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FRUCTOSE DURING PREGNANCY AFFECTS MATERNAL AND FETAL LEPTIN SIGNALING

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Abstract

Fructose intake from added sugars correlates with the epidemic rise in obesity, metabolic syndrome and cardiovascular diseases. Fructose intake also causes features of metabolic syndrome in laboratory animals. Therefore, we have investigated whether fructose modifies lipidemia in pregnant rats and produces changes in their fetuses. Thus, fructose administration (10% wt/vol) in the drinking water of rats throughout gestation, leads to maternal hypertriglyceridemia. This change was not observed in glucose-fed rats, although both carbohydrates produced similar changes in liver triglycerides and in the expression of transcription factors and enzymes involved in lipogenesis. After fasting overnight, mothers fed carbohydrates were found to be hyperleptinemic. However, after a bolus of glucose, leptinemia in fructose-fed mothers showed no-response, whereas it increased in parallel in glucose-fed and control mothers. Fetuses from fructose-fed mothers showed hypotriglyceridemia and a higher hepatic triglyceride content than fetuses from control or glucose-fed mothers. A higher expression of genes related to lipogenesis and a lower expression of fatty acid catabolism genes were also found in fetuses from fructose-fed mothers. Moreover, although hyperleptinemic, these fetuses exhibited increased tyrosine phosphorylation of the signal transducer and activator of transcription-3 (STAT-3) protein, without a parallel increase in the serine phosphorylation of STAT-3 nor in the suppressor of cytokine signaling-3 (SOCS-3) protein levels whose expression is regulated by leptin through STAT-3 activation. Thus, fructose intake during gestation provoked a diminished maternal leptin-response to fasting and re-feeding, and an impairment in the transduction of the leptin signal in the fetuses which could be responsible for their hepatic steatosis.

Key words: Fructose; pregnancy; leptin; rat; triglycerides.

1. Introduction

In the last few decades, obesity, metabolic syndrome, insulin resistance and diabetes have escalated to epidemic proportions in many countries worldwide. These metabolic diseases are multifactorial resulting from genetic, physiological, behavioural, and environmental influences. Genetic influence alone does not suffice to explain the rate at which these diseases have increased [1]. Moreover, nowadays several experimental and epidemiological studies clearly demonstrate that metabolic events during pre- and postnatal development markedly modulate metabolic diseases risks in later life [2]. This phenomenon called early nutritional or metabolic programming of adult health is strongly supported by animal experiments. In fact, it has been observed that fetal overnutrition may lead to the same outcomes as fetal undernutrition in terms of increased risk of obesity, hypertension, insulin resistance and cardiovascular disease in adult life [3, 4, and references therein].

Fructose, present in added sugars such as sucrose and high fructose corn syrup, has been linked with obesity and metabolic syndrome [5-7]. Experimental studies have shown that fructose can induce leptin resistance and virtually all the features of metabolic syndrome in rats, whereas glucose intake does not [8]. In fact, rodents fed chronically with solid diets containing high percentages of fructose (60–66%) develop hypertriglyceridemia and insulin resistance [9]. However, diets incorporating 10% wt/vol of fructose in drinking water induce hypertriglyceridemia and fatty liver without modifying plasma glucose and insulin levels [10, 11]. These changes were not observed in glucose-administered rats (10% wt/vol) and they were related to a fructose-induced impairment of the hepatic transduction in the leptin signal, a reduction in the amount of PPARα mRNA and a diminished fatty acid β-oxidation [12]. Clinical studies also support fructose as a cause of metabolic syndrome, especially in overweight individuals. Thus, overweight subjects that consumed a 25% fructose-based diet

