High-Fat, Carbohydrate-Free Diet Markedly Aggravates Obesity but Prevents β -Cell Loss and Diabetes in the Obese, Diabetes-Susceptible *db/db* Strain

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Key Words

Diabetes · Diet · β -Cell · Carbohydrates · Glucotoxicity

Summary

Objective: We have previously reported that a high-fat, carbohydrate-free diet prevents diabetes and β -cell destruction in the New Zealand Obese (NZO) mouse strain. Here we investigated the effect of diets with and without carbohydrates on obesity and development of β -cell failure in a second mouse model of type 2 diabetes, the *db/db* mouse. **Results:** When kept on a carbohydrate-containing standard (SD; with (w/w) 5.1, 58.3, and 17.6% fat, carbohydrates and protein, respectively) or high-fat diet (HFD; 14.6, 46.7 and 17.1%), *db/db* mice developed severe diabetes (blood glucose >20 mmol/l, weight loss, polydipsia and polyurea) associated with a selective loss of pancreatic β -cells, reduced GLUT2 expression in the remaining β -cells, and reduced plasma insulin levels. In contrast, *db/db* mice kept on a high-fat, carbohydrate-free diet (CFD; with 30.2 and 26.4% (w/w) fat or protein) did not develop diabetes and exhibited near-normal, hyperplastic islets in spite of a morbid obesity (fat content >60%) associated with hyperinsulinaemia. **Conclusion:** These data indicate that in genetically different mouse models of obesity-associated diabetes, obesity and dietary fat are not sufficient, and dietary carbohydrates are required, for β -cell destruction.

Introduction

Type 2 diabetes represents one of the major complications of obesity. It is generally accepted that it is the consequence of insulin resistance associated with a progressive failure of insulin-secreting β -cells. As a pathophysiological scenario, it has been suggested that insulin resistance causes postprandial hyperglycaemia exerting oxidative stress ('glucotoxicity') and damage of the β -cell [1–3]. In addition, it has been proposed that obesity-induced ectopic fat accumulation in the pancreas ('lipotoxicity') causes apoptosis of β -cells [4, 5]. These mechanisms are not mutually exclusive and may well function in combination [6, 7].

In animal models, diabetes can be accelerated or delayed by dietary modifications. We have previously demonstrated that the dietary fat content, in combination with the presence of diabetogenic alleles, markedly increases the prevalence of diabetes in New Zealand Obese (NZO) mice as well as in an out-cross population of NZO with the lean SJL strain [8]. In addition, we have shown that a high-fat, carbohydrate-free diet fully prevented β -cell destruction in NZO mice in spite of an extreme insulin resistance and a marked inflammatory state of adipose tissue [9]. This finding is consistent with the conclusion that dietary carbohydrates play an essential role in the pathogenesis of islet cell failure.

NZO mice represent a polygenic model for the metabolic syndrome [10] including obesity-associated diabetes ('diabesity'), with 2–4 diabetogenic quantitative trait loci (QTLs) being responsible for the decompensation of glucose homeostasis [11–13]. Thus, it remained to be determined whether the dietary effect on β -cell function was restricted to the NZO mouse model, or whether it can also be observed in genetically different diabetes-susceptible strains such as the *db/db* mouse. Indeed, it was reported previously that the prevalence of diabetes could markedly be reduced by substitution of

dietary protein for carbohydrates [14]. Here, we followed up on this study and exposed *db/db* mice to a carbohydrate-free diet where fat was substituted for the carbohydrates. Our data indicate that this diet produced extreme obesity with a body fat mass of >60% but markedly ameliorated hyperglycaemia, and fully prevented β -cell destruction as well as the manifestation of glucosuria. Thus, the data demonstrate the essential role of dietary carbohydrates for the development of β -cell destruction in two genetically different mouse models.

