Long-term ketogenic diet causes glucose intolerance and reduced β- and α-cell mass but no weight loss in mice

Johanne H. Ellenbroek,1 Laura van Dijck,2 Hendrica A. Töns,1 Ton J. Rabelink,1 Françoise Carlotti,1 Bart E. P. Ballieux,2 and Eelco J. P. de Koning1,3,4

1Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands; 2Department of Clinical Chemistry, Leiden University Medical Center, Leiden, The Netherlands; 3Department of Endocrinology, Leiden University Medical Center, Leiden, The Netherlands; and 4Hubrecht Institute, Utrecht, The Netherlands

Submitted 21 August 2013; accepted in final form 30 December 2013


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Address for reprint requests and other correspondence: E. J. P. de Koning, Leiden University Medical Center, Postal Zone C7-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands (e-mail: edekoning@lumc.nl).

HIGH-FAT, LOW-CARBOHYDRATE KETOGENIC DIETS (KD) are used in

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Am J Physiol Endocrinol Metab

erides, leptin, monocyte chemotactic protein-1, IL-1

In this study we investigate the effects of long-term KD on glucose tolerance and β- and α-cell mass in mice. Despite an initial weight loss, KD did not result in weight loss after 22 wk. Plasma markers associated with dyslipidemia and inflammation (cholesterol, triglycerides, leptin, monocyte chemotactic protein-1, IL-1β, and IL-6) were increased, and KD-fed mice showed signs of hepatic steatosis after 22 wk of diet. Long-term KD resulted in glucose intolerance that was associated with insufficient insulin secretion from β-cells. After 22 wk, insulin-stimulated glucose uptake was reduced. A reduction in β-cell mass was observed in KD-fed mice together with an increased number of smaller islets. Also α-cell mass was markedly decreased, resulting in a lower α- to β-cell ratio. Our data show that long-term KD causes dyslipidemia, a proinflammatory state, signs of hepatic steatosis, glucose intolerance, and a reduction in β- and α-cell mass, but no weight loss. This indicates that long-term high-fat, low-carbohydrate KD lead to features that are also associated with the metabolic syndrome and an increased risk for type 2 diabetes in humans.

Pancreatic islet; ketogenic diet; glucose intolerance; β-cell; α-cell

Materials and Methods

Animals. Male C57BL/6J mice, 10 wk old (Charles River Laboratories, Wilmington, MA), were fed a KD (Research Diets, New Brunswick, NJ) or regular chow (control; Special Diets Services, Essex, UK) for 22 wk. The proportion of calories derived from nutrients for the KD, which is similar to other studies (2, 12, 16, 18), and control diet is described in Table 1. In addition, 8-wk-old male C57BL/6J mice were fed a KD or a normal diet (D12450B; Research Diets) for 1 wk. Before euthanization, mice were anesthetized by isoflurane inhalation. Animal experiments were approved by the ethical committee on animal care and experimentation of the Leiden University Medical Center.

Glucose and insulin tolerance test. Glucose tolerance was assessed after 1, 5, 12, and 20 wk of diet. An intraperitoneal glucose tolerance test (GTT) was performed in overnight-fasted mice. Blood samples were drawn from the tail vein before injecting 2 g/kg glucose and after 15, 30, 60, and 120 min. Insulin tolerance was assessed after 22 wk of diet. An intraperitoneal insulin tolerance test (ITT) was performed in animals that had been fasted for 6 h. After measuring basal blood glucose concentration from the tail vein, 1.0 U/kg insulin was injected followed by monitoring of the blood glucose concentrations after 15, 30, 60, and 90 min. Blood glucose concentrations were measured using a glucose meter (Accu Chek, Roche, Basel, Switzerland) and β-hydroxybutyrate concentrations using a ketone meter (Precision Xtra System; Abbot Diabetes Care, Alameda, CA). Insulin concentrations were measured by ELISA (Ultra Sensitive Mouse Insulin ELISA kit; Chrystal Chem, Downers Grove, IL).

Plasma analysis. Plasma leptin, IL-6, IL-1β, and monocyte chemotactic protein (MCP)-1 were detected using a custom Cytokine/Metabolic multiplex assay (Mesoscale Discovery, Gaithersburg, MD).
Table 1. Diet composition

