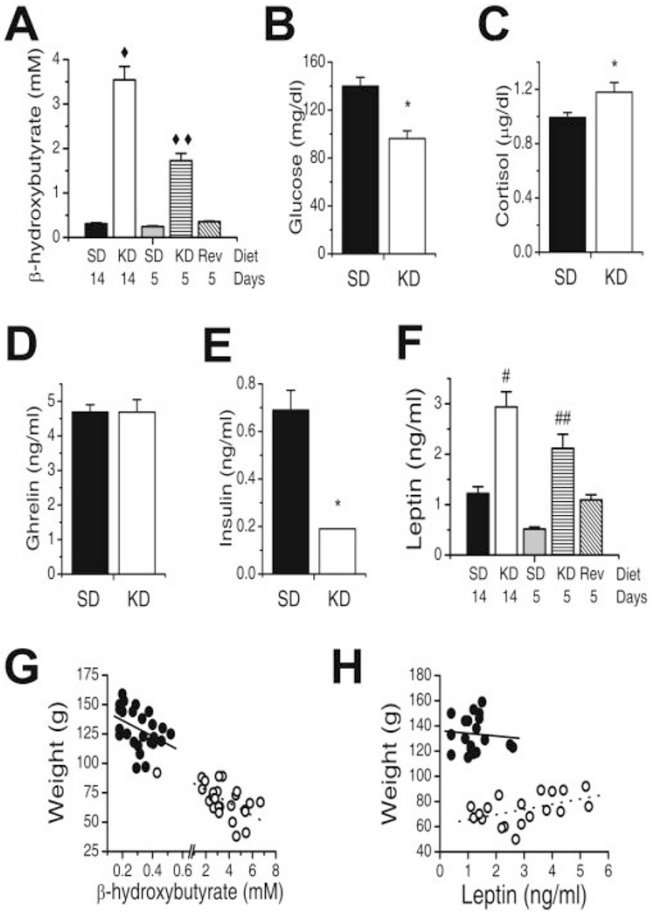

Figure 2. KD induced metabolic and hormonal changes. Mean serum βHB (*A*), glucose (*B*), cortisol (*C*), ghrelin (*D*), insulin (*E*), and leptin (*F*) levels in PD 35 rats fed the SD for 14 d (*filled columns*, $n = 11$–25), PD 35 rats fed the KD for 14 d (*open columns*, $n = 14$–25), PD 26 rats fed the SD for 5 d (*shaded columns*, $n = 6$–12), PD 26 rats fed the KD for 5 d (*horizontally striped columns*, $n = 6$–14), or PD 36 rats fed the KD from PD 26–30 (labeled *Rev*, *diagonally striped columns*, $n = 10$–23). ●, ◆◆ $p < 0.05$ *vs* all other columns. *$p < 0.05$ *vs* SD. #$p < 0.05$ *vs* left, middle, and right columns. ##$p < 0.05$ *vs* middle column. Error bars show SEM. PD 35 weight *vs* PD 35 serum βHB (*G*) and leptin (*H*) in rats fed the SD (*filled circles*) or the KD (*open circles*) for 14 d. Each point represents one rat. The lines in *G* show a linear fit for the SD (*solid line*, $R = -0.40$, $p = 0.045$) and the KD (*dotted line*, $R = -0.58$, $p = 0.002$). The lines in *H* show a linear fit for the SD (*solid line*, $R = -0.09$, $p = 0.72$) and the KD (*dotted line*, $R = 0.47$, $p = 0.04$).