for 10 wk developed insulin resistance, postprandial hypertriglyceridemia, and visceral obesity, unlike subjects given a glucose-based diet [13]. Furthermore, a recent report has shown that consumption of fructose-sweetened, but not glucose-sweetened beverages, with usual diet could induce postprandial hypertriglyceridemia in young subjects [14]. These data are consistent with a recent meta-analysis study that found sugar-sweetened drinks to be an independent risk factor for obesity, metabolic syndrome and diabetes [15]. Moreover, in a 20-year follow-up study including more than 40,000 men, it has been demonstrated that consumption of sugar-sweetened beverages is associated with increased risk of coronary heart disease and some adverse changes in lipids, inflammatory factors, and leptin [16]. Interestingly, a recent report has shown that alterations in VLDL-triglycerides induced by a 7-d high-fructose diet appear to be of greater magnitude in the healthy offspring of patients with type 2 diabetes than in control subjects, which suggests that these individuals might be more prone to developing dyslipidemia when challenged by high fructose intakes [17].

Since fructose intake modifies lipidemia in laboratory animals and humans [12, 18], we speculated that fructose administration during gestation would also cause changes in the perinatal development of fetuses. These changes could affect glycemia, insulinemia, lipidemia and/or the leptin signal and so, negatively influencing in morphological and physiological aspects of those pups [19, 20]. In fact, the effects of fructose intake during gestation have previously been studied in our laboratory by use of sucrose-rich diets. Thus, a high amount of sucrose (63% wt/wt) present in a pelleted diet produced maternal hypertriglyceridemia with negative effects on fetal growth [21, 22]. We have long-experience in perinatal development [19, 23, 24] and have studied the molecular events that might be disturbed as a consequence of altered perinatal nutrition which would cause long-standing consequences in adult metabolism [20, 25, 26].

Altogether, the connection between a greater intake of fructose-containing beverages and the increase in the prevalence of obesity, cardiovascular diseases, etc [16, 27], has been established. However, consumption of those beverages and fruit juices is allowed during gestation. Since there is solid evidence that much of our predisposition to adult illness is determined by the time of birth [28], in the current study we have investigated the effects of fructose intake (10% wt/vol) throughout gestation in glucose and lipid metabolism both in mothers and their fetuses.

2. Materials and methods

2.1. Animals and experimental design

Study I: Female Sprague-Dawley rats weighing 200-220 g were fed *ad libitum* standard rat chow (B&K Universal, Barcelona, Spain) and housed under controlled light and temperature conditions (12-h light-dark cycle; 22 ± 1ºC). The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU, Madrid, Spain. Animals were mated, and day 0 of pregnancy was determined by the appearance of spermatozoids in vaginal smears. Then, the animals were randomly separated into a control group, a fructose-supplemented group (Fructose), and a glucose-supplemented group (Glucose) (six to seven rats per group). Fructose and glucose were supplied as a 10% (wt/vol) solution in drinking water throughout gestation. Control animals received no supplementary sugar. Intake of solid food and liquid per cage were recorded daily; daily data for each parameter was used to calculate of the area under the curve (AUC) values for the duration of the experimental procedure.

On the morning of the 21st day of pregnancy, rats were decapitated and blood collected using tubes containing Na_2-EDTA. Liver was immediately removed, placed in liquid nitrogen and kept at -80 ºC until analysis. The conceptus was dissected, and, after being weighed,

fetuses were counted and weighed. Placentas were also obtained, weighted and frozen. Fetuses were decapitated, and blood from all pups of the same mother was collected and pooled into receptacles containing Na_2-EDTA for immediate separation of plasma at 4 ºC. The livers of the fetuses were obtained, and those coming from the same mother were pooled and placed in liquid nitrogen to be stored at −80 ºC until processed for further analysis.

Study II: A second set of pregnant rats were subjected to the same protocol as mentioned above (five rats per group), and on the morning of the 20th day of pregnancy were subjected to an oral glucose tolerance test (OGTT) in fasted conditions (12 h fasting). After drawing a basal blood sample from the tail vein, a bolus of glucose (2 g/kg) was administered orally to the animals. Subsequently, blood samples were collected into EDTA tubes at 7.5, 15, 20, 30, 45, and 60 min after glucose administration and placed on ice. Samples were then centrifuged, and plasma was stored at -20 ºC until processed for glucose, insulin and leptin determinations, and the AUC for glucose and insulin were calculated.

