Glucose and Lipid Dysmetabolism in a Rat Model of Prediabetes Induced by a High-Sucrose Diet

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Abstract: Glucotoxicity and lipotoxicity are key features of type 2 diabetes mellitus, but their molecular nature during the early stages of the disease remains to be elucidated. We aimed to characterize glucose and lipid metabolism in insulin-target organs (liver, skeletal muscle and white adipose tissue) in a rat model treated with high-sucrose (HSu) diet. Two groups of 16-week-old male Wistar rats underwent a 9-week protocol: HSu diet (n=10) – received 35% of sucrose in drinking water; Control (n=12) – received vehicle (water). Body weight, food and beverage consumption were monitored and glucose, insulin and lipid profiles were measured. Serum and liver triglyceride concentrations, as well as the expression of genes and proteins involved in lipid biosynthesis were assessed. The insulin-stimulated glucose uptake and isoproterenol-stimulated lipolysis were also measured in freshly isolated adipocytes. Even in the absence of obesity, this rat model already presented the main features of prediabetes, with fasting normoglycemia but reduced glucose tolerance, postprandial hyperglycemia, compensatory hyperinsulinemia, as well as decreased insulin sensitivity (resistance) and hypertriglyceridemia. In addition, impaired hepatic function, including altered gluconeogenic and lipogenic pathways, as well as increased expression of ACC1 and FAS in the liver, were observed, suggesting that liver glucose and lipid dysmetabolism may play a major role at this stage of disease.

Keywords: high-sucrose diet; prediabetes; glucose; lipid; metabolism; hypertriglyceridemia

1. Introduction

The Type 2 diabetes (T2DM) has become the tsunami of noncommunicable diseases, with 415 million people worldwide currently living with diabetes [1]. According to the International Diabetes Federation, about 5 million people died from DM in 2015 and the estimates indicate that there will be about 642 million people living with DM by 2040. In addition, there are about 318 million adults with impaired glucose tolerance (IGT), which puts them at high risk for the disease.

Prediabetes (or intermediate hyperglycemia) already displays metabolic alterations and is a high risk state for developing T2DM. According to the American Diabetes Association, prediabetes is distinguished by having impaired fasting glucose (IFG) (100-125 mg/dL glucose), impaired glucose tolerance (IGT) (140-199 mg/dL glucose 2 hours after a 75-g oral glucose tolerance test) and glycated hemoglobin (HbA1c) levels between 5.7-6.4%. The prevalence of prediabetes is rapidly increasing with over ≥470 million people projected with prediabetes by 2030 [2]. This likely anticipates increased morbidity, mortality and healthcare costs in the near future with DM management. Thus, preventing
the progression of IGT and/or IFG to T2DM is the most rational and effective way to combat the DM epidemic and lessen healthcare costs. However, before we can succeed, we need to unravel glucose and lipid metabolism at this stage of the disease.

Diet enriched in sugars including intake of sugar-sweetened beverages have been consistently linked to the increased risk of hypertriglyceridemia, obesity, T2DM and cardiovascular disease [3]. Particularly, chronically increased levels of plasma nonesterified fatty acids (NEFA) and triglyceride (TG)-rich lipoproteins impair lipid metabolism, a process referred to as lipotoxicity [4]. Furthermore, when NEFA supply exceeds metabolic capacity, lipids accumulate in peripheral tissues, such as liver and muscle, inducing organ dysfunction [4]. Importantly, lipotoxicity and glucotoxicity are associated to the progression of DM and its micro- and macrovascular complications. However, the nature of glucose and lipid deregulation in the prediabetic state remains to be elucidated.

Therefore, we aim to evaluate glucose and lipid metabolism in a prediabetic rat model, induced by high-sucrose (HSu) diet [5,6], focusing on the main insulin-target organs: liver, skeletal muscle and white adipose tissue.