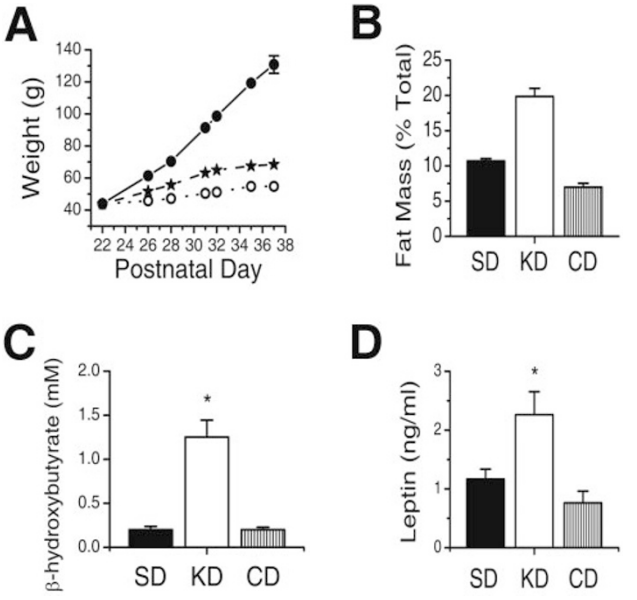

Figure 3. CD does not increase βHB and leptin. (*A*) Weight curves for rats on the SD (*filled circles*, $n = 5$), the KD (*open circles*, $n = 5$), or the CD (*stars*, $n = 4$) from PD 22 to PD 37. Symbols show mean body weight. Error bars show SEM if larger than the symbol. Weight curves on the SD, KD, and CD are significantly different from one another ($p < 0.05$). (*B*) Mean fat mass presented as a percentage of the total body mass excluding the head in PD 37 rats fed the SD (*filled column*, $n = 5$), KD (*open column*, $n = 5$), or CD (*vertically striped bar*, $n = 4$) for 16 d. All bars differ from each other ($p < 0.05$). Mean serum βHB (*C*) and leptin (*D*) levels in PD 35–37 rats fed the SD (*filled columns*, $n = 5$), the KD (*open columns*, $n = 5$), or the CD (*vertically striped columns*, $n = 4$) for 14–16 d. Error bars in *B* and *D* show SEM. *$p < 0.05$ *vs* SD and CD.

sacrifice, as did the rats on the SD and KD. For the rats on the CD, access to chow just before sacrifice should produce maximal leptin levels (11) and may decrease βHB levels. These results demonstrate that the increase in serum βHB and leptin induced by the KD does not result from slower growth.

Juvenile ob/ob and db/db mice did not gain weight more slowly on a KD. We further examined the importance of leptin and βHB to the slower weight gain induced by the KD by using leptin deficient *ob/ob* and leptin receptor deficient *db/db* mice, which are both obese. If the KD requires leptin to slow weight gain, then it should not slow weight gain in *ob/ob* and *db/db* mice. Juvenile *ob/ob* and *db/db* mice fed the KD for 12–14 d gain weight at the same rate as *ob/ob* and *db/db* mice fed the SD (Figs. 4*A* and 5*A*). These results suggest that the lower protein content and possible lower palatability of the KD do not completely explain the slower weight gain on the KD in rats and control mice (see below). The *ob/ob* and *db/db* mice maintained their weight gain despite having lower normalized caloric intakes on the KD (Figs. 4*B* and 5*B*). *ob/ob* mice on the KD were ketotic since they had 360% higher midday serum βHB levels than *ob/ob* mice on the SD (Fig. 4*C*). As expected, *ob/ob* mice on the KD and the SD had comparable low levels of serum leptin immunoreactivity (Fig. 4*D*), which probably reflects cross-immunoreactivity with the secretory form of the leptin receptor (21). Likewise, *db/db* mice on the KD had 430% higher serum βHB levels and tended to have higher serum leptin levels than *db/db* mice on the SD (Fig. 5*C* and *D*). The much higher leptin levels in *db/db* mice compared with control mice (see below) are expected (20). These results suggest that the KD requires leptin and its receptor to slow weight gain.

The background strain of the *ob/ob* and *db/db* mice did not influence the effects of the KD. C57BL/6J (background strain for *ob/ob* mice) and C57BLKS/J (background strain for *db/db* mice) mice fed the KD gained weight more slowly than those fed the SD (Figs. 4*A* and 5*A*). The KD decreased normalized caloric intakes in the C57BLKS/J but not in the C57BL/6J mice (Figs. 4*B* and 5*B*). The KD increased midday serum βHB levels in C57BL/6J mice by 10-fold and in C57BLKS/J mice by sixfold (Figs. 4*C* and 5*C*). The KD increased serum leptin levels by at least 130% in C57BL/6J and C57BLKS/J mice (Figs. 4*D* and 5*D*).