2.2. Determinations

Plasma aliquots, kept at -20 ºC, were used to measure glucose by an enzymatic colorimetric test [GOD/POD (glucose oxidase-peroxidase) method] (Spinreact, Girona, Spain). NEFA (non-esterified ('free') fatty acids) (Wako, Neuss, Germany), cholesterol and triglycerides were measured using commercial kits (Spinreact). Insulin was determined in plasma samples using a specific ELISA kit for rats (Mercodia, Uppsala, Sweden). Leptin and adiponectin were assayed in plasma samples using a specific enzyme immunoassay (EIA) kit for rats (Biovendor, Brno, Czech Republic; and Millipore, Bedford, MA, respectively).

Estimates of insulin resistance were calculated as previously described [29, and references therein], by determination of the following indexes from the short-term fasting plasma glucose and insulin values: homeostasis model assessment of insulin resistance

(HOMA-IR), quantitative insulin sensitivity check index (QUICKI), and fasting glucose to insulin ratio (FGIR). The HOMA-IR was calculated as the product of the fasting plasma glucose (FPG) and insulin (FPI) divided by a constant, 22.5. FPI was in micro-units per milliliter and FPG in millimoles per liter [30]. The QUICKI was calculated according to the following equation: the inverse log sum of fasting insulin in micro-units per milliliter and fasting glucose in milligrams per deciliter. Finally, the FGIR was calculated as the ratio of fasting plasma glucose divided by fasting plasma insulin, where FPG was in milligrams per deciliter and FPI in micro-units per milliliter.

A further index of insulin sensitivity was determined from the OGTT. The whole body insulin sensitivity index (WBISI) is based on values of insulin (microunits per milliliter) and glucose (milligrams values per deciliter) obtained from the OGTT and the corresponding fasting values, as originally described by Matsuda and DeFronzo [31]. The WBISI was calculated by the following equation: the ratio between a constant, 10,000; and the square root of the product of the FPG, the FPI, the mean OGTT glucose concentration and the mean OGTT insulin concentration.

Two hundred milligrams of frozen liver (or placenta) were homogeneized into 2.5 mL of 0.9% ClNa, and used for lipid extraction following of the Bligh and Dyer [32] method. Aliquots of lipid extracts were dried and the remaining residue weighted. Ten microliters (liver) or twenty microliters (placenta) of these homogenates were used to determine triglycerides as above mentioned.

2.3. Total RNA preparation and analysis

Total RNA was isolated from liver or placenta by using Ribopure (Ambion Inc., USA). RNA was prepared either from the liver of individual animals or from liver pools of fetuses of the same litter. The 260/280 absorption ratio of all samples was between 1.8 and 2.0.

Total RNA was subjected to DNase I treatment using Turbo DNA-free (Ambion Inc., USA), and RNA integrity was confirmed by agarose gel electrophoresis. Genomic DNA contamination was discarded by PCR using primers for ribosomal protein S29 (Rps29), resulting in a negative reaction. Later, cDNA was synthesized by oligo(dT)-primed reverse transcription with Superscript II (Invitrogen, USA). qPCRs were performed using a LightCycler 1.5 (Roche, Germany). The reaction solution was performed in a volume of 20 μl, containing 10 pmol of both forward and reverse primers, 10x SYBR Premix Ex Taq (Takara Bio Inc., Japan) and appropriate nanograms of the cDNA stock. Rps29 was used as a reference gene for qPCR. The sense and antisense primers sequences were: 5'-GAAGGCAAGATGGGTCACCAGCAGC-3' and 5'-CAGGGTAGACAGTTGGTTTCATTGGG-3' for Rps29 (BC058150). The rest of the primer sequences were obtained from Atlas RT-PCR Primer Sequences (Clontech, CA, USA). Samples were analyzed in duplicate on each assay. Amplification of non-specific targets was discarded using the melting curve analysis method for each amplicon. qPCR efficiency and linearity were assessed by optimization of standard curves for each target. The transcription was quantified with LightCycler Software 4.05 (Roche, Germany) using the relative quantification method.