2. Materials and Methods

2.1. Animals and diets

Male Wistar rats (16 week-old; Charles River Laboratories Barcelona, Spain) were housed, two per cage, under controlled conditions [12h light/dark cycle schedule and controlled temperature (22±1°C) and humidity]. After an adaptation period of 1 week, rats were randomly divided into two groups and submitted to a 9-week protocol: 1) control - receiving tap water as vehicle; 2) HSu - receiving 35% sucrose (S0389; Sigma–Aldrich) in the drinking water. All animals were fed standard rat chow, containing 16.1% of protein, 3.1% of lipids, 3.9% of fibers and 5.1% of minerals (AO4 Panlab, Barcelona, Spain), ad libitum (except during fasting periods). Body weight, food and beverage consumption were weekly monitored. All experiments were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and with the National and Local Authorities.

2.2. Chemicals

Used chemicals are listed in supplemental material.

2.3. Metabolic characterization

Metabolic characterization was performed as previously described [5] and is detailed in supplemental material. This included a glucose tolerance test (GTT), an insulin tolerance test (ITT), fasting insulin levels and insulin sensitivity (HOMA index). In addition, serum TG and high-density lipoprotein cholesterol (HDL-c) (TG/HDL) ratio, TG-glucose (TyG) index, as well as fed and fasted alanine aminotransferase [4] and aspartate aminotransferase (AST) assessment. Caloric intake was calculated based on the daily food intake, and in HSu-treated animals, the energy related to sucrose consumption was also calculated.

2.4. Blood and tissues collection

After a 9-week treatment, blood and tissues (liver, skeletal muscle and epididymal adipose tissue) were snap frozen and stored at -80°C. Details are available in the supplemental material.

2.5. Hepatic triglyceride extraction and quantification

Hepatic TG were extracted and quantified as previously reported [7]. Briefly, 50 mg of liver samples were homogenized in 0.5 ml of chlorophorm/methanol (2:1) and incubated for 3 hours with agitation at 4°C. 300 uL of milliQ water were then added to the homogenate and centrifuged (13.000
rpm for 20 minutes, room temperature). The organic phase was transferred to a clean eppendorf, allowed to evaporate at 4º C and finally stored at -20ºC.

Liver TG quantification was performed with a specific commercial kit from Spinreact. Colorimetric determination was performed in a spectrophotometer (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, California, United States) at a wavelength of 505 nm or 490 nm after TG resuspension in 500 uL of chloroform. Intensity of the formed color was proportional to TG concentration in each sample.

2.6. Cell size and weight, glucose uptake and lipolysis in isolated epididymal adipocytes

Epididymal adipocyte size and weight, insulin-stimulated D-[U-14C] glucose uptake and isoproterenol-stimulated lipolysis in isolated adipocytes were performed as previously described [8, 9]. Briefly, for the insulin-stimulated glucose uptake, the isolated adipocytes were diluted ten times and were stimulated or not with human insulin (1000 μU/ml), for 10 minutes, at 37º C, in a shaking water-bath. Subsequently, 0.86 μM D-[14C(U)] glucose was added to the medium and the accumulation of glucose was followed for 30 minutes. The cell suspension was then transferred to pre-chilled tubes, containing silicone oil, allowing cells to be separated from the buffer by centrifugation. Cell-associated radioactivity was determined by liquid scintillation counting, which allowed us to calculate the rate of transmembranar glucose transport, according to the formula: cellular clearance of medium glucose = (c.p.m. cells x volume)/(c.p.m. medium x cell number x time). For isoproterenol-stimulated lipolysis, the isolated adipocytes were diluted ten times and were incubated in the presence or absence of insulin (1000 μU/ml), in a shaking water bath, at 37º C, for 60 min. The medium was also supplemented or not with isoproterenol (1 μM). Following incubation, cells were separated from the medium by centrifugation and glycerol levels were measured in the medium using an assay kit (Zen Bio, Inc.). Further details are available in the supplemental material.

2.7. Liver, skeletal muscle and adipose tissue gene and protein expression

Total RNA from liver, skeletal muscle and epididymal adipose tissue was extracted, and cDNA synthesis and relative mRNA levels for glucose transporter-1(Glut1), -2 (Slc2a2) and -4 (Glut4), phosphoenolpyruvate carboxykinase (Pepck), glucose-6-phosphatase (G6pc), acetyl-CoA carboxylase 1 (Acc1), fatty acid synthase (Fasn), diglyceride acyltransferase (Dgat1), carbohydrate-responsive element-binding protein (MLXipl/Chrebp), sterol regulatory element-binding transcription factor 1 (Srebf1) and hormone-sensitive lipase (Hsl) were measured (by real time-PCR), as previously described [10].