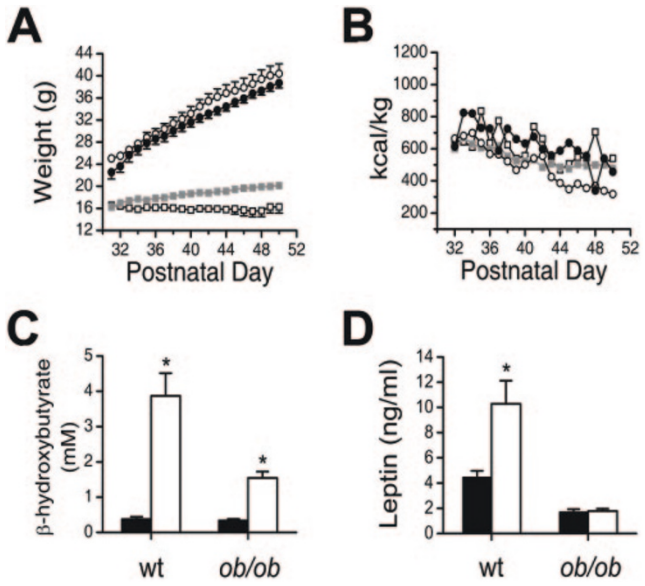

Figure 4. KD does not slow weight gain in leptin deficient *ob/ob* mice. (*A*) Weight curves for *ob/ob* mice fed the SD (*filled circles*, $n = 7$) or the KD (*open circles*, $n = 8$). Weight curves for control (C57BL/J6) mice fed the SD (*shaded squares*, $n = 8$–14) or the KD (*open squares*, $n = 7$–14). Symbols show mean body weight. Error bars show SEM if larger than symbol. Curves for the *ob/ob* mice are comparable ($p = 0.29$) while the control mice show slower weight gain on the KD ($p < 0.05$). (*B*) Curves for caloric intake in *ob/ob* mice on the SD and KD differ ($p < 0.05$), but the corresponding curves for control mice do not ($p = 0.08$). Symbols show mean caloric intake normalized to body weight for animals and diets in *A*. Error bars omitted for clarity. Mean serum βHB (*C*) and leptin (*D*) levels at PD 44–53 in control (*wt*) and *ob/ob* mice fed the SD (*filled columns*, $n = 5$-14) or the KD (*open columns*, $n = 4$–13) for 2–3 wk. Error bars show SEM. *$p < 0.05$ *vs* SD.

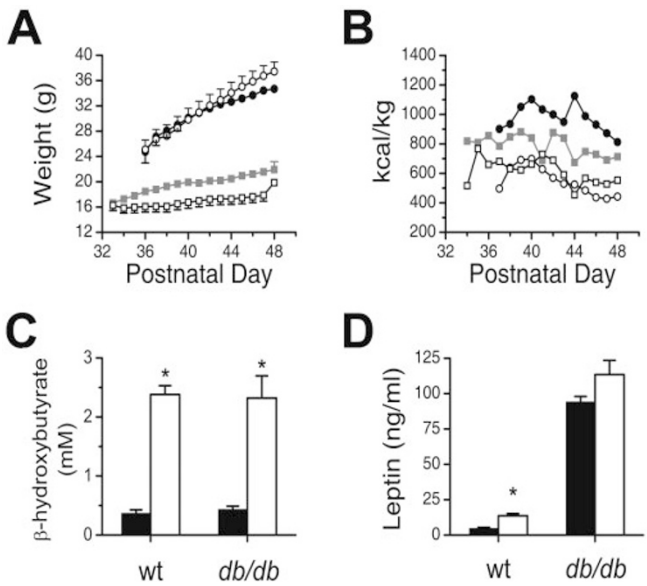

Figure 5. KD does not slow weight gain in leptin receptor deficient *db/db* mice. (*A*) Weight curves for *db/db* mice fed the SD (*filled circles*, $n = 7$) or the KD (*open circles*, $n = 8$). Weight curves for control (C57BLKS/J) mice fed the SD (*shaded squares*, $n = 4$–7) or the KD (*open squares*, $n = 5$–8). Symbols show mean body weight. Error bars show SEM if larger than symbol. Curves for the *db/db* mice are comparable ($p = 0.68$) while the control mice show slower weight gain on the KD ($p < 0.05$). (*B*) Curves for caloric intake in *db/db* mice on the SD and KD differ ($p < 0.05$) as do the corresponding curves for control mice ($p < 0.05$). Symbols show mean caloric intake normalized to body weight for animals and diets in *A*. Error bars omitted for clarity. Mean serum βHB (*C*) and leptin (*D*) levels at PD 43–51 in control (*wt*) and *db/db* mice fed the SD (*filled columns*, $n = 6$–7) or the KD (*open columns*, $n = 4$–11) for about 14 d. Error bars show SEM. *$p < 0.05$ *vs* SD.