2.4. Western Blot Analysis

Thirty micrograms postnuclear supernatant (for phospho (P)-Tyr-STAT3) or 40 μg crude nuclear extract (for P-Ser-STAT3) from the liver were subjected to 10% SDS-polyacrylamide gel electrophoresis (8% for P-Tyr-STAT3 and P-Ser-STAT3), as described previously [12]. Briefly, proteins were transferred to Immobilon polyvinylidene diflouride transfer membranes (Millipore, Bedford, MA) and blocked for one hour at room temperature with 5% non-fat milk solution in TBS-0.1% Tween-20. Membranes were then incubated with

the primary polyclonal antibody raised against P-Tyr-STAT3, and P-Ser-STAT3 (1:1,000 dilution), and SOCS-3 (1:200 dilution), in TBS–0.1%Tween-20 with 5% non-fat milk (except for P-STAT3 determination, which used 5% bovine serum albumin) at 4°C overnight. After several washes, they were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:3,000 dilution). Detection was achieved using the ECL chemiluminescence kit for horseradish peroxidase (Amersham Biosciences). To confirm the uniformity of protein loading in each lane, the blots were stained with Ponceau S. Values were normalized to β-actin (Sigma-Aldrich) levels. The size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Life Technologies). SOCS-3 and protein kinase B (Akt) antibodies were obtained from Santa Cruz Technologies, and those for STAT-3 and for P-Akt were obtained from Cell Signaling (Danvers, MA).

2.5. Statistical analysis

Results were expressed as means ± S.E., except where indicated. Treatment effects were analyzed by one-way analysis of variance (ANOVA). When treatment effects were significantly different ($P < 0.05$), means were tested by Tukey multiple range test. For non-parametric data, the Mann-Whitney *U* test was used with differences between the two groups analyzed by Student *t* test.

3. Results

3.1. Ingestion of a 10% w/v fructose solution throughout gestation does not affect glucose tolerance in pregnant rats

As shown in Table 1, fructose-fed rats showed a non-significant increase in the ingestion of liquids, and a reduction in the amount of ingested solid food. Accordingly, the total amount of ingested energy did not differ significantly between fructose-supplemented

and control rats (4168 vs. 3652 kcal/21 days/2 rats, respectively). Consequently, no changes were observed in the maternal body weight increase (free of conceptus) nor in the conceptus weight by the end of the gestation, after 21 days of fructose administration (Table 1).

In parallel, one group of mothers was fed glucose. In comparison to the control mothers, glucose-supplemented rats showed a marked increase in the ingestion of liquids (Table 1). However, the reduction in the amount of ingested solid food was non-significant and therefore, the total amount of ingested energy did not become significantly different between glucose-fed and control rats (4716 vs. 3652 kcal/21 days/2 rats, respectively). As a result, glucose solution ingestion throughout gestation did not significantly affect the maternal body weight increase nor the conceptus weight, in comparison to the control rats (Table 1).

Interestingly, neither fructose-fed nor glucose-fed pregnant rats showed alterations in the number or body weight of their fetuses (Table 1).

Fructose-fed pregnant rats were hypertriglyceridemic compared to control rats (Table 2). However, both fructose- and glucose-supplemented rats showed an increase in their plasma leptin concentrations (Table 2), this effect only being significant in the glucose-fed group. Similar findings were recorded for plasma adiponectin levels (Table 2). Therefore, only glucose-fed rats presented an increase in the leptin/adiponectin ratio (LAR) versus the control values (Table 2).

Both glycemia and insulinemia showed no-differences between the three groups (Table 2) and thus, glucose/insulin ratios turned out to be similar (12.5 ± 2.5, 10.3 ± 3.0, and 9.7 ± 2.9) for control, fructose-fed and glucose-fed rats, respectively. Plasma non-esterified fatty acid (NEFA) concentrations were similar in the carbohydrate-fed groups with respect to control values at the end of the feeding period (Table 2).