Material and Methods

Experimental Animals

Male *db/db* mice (BKS.Cg-*m*+/*+*Lepr^{db}/J) and lean C57BL/KsJ littermates (BKS.Cg-*m*+/*+*J) (Charles River, Sulzfeld, Germany) were housed in groups of 5 per cage (type II macrolon) at a temperature of $21 \pm 1^\circ\text{C}$ with a 12:12 hours light-dark cycle (lights on at 6:00 a.m.). Animals had free access to food and water, and were kept in accordance with the NIH guidelines for the care and use of laboratory animals. All experiments were approved by the Ethics Committee of the State Ministry of Agriculture, Nutrition and Forestry (State of Brandenburg, Germany).

Diets and Study Design

All diets were purchased from Altromin (Lage, Germany). The standard diet (SD, Art. No. C 1000) contained (w/w) 5.1% fat, 58.3% carbohydrates, and 17.6% protein, with a total metabolisable energy of 14.7 kJ/g according to the information provided by the manufacturer. The high-fat diet (HFD, Art. No. C1057) contained (w/w) 14.6% fat, 46.7% carbohydrates, and 17.1% protein, with a total metabolisable energy of 15.3 kJ/g. The carbohydrate-free high-fat diet (CFD, Altromin No. C 1057 without carbohydrates, custom made by the manufacturer) contained (w/w) 30.2% fat and 26.4% protein with a total metabolisable energy of 23 kJ/g. Fat content normalised per total energy was 13.3, 34.1 and 72.2%, protein content was 20.1, 17.6 and 27.8%, carbohydrate content was 66.7, 48.2 and 0% in the SD, HFD and CFD, respectively. Because of the soft texture of HFD and CFD, mice had access to wooden gnawing sticks in order to avoid excessive teeth growth.

The dietary intervention was started at the age of 5 weeks and continued for 22 weeks or until the development of severe hyperglycaemia (blood glucose > 25 mmol/l) and weight loss > 10%. Blood samples were collected weekly from the tail vein of fed mice between 9:00 and 11:00 am. Blood glucose was determined with an Ascensia ELITE XL glucose meter (Bayer Health Care, Leverkusen, Germany). Plasma insulin was assayed with the Mouse Insulin Ultrasensitive ELISA Kit (Cat. No. EIA-3440, DRG Instruments GmbH, Marburg, Germany). Plasma triglycerides were determined with the SOP 7-Triglyceride/Glycerol Assay (Wako Chemicals GmbH, Neuss, Germany). Body composition (fat and lean mass) was measured weekly by nuclear magnetic resonance (NMR) with a Echo-MRI™ Whole Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) as previously published [9, 15, 16].

Immunohistochemistry of Pancreatic Islets

Pancreatic tissue excised immediately after exsanguination under isofluran anaesthesia at the study endpoint was fixed in 4% formaldehyde for 24 h and embedded in paraffin according to standard procedures. Longitudinal serial sections (4 μm , at sampling intervals of 300 μm) were prepared. The following antibodies were used for immunostaining: sheep anti-insulin antiserum (1:6,000; Vector Laboratories, Burlingame, CA, USA), rabbit anti-glucagon antibody (1:2,000; DAKO Diagnostika, Hamburg, Germany), rabbit antiserum against the C-terminus of rat GLUT2 (1:1,000) [17]. Secondary antibodies were Histofine® Anti-Rabbit Im-

muno-POD Polymer for mouse tissues (Nichirei Biosciences Inc., Tokyo, Japan) or sheep IgG-antiserum (1:800, Dianova, Hamburg, Germany). For immunocytochemistry of GLUT2, biotin-conjugated donkey anti-rabbit IgG (Dianova) was used, it was detected by incubation with streptavidin-biotin-horseradish peroxidase complex (StreptAB-complex/POD; Dako, Hamburg, Germany) and diaminobenzidine as substrate according to the manufacturer's specifications. Specificity of staining was tested with pre-immune serum. Microscopic investigation and photo documentation were performed with the combined light and fluorescence microscope ECLIPSE E-100 (Nikon, Düsseldorf, Germany) in combination with the video camera CCD-1300CB (Vosskühler, Osnabrück, Germany) and the Analysis System LUCIA (Nikon).