DISCUSSION

We reproduced the KD induced slower weight gain in epileptic children in several strains of juvenile rats and mice. Animals on the KD had higher serum leptin levels, lower insulin levels, slightly increased cortisol levels, and similar ghrelin levels compared with animals fed the SD. Only the change in leptin is consistent with slower weight gain. The failure of a KD to slow weight gain in *ob/ob* and *db/db* mice supports a role for leptin in this process.

The metabolic and hormonal effects of the KD appear to provide the brain with conflicting information regarding energy status. The high leptin levels indicate an energy-replete state and probably reflect a relative increase in fat mass, which correlates with leptin levels (20). Although another study concurs with our observation of higher leptin and lower insulin levels in KD-fed rats compared with SD-fed rats (22), we cannot explain the contrasting observations in humans with rheumatoid arthritis or type 2 diabetes who exhibit lower leptin levels on a KD (16,23). The lower insulin levels on a KD signal lower energy supplies and may result from decreased carbohydrate intake or increased insulin sensitivity (16,22). The resulting change in the leptin:insulin ratio may have a role in the slower weight gain because leptin and insulin levels typically change in the same direction (8). The inverse relationship between βHB and weight suggests an important role for ketosis. The observation that rodents on nonketotic, high-fat diets gain weight faster than those on ketotic, high-fat diets supports this idea (22,24,25). Furthermore, exogenously administered βHB causes weight loss (26). Despite these observations, our results suggest that ketosis alone cannot slow weight gain because the KD caused ketosis in *ob/ob* and *db/db* mice without slowing weight gain. We do not know the reason for this discrepancy, but βHB may require leptin to alter energy balance. Overall, our results indicate that an intact leptin signaling system is necessary and that ketosis is insufficient for the KD to slow weight gain.

Our results differ from those of a recent study that found increased ghrelin levels in rats fed a KD (22). We might predict such an increase because ghrelin levels rise in association with the slower weight gain observed in rats on protein-restricted diets (19). Although the low protein content of the KD we used may have contributed to our findings (7), the lack of a change in ghrelin and three other observations suggest that it is not the only factor involved. First, the KD maintained normal weight gain in *ob/ob* and *db/db* mice. Second, rats fed the KD showed relatively well-preserved lean mass when expressed as a percentage of body mass. Third, peak leptin levels on the CD did not exceed trough leptin levels on the SD despite the slower weight gain on the CD.

Ultimately, the KD must increase energy expenditure over energy intake to slow weight gain. The KD had variable effects on normalized caloric intake despite consistently slowing weight gain. It decreased caloric intake in C57BLKS/J,

db/db, and *ob/ob* mice, had no effect on caloric intake in C57BL/J6 mice, and increased caloric intake in Sprague-Dawley rats. Although the oily consistency of the KD chow made caloric intake measurements difficult, overall our findings suggest that the KD can decrease food intake and increase energy expenditure, which are known effects of leptin (8).

Our results support the hypothesis that the KD exerts its anticonvulsant effects by activating a variety of potassium channels *via* the metabolic changes it induces (27). We speculate that the KD-induced rise in serum leptin increases brain leptin levels because brain levels are proportional to serum levels (28). We expect a diffuse increase in brain levels because leptin transporters exist throughout the brain (28) and leptin increases throughout the brain when administered exogenously (29). Leptin can act as an anticonvulsant throughout the brain because leptin receptors exist throughout the brain (28) and activate calcium-activated potassium channels (9). However, the decline in insulin may counteract some of the effects of the rise in leptin because insulin activates the same channel (10). In summary, we hypothesize that the KD produces a net anticonvulsant effect in part because increased brain leptin activates potassium channels. This mechanism may be unique among antiepileptic drugs, which do not increase leptin independent of weight gain (30–32). Our results do not exclude leptin modulation of neuronal or glial energy homeostasis or other mechanisms involving the complex metabolic and cellular effects of the KD from contributing to its anticonvulsant properties (27).

Despite our focus on leptin, other neurohormones may contribute to the anticonvulsant and weight effects of the KD. For example, the increased cortisol levels we found in rats on the KD mirrors the increase seen in children on the KD and may contribute to the anticonvulsant effect of the diet (33). Others have considered the possibility that changes in neuropeptide Y and galanin expression contribute to the effects of the KD, but the diet does not change their brain mRNA levels (34). Rather than excluding a contribution of other neurohormones, our results indicate that studying KD-induced changes in neurohormones involved in energy homeostasis may help elucidate the mechanisms underlying the anticonvulsant and weight effects of the KD.

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