As shown in Table 2, carbohydrate-fed pregnant rats showed hepatic steatosis in comparison to the control rats, with it being more pronounced in the fructose-fed animals.

Related to this, hepatic expression of transcription factors and enzymes involved in fatty acid synthesis was increased in both fructose and glucose-fed rats. More precisely, fructose and glucose feeding induced an increase in the mRNA levels of the carbohydrate response element binding protein (ChREBP) (Table 2), which is a transcription factor directly involved in the hepatic transcriptional regulation of lipogenic genes by carbohydrates. According to this, the expression of lipogenic genes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) was significantly increased in fructose-fed and glucose-fed rats versus control values (Table 2). As expected, the hepatic fatty acid catabolism was diminished by carbohydrate ingestion. Thus, fructose and glucose-fed rats showed a non-significant reduction in mRNA levels for liver-carnitine palmitoyl transferase-I (L-CPT-I) and a marked diminution in uncoupling protein 2 (UCP2) levels in comparison to control rats (Table 2).

To further investigate the insulin-glucose relationships after feeding carbohydrate solution throughout gestation, we supplemented three groups of pregnant rats with either plain water, a fructose solution (10 % w/v) or a glucose solution (10 % w/v), and on the morning of the 20th day, we performed a glucose tolerance test, as described in the Material and Methods section. At time zero, the HOMA-IR, QUICKI and FGIR indexes were unchanged in fructose and glucose-fed 20-d pregnant animals versus controls (Table 3) and, consequently, fasting glucose and insulin were not different between the three groups. Glucose plasma concentrations were unchanged during the different time points of the experiment in the three groups (data not shown), although glucose-fed rats showed a non-significant increase in the AUC of plasma glucose concentration (Table 3). A similar profile was found for the insulin plasma levels: the curve corresponding to the glucose-fed group remained higher than those of the other two groups during all the time points of the experiment (data not shown). This is why glucose-fed rats showed a substantial but non-significant increase in the AUC of plasma insulin concentration (Table 3). In fact, the WBISI index which considers both fasting

glycemia and insulinemia and medium values of insulin and glucose during the OGTT, was not significantly lower in the glucose-fed pregnant rats (Table 3).

Interestingly, we found an unexpected finding in leptin response to fasting and refeeding in the animals used for the glucose tolerance test. As shown in Figure 1, after fasting overnight, mothers fed carbohydrates turned out to be hyperleptinemic in comparison to the control rats. However, after a bolus of glucose (2 g/kg), fructose-fed pregnant rats showed no-response at 30 min (slope near to 1, that is, an average increase in leptin of 1.16 pg/ml per minute), whereas in glucose-fed and control mothers leptinemia increased almost in parallel (slope = 15.65 for glucose-fed rats, and slope = 11.69 for controls). Consequently, at 60 min after the bolus of glucose, leptinemia had similarly increased in glucose-fed and controls rats (Figure 1) while it had hardly been modified in fructose-fed rats.

3.2. Fructose induced hepatic steatosis and modified leptin signal transduction in liver of fetuses from fructose-fed pregnant rats

One of the most important aims of the present work was to determine whether the ingestion of fructose solution (10% w/v) by pregnant rats throughout gestation would affect their fetuses. As shown in Table 4, only fetuses from glucose-fed mothers showed a slightly higher glycemia and insulinemia than fetuses from control rats. Thus, glucose/insulin ratios were similar in the fetuses from the three experimental groups (data not shown). Fetuses from carbohydrate-supplemented rats showed a non-significant increase in their plasma leptin concentrations (Table 4), but only fetuses from glucose-fed rats also presented a significant increase in plasma adiponectin concentrations.