Protein extraction and Western blot analysis of GLUT1, GLUT2, GLUT4, PEPCK, G6PC, ACC1, FASN, DGAT1, ChREBP, SREBP and HSL were performed as previously described [8, 9]. Supplemental material details the protocols and lists the primer sequences (Table 1) and antibodies (Table 2) used.

2.8. Statistical analysis

Results were expressed as mean ± standard error of the mean, using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Student’s t-test for normal distributed data or Mann Whitney test for non-normal distributed data were performed when two groups were considered. One-way or two-way ANOVA, followed by Tukey post hoc test, for multiple comparisons, was used as appropriate. Repeated measures ANOVA, followed by the Tukey post-hoc test, was used to access differences between groups and between basal and insulin-stimulated glucose uptake. Differences were considered significant when * p≤0.05, ** p≤0.01, *** p≤0.001 or **** p≤0.0001.

3. Results

3.1. HSu diet increases beverage consumption and caloric intake, maintaining body weight
During 9 weeks of treatment, HSu-treated rats had a higher beverage (35% sucrose) consumption and caloric intake but lower food ingestion, thus maintaining body weight gain identical to that of control rats (Figure 1A-D), even though they had increased fat pad weight (Figure 4A).

Figure 1. Food (A), beverage (B) and total caloric (C) intake, as well as body weight (D), throughout the 9 weeks of treatment. Control (n=12) and HSu (n=10). ** p<0.01, *** p<0.001, **** p<0.0001.

3.2. HSu diet increases serum triglyceride levels and impairs liver function

Serum TG content in HSu-treated rats was significantly higher than in control animals in the fed state. Also, fed HSu-treated rats presented upper TG concentration than in fasted HSu animals (Figure 2A). However, no significant differences were found in liver TG levels (Figure 2B). Regardless of the nutritional status (fasted or fed), serum ALT levels were reduced in the HSu-treated rats (Figure 2C); however, no significant differences were found in serum AST levels (Figure 2D). In addition, the liver weight/body weight ratio was increased in HSu-treated animals (Figure 2E).

Figure 2. Serum (A) and liver (B) triglyceride (TG) content, serum alanine aminotransferase (ALT), (C) and aspartate aminotransferase (AST) (D) levels, as well as liver weight/body weight ratio (E) at the end of treatment. Control (n=12) and HSu (n=10). *p<0.05, **p<0.01, ***p<0.001.

3.3. HSu diet impairs glucose tolerance and causes insulin resistance
Although both groups showed similar fasting glucose levels (Figure 3A, C and E), the HSu-treated rats had significantly slower glucose excursion during a GTT (2 g/kg body weight of glucose), compared to control animals (Figure 3A-B), revealing glucose intolerance. Blood glucose levels were significantly elevated in the HSu-treated rats, 120 min after an insulin injection (0.75 U/kg body weight of insulin) (Figure 3C-D). In addition, serum fasting insulin concentration was significantly elevated in HSu-treated animals (Figure 3F). Moreover, TG/HDL-c, TyG and HOMA-IR indexes, known markers of insulin resistance, were significantly elevated in the HSu-treated rats, compared to controls (Figure 3G-I).

**Figure 3.** Glucose tolerance test (GTT) and area under the curve (A-B), insulin tolerance test (ITT) and area under the curve (C-D), fasting glucose (E) and insulin (F) levels, as well as insulin resistance markers: TG/HDL ratio (G), TyG index (H) and HOMA-IR index (I) at the end of treatment. Control (n=12) and HSu (n=10). * p <0.05, ** p<0.01, *** p<0.001 and **** p ≤ 0.0001.