Quantitative Analysis of Islet Morphology

Longitudinal sections of pancreas co-stained with anti-insulin and anti-glucagon antiserum were analysed with the Lucia G image analysis software (Laboratory Imaging, Prague, Czech Republic) via an unbiased stereological approach. In 7–12 randomly selected islets per section from 4 mice per treatment, the number of cells immunoreactive for glucagon (α -cells) and insulin (β -cells) as well as islet and total pancreas area were determined. The β - and α -cell counts in one section were normalised per islet, and means $\pm$ SEM were calculated.

Statistical Analysis

Differences in body weight, body fat and blood glucose under the different dietary regimens were tested with a one-way analysis of variance (one-way ANOVA) followed by a post-hoc test (homogeneous variances by Bonferroni type and inhomogeneous variances Games-Howell type). Differences in insulin levels were determined by a two-tailed Student's *t*-test. The number of β -cells per islets and its proportion to α -cells were compared by the non-parametric Mann-Whitney U-test. All statistical analyses were performed with the software package SPSS 14.0 (SPSS Inc., Chicago, IL, USA), and differences were considered significant at $p < 0.05$.

Results

Development of Body Weight under the Different Diets

Figure 1 illustrates the time course of body weight (panel A) and body fat (panel B) increment in *db/db* mice raised on SD, HFD and CFD, starting at 5 weeks of age. Mice exhibited a rapid increase of body fat that was initially higher under the high fat-containing diets (HFD with 14.6% and CFD with 30.2% fat, w/w) than under the standard diet (SD with 5.1% fat). In mice receiving a carbohydrate-containing diet (SD and HFD), body weight and fat gain reached a plateau at week 4–8 when diabetes (defined as blood glucose levels > 25 mmol/l or 450 mg/dl) commenced. Because of the severity of their diabetes, all mice in the HFD group were euthanised until week 16. In contrast, mice on the carbohydrate-free diet (CFD) continued to gain weight and accumulate body fat until the end of the observation period (22 weeks).

In a parallel series of experiments, lean C57BL/KsJ mice were exposed to the three diets for 22 weeks (fig. S1 see online supplemental material at www.karger.com/doi/10.1159/000176064). As expected, HFD and CFD increased the body weight by approximately 2–3 g but failed to cause obesity, indicating that the C57BL/KsJ background strain was resistant to the high-fat diets.

Fig. 1. Time course of **a** body weight and **b** fat mass increase in *db/db* mice on SD, HFD, CFD. Mice were 5 weeks old at the start of the experiment. Data represent means $\pm$ SEM of 6–8 mice per group.

Time Course of Blood Glucose and Plasma Insulin Levels under the Different Diets

As is illustrated in figure 2, *db/db* mice on SD and HFD developed severe hyperglycaemia, with blood glucose levels above 400 (SD) or 500 mg/dl (HFD). HFD appeared to accelerate the onset of diabetes, consistent with similar results obtained in NZO mice [9]. In contrast, hyperglycaemia was much less pronounced in mice exposed to the CFD, except for a transient increase between weeks 4 and 6 which might reflect a reduced insulin sensitivity which was later compensated by β -cell hyperplasia. *db/db* mice in all groups were hyperinsulinaemic in week 2 (8–10 ng/ml as compared with levels in lean mice (<1 ng/ml)) when blood glucose levels were normal, consistent with previous data demonstrating an early, severe insulin resistance in the *db/db* strain [18]. At the study end point, plasma insulin levels were markedly reduced in the SD and HFD groups, and were 4- to 6-fold lower than in mice on CFD. It should be noted that insulin levels at 6 weeks were lower in HFD than in SD, confirming the observation that decompensation of blood sugar homeostasis was accelerated by

Fig. 2. CFD ameliorates **a** hyperglycaemia and **b** hyperinsulinaemia in *db/db* mice. Data represent means $\pm$ SEM of 6–8 mice per group.