Interestingly, fetuses from fructose-fed mothers presented, in contrast to the findings observed in their mothers, a clear hypotriglyceridemia in comparison to controls (Figure 2). Plasma NEFA levels were similar in fetuses from fructose-fed mothers versus fetuses from

the other two groups (Table 4). Nevertheless, although triglyceride content in placenta of fructose-fed rats (1.43 ± 0.05 mg/g of tissue) was slightly lower than in placenta from glucose-fed and control rats (1.61 ± 0.13, and 1.69 ± 0.13, respectively), the placental LPL expression showed no differences between the three groups (12.8 ± 3.4, 14.1 ± 4.3, and 18.2 ± 2.1 a.u., for control, fructose-fed and glucose-fed mothers, respectively).

As shown in Figure 2, fetuses from fructose-fed pregnant rats showed hepatic steatosis in comparison to the fetuses from glucose-fed and control rats. Accordingly, as shown in Figure 3, the fetal livers of fructose-fed mothers presented marked increases in the mRNAs for sterol response element-binding protein-1 (SREBP-1), and for stearoyl-CoA desaturase 1 (SCD1) versus livers from fetuses of glucose-fed and control mothers. Moreover, the hepatic fatty acid catabolism was also diminished in the fetuses from fructose-fed rats. In fact, these fetuses showed a marked diminution in the hepatic UCP2 mRNA levels in comparison to the fetuses from glucose-fed and control mothers (Figure 3).

Related to the findings observed in the hepatic gene expression, fetuses from fructose-fed rats, but not from glucose-fed or control mothers, exhibited an increased tyrosine phosphorylation of the signal transducer and activator of transcription-3 (STAT-3) transcription factor, although they did not present a similar increase in the serine phosphorylation of nuclear STAT-3 (Figure 4). Finally, the protein levels for suppressor of cytokine signaling-3 (SOCS-3) remained unchanged in fetal liver samples obtained from fructose-fed rats (Figure 4).

4. Discussion

A sucrose-rich diet during pregnancy in rats has been reported to reduce fetal or newborn weights [21], although other authors report no effect on this variable [33, and references therein]. Moreover, fetuses of dams fed a high carbohydrate diet in late or

throughout, but not in early, pregnancy had lower body and placental weights than controls [34]. Also, it has been described that changes in the type of carbohydrate eaten (high- vs. low-glycaemic sources) during pregnancy influences the rate of feto-placental growth [35]. In a recent report, it has been shown that a high-carbohydrate supplement ingestion during gestation leads to insulin and leptin resistance in adult offspring independently of birth weight, and it is known that these hormonal changes characterize obesity-prone animals [36]. In fact, Fergusson and Koski [37] concluded that, during pregnancy, it was the quantity rather than quality of carbohydrate that determined fetal and postnatal development.

In the present study, the ingestion of a fructose solution (10% w/v) by pregnant rats throughout gestation did not affect the number or body weight of their fetuses. Furthermore, despite the proposed role of maternal hypertriglyceridemia on fetal growth [23, and references therein], the maternal-augmented hypertriglyceridemia found here in fructose-fed pregnant rats did not affect the fetal weight. Hypertriglyceridemia was also reported both in females and males fed 10% of fructose in drinking water [38] for 14 days, in males drinking fructose in water for 21 days [39], and in pregnant and non-pregnant rats fed a sucrose-rich diet [21]. The increase in circulating triglycerides caused by fructose in drinking water could be due to an enhanced release of adipose tissue lipolytic products providing substrates (NEFA) for an increased liver triglyceride synthesis and release into the circulation in the form of VLDL-triglycerides [21, 23]. However, in the present study, plasma NEFA concentration did not differ from the values found in glucose-fed or control pregnant rats. Moreover, fructose-fed pregnant rats showed an accumulation of triglycerides in the liver. Since that accumulation was also found in glucose-fed pregnant rats, the hepatic steatosis produced by carbohydrate feeding would be probably due to, on the one hand, the increase in the hepatic gene expression of enzymes related to fatty acid synthesis (FAS and ACC) and ChREBP and, on the other hand, a diminution in fatty acid catabolism since UCP2 expression was reduced.