### 3.4. HSu diet increases fat mass while the insulin-stimulated glucose uptake in adipocytes is impaired

The epididymal fat pad weight/ body weight ratio was significantly higher in the HSu-treated rats when compared to controls (Figure 4A), while fat cell diameter and weight were unchanged between the groups (Figure 4C and D). Insulin-stimulated glucose uptake was measured in freshly
isolated adipocytes. Basal glucose uptake was not significantly different between groups (p>0.05). Adipocytes responded to the stimulatory effect of insulin in the uptake of glucose in both groups (Control: Basal – 20.31±2.59 vs. Insulin – 35.88±4.22, p<0.001; HSu: Basal – 8.55±1.09 vs. Insulin – 17.48±1.16, p<0.001); however, the insulin-stimulated glucose uptake was significantly reduced in adipocytes from HSu-treated compared to controls (17.48±1.16 vs. 35.88±4.22, p<0.01, respectively) (Figure 4B).

Figure 4. Epididymal fat pad weight/bw ratio (A), insulin-stimulated 14C-glucose uptake in isolated adipocytes (B), adipocyte diameter (C) and weight (D) at the end of treatment. Control (n=12) and HSu (n=10). ** p<0.01, *** p<0.001.

3.5. HSu diet decreased GLUT1 but increased G6Pase levels in liver

Gene and protein levels of mediators of glucose uptake and gluconeogenesis were quantified in liver, skeletal muscle and epididymal adipose tissue. There were no changes in glucose transporter gene levels in all tissues, in the fed state (Figure 5A). Moreover, GLUT1 protein levels were reduced in HSu-treated rats versus controls, with no change in GLUT2 expression, in liver. In addition, no differences were found in either GLUT1 or GLUT4 protein levels in skeletal muscle or epididymal adipose tissue. To assess hepatic gluconeogenesis, PEPCK and G6Pase gene and protein levels were evaluated. While there was no alteration in gene expression, there was a significant increase in only G6Pase protein levels in the HSu-treated group (Figure 5B).

A) Glucose transporters
3.6. **HSu diet increases hepatic lipid biosynthesis, without alterations in lipolysis**

Gene and protein levels of ACC1, FASN and DGAT1, that play a major role in lipid biosynthesis in liver and in fat, were analyzed, together with SREBP and ChREBP. Increased protein levels of ACC1, FASN and SREBP were observed in liver of HSu-treated animals, without changes in gene levels (Figure 6A). In addition, in adipose tissue, both gene and protein levels for ChREBP were increased in the HSu-treated rats, without further changes on the other measured mediators (Figure 6A).

Isoproterenol-stimulated lipolysis was performed to measure TG hydrolysis into glycerol and free fatty acids, and to evaluate antilipolytic effect of insulin in isolated adipocytes (Figure 6B1). Both groups responded similarly to the stimulatory effect of isoproterenol (Control: 0.66±0.11; HSu: 0.50±0.08) versus basal (Control: 0.30±0.03; HSu: 0.22±0.03) and insulin levels (Control: 0.34±0.04; HSu: 0.24±0.03).

Even though there is a tendency for an antilipolytic effect of insulin after isoproterenol stimulation (Control: 0.50±0.05; HSu: 0.39±0.05), it does not reach significance. Moreover, gene and protein expression levels for HSL were not different in either group (Figure 6B2).
A) Lipid biosynthesis

Liver

Epididymal Adipose Tissue
4. Discussion

The novel findings from this study indicate that a high-sugar diet induces early glucose and lipid dysmetabolism, in the absence of weight gain, only after 9 weeks of treatment. HSu-treated animals developed impaired insulin-stimulated glucose uptake and reduced insulin sensitivity (resistance), together with decreased GLUT1 but increased G6Pase protein levels in liver. These metabolic changes are paralleled by fed hyperglycemia and hypertriglyceridemia.

Throughout the 9-week treatment, the HSu-treated group had lower food consumption but higher beverage (35% sucrose) and caloric intake, while maintaining body weight, as previously described [11-13]. Similar body weight might have been maintained due to: first, the high-sugar treated rats may have a distinct energy requirement; second, differences in digestion and absorption may have modified the amount of ‘bioavailable’ energy, affecting the actual positive energy balance; third, the composition of weight gain (fat mass and lean mass) might be different, as the energy cost of protein deposition is higher than that of adipose tissue. In fact, we observed that HSu-treated animals have a greater adiposity translated by an increased epididymal fat pad weight/body weight ratio. Moreover, dietary protein content was suggested to be a critical determinant of weight gain during an ad libitum feeding [14].