<table>
<thead>
<tr>
<th></th>
<th>Control kcal/100 kcal</th>
<th>Control g/100 g</th>
<th>KD kcal/100 kcal</th>
<th>KD g/100 g</th>
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<tr>
<td>Protein</td>
<td>27.2</td>
<td>22.5</td>
<td>5.9</td>
<td>10.0</td>
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<tr>
<td>Carbohydrate</td>
<td>61.2</td>
<td>50.4</td>
<td>1.0</td>
<td>2.0</td>
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<tr>
<td>Fat</td>
<td>11.5</td>
<td>4.2</td>
<td>93.1</td>
<td>72.0</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>16.2</td>
<td></td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.37</td>
<td></td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Choline</td>
<td>0.14</td>
<td></td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>1.9</td>
<td>0.7</td>
<td>29.6</td>
<td>22.8</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>3.0</td>
<td>1.1</td>
<td>33.7</td>
<td>25.9</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td>4.2</td>
<td>1.6</td>
<td>24.6</td>
<td>18.9</td>
</tr>
<tr>
<td>ω-3 fatty acids</td>
<td>0.5</td>
<td>0.2</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Energy density</td>
<td>3.3</td>
<td></td>
<td>6.9</td>
<td></td>
</tr>
</tbody>
</table>

KD, ketogenic diet.

Plasma cholesterol, triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured on a Roche Modular P800 analyzer (Roche).

Liver triglyceride analysis. Following euthanization, the liver was dissected, weighed, and stored at −80°C. Lipid extraction was performed using a modified protocol of Bligh and Dyer (5). Briefly, liver tissue was homogenized in ice-cold methanol. Lipids were extracted by addition of ice-cold chloroform. After centrifugation, the supernatant was dried with nitrogen gas. Lipids were dissolved in chloroform by addition of ice-cold chloroform. After centrifugation, the supernatant was dried with nitrogen gas. Lipids were dissolved in 100 μL H2O. Triglyceride content was measured using an enzymatic kit (Roche), and protein content was measured using the BCA protein assay kit (Pierce, Rockford, IL). Liver triglyceride content was defined as total triglyceride content per milligram of protein.

Islet morphometry. The pancreas was dissected and weighed after euthanization. To obtain representative samples of the entire organ, pancreata from each mouse (6/group) were cut in three pieces (duodenal, gastric, and splenic region) (13) that were fixed by immersion in a 4% paraformaldehyde solution, embedded in paraffin blocks, and sliced into 4-μm sections. From each block four sections, with an interval of at least 200 μm between sections, were immunostained and analyzed. The average of the three regions was taken as a measure for the entire organ.

For the identification of β-cells, sections were immunostained with guinea pig anti-insulin IgG (Millipore, Billerica, MA) or rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h followed by horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. α-Cells were identified by immunostaining by rabbit anti-glucagon IgG (Vector Laboratories, Burlingame, CA) for 1 h followed by HRP-conjugated secondary antibody for 1 h. Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride or liquid permanent red (LPR, Dako, Denmark) and counterstained with hematoxylin. Stained sections were digitally imaged (Panoramic MIDI; 3DHISTECH).

β-Cell area and pancreas area were determined using an image analysis program (Stacks 2.1; LUMC), excluding large blood vessels, larger ducts, adipose tissue, and lymph nodes as previously described (11). β-Cell mass was determined by the ratio of β-cell area to pancreas area multiplied by the pancreas weight. β-Cell cluster size was determined as the median size of β-cell clusters (defined as ≥4 β-cells per cluster) per mice. α-Cell mass was determined as previously described (14) by calculating the ratio of α-cell area to β-cell area, using Image J software (Image J; National Institutes of Health, Bethesda, MD) multiplied by the β-cell mass.

Statistical analysis. Data are presented as means ± SE. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA). The statistical significance of differences was determined by an unpaired Student’s t-test, Mann-Whitney test, or two-way ANOVA, followed by Bonferroni’s multiple-comparisons test, as appropriate. P < 0.05 was considered statistically significant.

RESULTS

No weight loss after 22 wk of KD. The KD was not associated with weight loss after 22 wk of diet, despite an initial weight loss during the first weeks of diet (Fig. 1A). After 22 wk, body weight in the KD-fed mice was 34.8 ± 1.2 vs. 32.2 ± 0.4 g in the control mice (P < 0.05). After 5 wk, increased concentrations of circulating β-hydroxybutyrate were measured and remained elevated after 20 wk of diet, which indicates a ketotic state in KD-fed mice (Fig. 1B).

KD leads to dyslipidemia, a proinflammatory state, and hepatic steatosis. To assess the metabolic profile of KD-fed mice, several markers that are also associated with the metabolic syndrome in humans were measured. After 22 wk, there was a significant increase of plasma cholesterol, triglyceride, leptin, MCP-1, IL-1β, and IL-6 concentrations in plasma of KD-fed mice (Table 2). To assess whether these systemic markers were related to metabolic changes in the liver, the intrahepatic triglyceride levels were determined as a measure of hepatic steatosis. Liver triglyceride content was increased twofold after 22 wk of KD [379 ± 41 nmol/mg protein (KD) vs. 159 ± 19 nmol/mg protein (Control)].