HFD. In lean C57BL/KsJ, blood glucose levels were unaltered by the different diet and remained between 100 and 140 mg/dl throughout the observation period (fig. S2 see online supplemental material at www.karger.com/doi/10.1159/000176064). Plasma triglycerides at week 6 were not significantly different under the three diets (fig. S3 see online supplemental material at www.karger.com/doi/10.1159/000176064).

Pancreas Histology and Immunohistochemistry of GLUT2

In order to support the conclusion that carbohydrate-free diet prevented loss of β -cells in the course of the development of diabetes in *db/db* mice, histology of pancreatic islets was performed (fig. 3). As compared with islets from lean litter mates (C57BL/KsJ) or from CFD-fed *db/db* mice, mice on SD or HFD appeared smaller and exhibited fewer insulin-positive cells. Morphometric analysis (fig. 4a) confirmed this observation and demonstrated a 70–80% reduction of β -cells in SD/HFD compared with CFD mice, leading to an altered ratio of α - to β -cells (fig. 4b). When β -cell area was normalised per islet area, the effect was still significant but smaller, since islet

Fig. 3. CFD prevents loss of β -cells and GLUT2 in *db/db* mice. For comparison, lean non-diabetic C57BL/KsJ litter mates (+/+) were studied. Light microscopical sections depict hematoxylin-stained pancreatic islets immunostained for insulin (**a**, red), glucagon (**a**, brown) or GLUT2 (**b**, brown).

area decreased with the reduction of β -cells in the diabetic mice. As is also shown in figure 3, GLUT2 immunoreactivity as a marker of β -cells was markedly decreased in islets from diabetic SD and HFD mice as compared with CFD mice.

Discussion

The present data confirm and extend our previous results, indicating that carbohydrate restriction prevents obesity-associ-

Fig. 4. CFD prevents loss of β -cells in islets from *db/db* mice. Morphometric determination of **a** the number of β -cells and **b** ratio of α - and β -cell under the different diets. Data represent means $\pm$ SEM of 32–48 randomly selected islets from 4 mice per group.

ated diabetes ('diabesity') in the NZO mouse [9]. Here we show a similar protection in the diabetes-susceptible *db/db* strain: whereas a conventional HFD (14.6% (w/w) fat) with carbohydrates accelerated the development of diabetes, a CFD with an even higher fat content (30.2%) ameliorated hyperglycaemia and prevented β -cell loss. Conclusions based on these data are consistent with an earlier study, reporting that substitution of carbohydrates with proteins prevented diabetes in the *db/db* mouse [14]. Thus, an essential role of dietary carbohydrates for the development of β -cell failure has been demonstrated in two genetically diverse mouse models of diabesity under two different dietary regimens. These data provide further proof of the concept that glucose toxicity plays an essential role in β -cell failure of type 2 diabetes [1–3, 7]. The effect of carbohydrate restriction on β -cell function as well as on the development of adiposity appeared similar, if not identical, in the two mouse models NZO and *db/db*. In addition, the degree of β -cell dysfunction appeared similar in *db/db* and NZO, with a reduction of insulin-immunoreactive β -cells by 75–85% after 16–20 weeks on HFD [9]. Diabetes in NZO is determined by 2–4 susceptibility loci on chromosomes 1, 5, 13, and 15 that were identified by genome-wide scans of outcross populations [11, 12] and generation of congenic lines [13]. In the C57KsJ-*db/db* strain investigated here, the adipogenic mutation in the leptin receptor gene is necessary but

not sufficient for the development of diabetes since C57BL/6J mice carrying the *db/db* allele are not diabetic [19]. Rather, diabetogenic alleles contributed by the C57BL/KsJ background are responsible for the β -cell loss of the *db/db* strain. Recently, the major diabetogenic allele of the C57BL/KsJ background on chromosome 1 has been identified as a variant of the *Lisch-like* gene [20]. At present, it cannot be excluded that the NZO strain carries a different diabetogenic allele of this gene. However, a meta-analysis of the published linkage studies [21] together with unpublished data (Vogel, Scherneck, Schmolz et al., unpublished) indicates that the genetic basis of diabetes in *db/db* is clearly different from that in NZO. Consequently, the different genetic basis of diabetes in *db/db* and NZO mice led to a distinct phenotype, but the protective effect of carbohydrate restriction appears to be independent of the specific genetic basis of the diabetes.