This prediabetic animal model presented low food intake, which is enriched in 16.1% protein, and increased fluid intake of sucrose-enriched water, in agreement with previous studies [12]. This means that HSu-treated animals ingested a smaller amount of protein and a greater amount of carbohydrates, which can be translated by higher fat mass and smaller lean mass, which allowed the maintenance of body weight. Indeed, epididymal fat pad weight/body weight ratio was significantly higher in HSu-treated rats. Moreover, high-protein diets are known to reduce adiposity/lipogenesis.
in the context of high carbohydrate consumption in Western diets [15]. In fact, high-sucrose diets may increase adiposity by stimulating liver lipogenesis [16].

After 9 weeks of treatment, the HSu-treated group had higher serum TG levels, in agreement with previous reports [12,17-19]. In addition, ALT levels were significantly reduced in HSu-treated rats, while a significant increase in the liver weight/body weight ratio was observed, suggesting altered liver function. The strongest hypothesis to explain the reduced liver function and ALT low levels is malnutrition or an altered nutritional pattern. This prediabetes model had lower food consumption (constituted by 16.1% protein) and increased fluid intake (sucrose-enriched water). Thus, the low serum ALT levels, indicative of liver dysfunction, observed in the HSu-treated rats might be caused by a diet poor in essential nutrients/macromolecules, i.e. malnutrition.

This prediabetes model presented impaired glucose homeostasis. Already at the early stages of the disease, there were initial metabolic deregulations, evidenced by alterations in glucose tolerance during the GTT and ITT, as well as the glucose and insulin levels, indicative of insulin resistance, in agreement with other studies [12,20,21]. At this stage, although fasting glucose levels are maintained within normal values (normoglycemia), due to compensatory hyperinsulinemia, there is already some degree of glucose intolerance and peripheral insulin resistance, in agreement with other reports [12,22-24].

Insulin resistance was demonstrated not only by the increased HOMA-IR index but also by augmented TG/HDL ratio and the TyG index, which have previously been used as surrogate measures of impaired insulin sensitivity in obese adolescents with normoglycemia, in prediabetes, as well as in T2DM [25].

In an attempt to explain the glucose, insulin and lipid dysmetabolism in this model at the prediabetic stage, we assessed possible alterations in glucose and lipid metabolism in fat. Accordingly with previous studies [12], HSu diet increased fat mass, which is closely linked to the development of severe peripheral IR and prediabetes [26,27]. Moreover, under the fed state, HSu diet impaired insulin-induced glucose uptake in isolated adipocytes, confirming that visceral adiposity is strongly associated with impaired glucose uptake and IR [28]. Additionally, under IGT, adipocytes are resistant to insulin, and its effectiveness may be impaired, contributing to postprandial hyperglycemia in prediabetic states. Furthermore, the decrease in hepatic GLUT1 in the HSu-treated rats might be mediated by the hyperinsulinemia observed in this animal model [29]. On the other hand, both GLUT2 (in the liver) and GLUT4 protein levels (in the adipose tissue and skeletal muscle), were unchanged. The translocation of both GLUT2 and GLUT4 to the plasma membrane is mediated by insulin; however, under IR conditions, this process is impaired [30,31], leading to decreased insulin-stimulated glucose uptake in hepatocytes (mostly by GLUT2), while increasing postprandial blood glucose levels. Even though we did not measure translocation of glucose transporters in this study, it may be possible that these processes are impaired, thus also contributing to the increased postprandial blood glucose levels.

Another factor that may also be contributing to the postprandial hyperglycemia observed in this animal model is that high sucrose consumption increased gluconeogenesis, as previously reported [32]. However, under IGT and/or IR conditions, the derangement of hepatic glucose handling, indicated by changes in GLUT1 and G6Pase may at least in part lead to postprandial hyperglycemia in prediabetic states [33]. Furthermore, in this animal model of IR, insulin may not inhibit the de novo glucose production by the liver, leading therefore to elevated gluconeogenesis, resulting in elevated blood glucose levels in the fed state, as previously reported [29], as well as altered glucose tolerance during a GTT.