Fig. 1. Effects of 22-wk control or ketogenic diet (KD) on weight gain and ketosis: A: weight gain during 22 wk of diet (n = 8–10 mice). B: β-hydroxybutyrate (βOHB) levels (n = 2–7 mice). **P < 0.01 and ***P < 0.001 vs. control.
In addition, plasma ALT and AST were significantly increased. Long-term KD leads to glucose intolerance. Glucose tolerance tended to be decreased after 5 wk, but KD-fed mice became markedly glucose intolerant after 12 wk of diet (Fig. 2, A–D). After 1 wk KD, insulin concentrations were increased during the GTT (Fig. 2, E and I). However, continuation of the diet for 5 wk or longer resulted in insufficient insulin secretion from β-cells to maintain glucose tolerance (Fig. 2, F–H and J–L). After 20 wk diet, glucose-induced insulin concentrations were significantly decreased in KD-fed mice (Fig. 2L). Insulin-dependent glucose uptake assessed by an ITT was also reduced in KD-fed mice compared with control mice after 22 wk (Fig. 3, A and B). Also, the fasting insulin-to-glucose ratio was significantly increased in KD-fed mice (Fig. 3 C).

KD leads to decreased β- and α-cell mass. After 22 wk, β-cell mass in KD-fed mice, determined by analyzing 175.1 ± 7.1 mm² pancreatic tissue/mouse, was decreased (Fig. 4, A–C). The density of islets was unchanged (Fig. 4D), but the median β-cell cluster size was significantly decreased (Fig. 4E). This was because of a relatively increased number of islets with a size smaller than 2,500 μm² in KD-fed mice (Fig. 4F). The α-cell mass was reduced by 50% in KD-fed mice after 22 wk, which resulted in a decreased α-cell/β-cell ratio in KD-fed mice (Fig. 5, A–D).

Table 2. Plasma markers of the metabolic syndrome in control mice and mice fed a ketogenic diet for 22 wk

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>92.7 ± 3.1</td>
<td>141.3 ± 9.5*</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>42.0 ± 1.5</td>
<td>64.9 ± 6.8*</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>0.63 ± 0.37</td>
<td>2.35 ± 0.99*</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>6.20 ± 0.73</td>
<td>12.65 ± 1.40*</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>1.19 ± 0.31</td>
<td>3.86 ± 1.17*</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>3.20 ± 2.11</td>
<td>19.84 ± 8.02*</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>31.3 ± 0.9</td>
<td>80.6 ± 15.8a</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>89.0 ± 10.7</td>
<td>155.2 ± 31.4a</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–10 mice. MCP, monocyte chemotactic protein; IL, interleukin; ALT, alanine aminotransferase; AST, aspartate aminotransferase. *P < 0.05, †P < 0.01, and ‡P < 0.001.

(control), P < 0.01. In addition, plasma ALT and AST were significantly increased.

Long-term KD leads to glucose intolerance. Glucose tolerance tended to be decreased after 5 wk, but KD-fed mice became markedly glucose intolerant after 12 wk of diet (Fig. 2, A–D). After 1 wk KD, insulin concentrations were increased during the GTT (Fig. 2, E and I). However, continuation of the diet for 5 wk or longer resulted in insufficient insulin secretion from β-cells to maintain glucose tolerance (Fig. 2, F–H and J–L). After 20 wk diet, glucose-induced insulin concentrations were significantly decreased in KD-fed mice (Fig. 2L). Insulin-dependent glucose uptake assessed by an ITT was also reduced in KD-fed mice compared with control mice after 22 wk (Fig. 3, A and B). Also, the fasting insulin-to-glucose ratio was significantly increased in KD-fed mice (Fig. 3 C).

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Fig. 2. Glucose tolerance in control and KD-fed mice. Blood glucose concentrations during the glucose tolerance test after 1 (A), 5 (B), 12 (C), and 20 wk (D) of diet (n = 5–10 mice). Insulin concentrations during the glucose tolerance test after 1 (E), 5 (F), 12 (G), and 20 (H) wk of diet (n = 3–10 mice). Area under the curve (AUC) 0–15 min insulin concentrations during the glucose tolerance test after 1 (I), 5 (J), 12 (K), and 20 (L) wk of diet (n = 4–10 mice). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.
DISCUSSION

High-fat, low-carbohydrate KD have been associated with beneficial effects on body weight and epileptic seizures. However, their effects on pancreatic endocrine cells and glucose metabolism on the long term are less clear. The main results of our study show that long-term KD in mice causes glucose intolerance and a reduction in both β- and α-cell mass, but no weight loss. This indicates that long-term KD leads to features that are also associated with the metabolic syndrome in humans and an increased risk for type 2 diabetes.