In both strains, β -cell failure was associated with a marked decrease in the immunoreactivity of the GLUT2. Reduced expression of GLUT2 in β -cells of NZO mice and in other models of diabetes (Chinese hamster and *Psammomys obesus*) has been reported earlier to precede the loss of insulin immunoreactivity [22–24].

The present data indicate that dietary carbohydrates are essential for the development of diabetes in obese mice. However, they do not exclude a major role of lipids. Obesity with a threshold body weight (approximately 45 g at 12 weeks) is required for the development of diabetes in NZO mice [11, 12]. Lean C57BL/KsJ mice do not develop diabetes although they carry the diabetogenic alleles responsible for the diabetes in *db/db* mice. Thus, obesity is absolutely required for diabetes in NZO and *db/db* mice, although apparently not sufficient. The present data are therefore fully consistent with a scenario in which the combination of fatty acids or triglycerides accumulating in the β -cells ('lipotoxicity') with elevated glucose concentration ('glucotoxicity') confers β -cell toxicity [1–3, 6, 7].

It has previously been reported that β -cell damage in NZO mice was associated with leucocyte infiltration in islets [25]. In contrast, our own data showed infiltration of islets only at a late stage of β -cell destruction, after cells had become necrotic [9]. In the present study (fig. 3), loss of functioning β -cells in *db/db* mice was detected without any lymphocytic infiltration. Thus, leucocyte infiltration does not appear to be a contributing factor in the pathogenesis of diabetes in *db/db* mice. Rather, hyperglycaemia-induced oxidative stress might ex-

plain the β -cell cytotoxicity that leads to diabetes in *db/db* and NZO mice [6]. Oxidative stress resulting from high concentrations of glucose has previously been investigated in cultured β -cell lines and is assumed to initially affect specific transcription factors controlling the insulin gene, such as Pdx1 and MafA, and subsequently the proliferation of the cells [26, 27]. The CFD has presumably caused major shifts in substrate fluxes such as an increase in fatty acid oxidation in tissues. Thus, the possibility has to be discussed that the effect of carbohydrate restriction is mediated by an increase in insulin sensitivity, or by a reduction in deposition of ectopic lipids. In NZO mice, we have previously shown that CFD markedly enhanced insulin resistance; it appears unlikely that CFD produces the opposite effect in *db/db* mice. However, in the absence of data on the metabolism of fatty acid in β -cells, it cannot be excluded that glucose depletion has reduced lipotoxicity by an increase in fat oxidation in β -cells.

The role of dietary carbohydrates in the development and course of human type 2 diabetes is a matter of intense debate. In prospective cohort studies, a diet with a high glycaemic load [28] as well as an increased consumption of soft drinks and white bread [29, 30] was associated with an increased risk of type 2 diabetes. Furthermore, the glucosidase inhibitor acarbose reduces the risk of conversion of impaired glucose tolerance to overt diabetes [31]. The results of these studies were interpreted as evidence for a role of postprandial glucose excursions in the development of human β -cell failure. Our present data are consistent with such a pathogenetic scenario. It should be noted, however, that the diet employed in our study is not palatable for humans and that we do not intend to translate our experimental data into nutritional recommendations. However, the data underscore the need to further investigate the role of carbohydrates in the development of human diabetes and to test preventive strategies accordingly.

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Disclosure

The authors declared no conflict of interest.

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