Furthermore, HSu diet increased liver lipid biosynthesis. Our results show that ACC1 and FASN enzymes, as well as the transcription factor SREBP expression levels were increased in the liver of HSu-treated rats, in agreement with previous studies [34-38]. The perturbed liver lipid metabolism observed might explain the hypertriglyceridemia present in this prediabetic animal model. In addition, high sucrose consumption may also be contributing to hypertriglyceridemia due to the increased ChREBP gene and protein levels observed in fat [39]. However, HSu diet did not induce changes in the isoproterenol-stimulated lipolysis in isolated adipocytes, and the HSL protein levels
were not different. HSL lipolytic activity is regulated by reversible phosphorylation on five critical residues [40]. Therefore the absence of alterations in lipolysis suggest that HSL phosphorylation was not changed by HSu diet. Finally, insulin did not showed significant antilipolytic effect at these insulin concentrations. However, it is important to note that we used supra-physiological insulin concentrations (1000 μU/ml). Testing physiological insulin concentrations are warranted in both control and HSu animals.

5. Conclusions

Our study shows that nine week of HSu-diet, which mimics at least in part western diets [5,6,41], can lead to impaired hepatic glucose and lipid metabolism, typical features of T2DM, already present in this prediabetic model, even in the absence of obesity. This animal model of diet-induced prediabetes shows reduced glucose tolerance, postprandial hyperglycemia, hiperinsulinemia, reduced insulin sensitivity (resistance) and hypertriglyceridemia, together with impaired gluconeogenesis and lipogenesis (Figure 7).

Figure 7. A molecular model describing insulin resistance and prediabetes development after a HSu diet. HSu diet increases serum triglyceride (TG) accumulation and triggers liver lipogenesis, inducing hypertriglyceridemia and interfering with liver function. However, no alterations were observed in lipolysis. HSu-diet alters hepatic proteins involved in basal glucose uptake and gluconeogenesis and impairs the insulin-stimulated glucose uptake in adipocytes, leading to impaired glucose homeostasis and increased IR markers. Legend: Apolipoprotein; CE – Cholesterol ester; IDL – Intermediate-density protein; LDL – Low-density lipoprotein; LDL – Lipoprotein lipase; TG – Triglycerides; VLDL – Very low-density lipoprotein.

This study should be viewed as an important wake up call for the lifestyle that general population have been gradually adopting, by consuming large amounts of simple sugars in soft drinks [42]. Importantly, exaggerated consumption of these drinks has been associated with an increased risk for T2DM development by about 26% if the average intake is one/two cans a day, or even more [43]. This feeding behavior is one of the main causes of the uncontrolled increase of IR, T2DM and associated complications, such as coronary heart disease [44,45]. We expect that new insights of glucose and lipid dysmetabolism at this early stage of the disease might contribute to disclose new therapeutic targets and strategies to counteract prediabetes and hinder the natural course of T2DM progression.
Supplementary Materials: The following are available online at www.mdpi.com/link, Supplemental methods; Table S1: Primer sequences for RT-PCR; Table S2: Antibodies used for Western blotting.

Acknowledgments: Authors kindly thank Novo Nordisk A/S for the human insulin (Actrapid), as well for funding support of: SPD/GIFT award, European Foundation for the Study of Diabetes (EFSD), EXCL/DTP-PIC/0069/2012, CNC.IBILI Strategic Project 2015-UID/NEU/04539/2013 funded by the Portuguese Foundation for Science and Technology (FCT) and by FEDER through Operational Programme Competitiveness Factors (COMPETE): FCOMP-01-0124-FEDER-028417 and POCI-01-0145-FEDER-007440 and; SFRH/BD/109017/2015 (Sara Nunes PhD scholarship by FCT). EC is partly supported by NIH P30AG028718, and NIH RO1 AG033761.

Author Contributions: A.B., F.C.P., F.R. and E.C. designed the study. All authors participated in rats housing/feeding and/or in the analytical assays. A.B., M.C., B.M.V-R. and SN performed statistical analyses. AB and M.C. prepared figures and drafted manuscript. A.B., F.C.P., F.R. and E.C. edited and revised manuscript. All authors read and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest.

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