KD resulted in weight loss in the first weeks of the diet, which is in line with previous reports in rodents (2, 20, 22). Also in patients with refractory epilepsy, short-term (3–4 mo) KD treatment resulted in decreased body weight (25, 28). However, we now show that a prolonged KD is not associated with weight loss. After 12 wk, weight gain is similar in both groups of mice. Long-term use of KD in children with epilepsy resulted in slowed growth but did not change the body mass index (17, 28, 39). In rats it has been shown that 4–6 wk KD leads to visceral fat accumulation (32) and increased leptin.
We did not assess the effect of long-term KD on body composition. However, the elevated plasma leptin concentrations observed in our study suggest that prolonged KD eventually leads to regaining weight because of an increase of body fat.

Long-term KD leads to increased plasma cholesterol and triglyceride levels indicative of dyslipidemia. In previous short-term studies, increased plasma cholesterol levels were observed after 9 wk of diet (2), whereas at that time point plasma triglyceride levels were lower. Also, in both adults and children with refractory epilepsy, KD was associated with increased plasma cholesterol and triglyceride levels (24, 38). Furthermore, the increased plasma cytokine concentrations in our study suggest a systemic proinflammatory state in long-term KD-fed mice. This is in line with the observation that short-term KD in mice is associated with increased inflammatory markers in liver and adipose tissue (2) and macrophage infiltration in the liver (16). Also, we show that KD-fed mice had increased plasma levels of the chemokine MCP-1, which is associated with increased macrophage recruitment to the liver (26). In patients with the metabolic syndrome, increased MCP-1 is associated with increased inflammatory markers in liver and adipose tissue (2) and macrophage infiltration in the liver (16). Also, we show that KD-fed mice had increased plasma levels of the chemokine MCP-1, which is associated with increased macrophage recruitment to the liver (26).

Dyslipidemia and the proinflammatory state induced by KD may be the consequence of the high content of saturated fatty acids. It was shown that a short-term polyunsaturated fat-enriched KD did not adversely alter lipid metabolism in adults (15). Also, supplementation of a high-fat diet with ω-3 polyunsaturated fatty acids has been shown to prevent high-fat diet-induced insulin resistance by reducing inflammasome-dependent inflammation in rodents (41). However, in our study, the higher content of ω-3 fatty acids in the KD could not prevent proinflammatory effects on the long term. Whether further modification of the fatty acid content of KDs can attenuate dyslipidemia and the proinflammatory effects on the long term needs further study.

Furthermore, KD-fed mice showed signs of steatohepatitis as indicated by the increased hepatic triglyceride content and elevated AST and ALT levels. This is in line with metabolic changes observed in previous studies in mice (2, 16, 20, 22) and may indicate an early stage of nonalcoholic fatty liver disease (16, 36). Recent short-term studies have shown that supplementation of KD with choline or methionine could limit hepatic steatosis and the proinflammatory state of the liver, respectively (31, 37). However, the similar methionine and higher choline content of KD in our study did not prevent signs of steatohepatitis in the long term. Altogether these data indicate that a prolonged KD leads to dyslipidemia, a proinflammatory state, and signs of hepatic steatosis.

In this study we show that long-term KD is associated with pronounced glucose intolerance and reduced insulin-stimulated glucose uptake. This was also observed in a recent study by Bielohuby et al. after 4 wk of KD in mice (4). We now show that insulin concentrations were increased to maintain normoglycemia after 1 wk of KD, but continuation of the KD resulted in insufficient insulin secretion to maintain glucose tolerance.

This inadequate insulin secretory response could be the result of β-cell dysfunction, an insufficient β-cell number, or a combination of these two mechanisms. The results in this study strongly indicate that long-term KD leads to an insulin secretory defect. Furthermore, not only after short-term diet (4) but also after long-term KD, β-cell mass is reduced. An inadequate function and/or number of β-cells leads to insufficient insulin...
secretion that results in glucose intolerance and ultimately type 2 diabetes in humans (21, 29). Interestingly, also the $\alpha$-cell mass was decreased considerably, which is in line with decreased glucagon levels that have been observed after 5 wk of KD in mice (20). In relative terms, this decreased $\alpha$-cell mass was even more pronounced than the reduction in $\beta$-cell mass, resulting in a major decrease of the $\alpha$-cell/$\beta$-cell ratio. Decreased glucagon levels lead to less gluconeogenesis from the liver, which may prevent hyperglycemia in KD-fed mice. Whether this change in $\alpha$-cell mass is a direct consequence of KD or a response to counteract glucose intolerance remains to be elucidated.

Altogether, the results of this study indicate that a long-term high-fat, low-carbohydrate KD in mice does not cause weight loss and leads to glucose intolerance and a reduction in both $\beta$- and $\alpha$-cell mass. In addition, dyslipidemia, a proinflammatory state, and signs of hepatic steatosis are observed. Effects of short-term diets cannot be automatically translated to metabolic effects after long-term diet use.

ACKNOWLEDGMENTS

We thank Dr. Bruno Guigas and Dr. Janna van Diepen for expert advice.

REFERENCES