ChREBP has been directly involved in the transcriptional regulation of lipogenic genes in the liver by carbohydrates [40]. Thus, similar results have been reported for male and female rats, where fructose and glucose ingestion induced a lipogenic effect in the livers of carbohydrate-supplemented rats [12, 38]. The UCP2 expression in liver can modify the efficiency of ATP production, leading to a diminution in the fatty acid catabolism [41] and, therefore, an augmented storing of fatty acids as triglycerides.

Since it had previously been demonstrated that fructose-fed female, but not male rats, showed hyperinsulinemia and an altered glucose tolerance test [38], we studied glucose homeostasis in control, fructose- and glucose-fed pregnant rats. However, none of the indexes for measuring insulin resistance here determined (HOMA, QUICKI, FGIR, and WBISI) were affected by fructose ingestion throughout gestation. Thus, insulin sensitivity was not modified by fructose in drinking water in late pregnancy. Accordingly, the hepatic insulin signal transduction was also unaltered by fructose ingestion since IRS2 and AKT proteins did not present (measured as total protein or as phosphorylated form) any differences in comparison to control rats (data not shown). In agreement with these results, a sucrose-rich diet did not modify either insulinemia and glycemia nor insulin sensitivity in pregnant rats, but not in virgin rats [21].

A very interesting and unexpected finding was observed in the leptin response to fasting and refeeding. Leptin response to refeeding has been observed in animals both with a high fasting leptin concentration and with low leptin levels [42]. Nevertheless, although fasting leptin levels were augmented in carbohydrate-fed pregnant rats in comparison to control rats, fasted fructose-fed pregnant rats, but not glucose-fed or control animals, showed no response in leptin concentrations to a bolus of glucose. This relevant finding, which has not been previously described, would indicate a fructose-induced leptin resistance in these pregnant rats.

Next, we aimed to study how the ingestion of a fructose solution (10% w/v) by pregnant rats throughout gestation could affect their fetuses. In spite of fructose-fed mothers

being hypertriglyceridemic, their fetuses showed hypotriglyceridemia. This protection against exaggerated maternal triglycerides observed here in both fetal triglyceridemia and growth would be a new example of the impermeability of the placenta for maternal circulating triglycerides [33, 43]. Thus, maternal triglycerides are unable to cross the placental barrier directly, but the presence of LPL in the placenta allows fatty acids to reach the fetus for use in triglyceride synthesis in the fetal liver [43]. Fetuses from fructose-fed mothers showed lower values of NEFA than fetuses from glucose-fed or control rats. However, placental LPL gene expression turned out to be similar in the three groups.

Interestingly, fetuses from fructose-fed pregnant rats presented a significantly higher accumulation of hepatic triglyceride than presented in those from dams fed glucose or controls. Related to this interesting result, we observed an increase in the hepatic gene expression of the enzymes and transcription factors related to triglyceride synthesis, namely, SCD1 and SREBP1. SCD1 is an enzyme that controls the biosynthesis of hepatic cholesterol esters and triglycerides [44], and SREBP1 is a key transcription factor controlling fatty acid synthesis in liver [40]. In a previous report, we had found a clear relationship between SCD1 expression and triglyceride accumulation in liver [24]. Therefore, fructose ingestion, but not glucose, in pregnant rats was producing a lipogenic effect in the liver of their fetuses. Moreover, hepatic fatty acid catabolism was diminished in fetuses from fructose-fed mothers since UCP2 mRNA was reduced. Uncoupling proteins dissipate the energy generated by fatty acid (or glucose) oxidation as heat [45]. Thus, a lower UCP content would indicate a higher efficiency of substrate oxidation for generating energy.

Leptin controls intracellular triglyceride homeostasis by increasing fatty acid oxidation and decreasing fatty acid synthesis and triglyceride accumulation [45, 46]. Although the fetuses from fructose-fed mothers had a higher plasma leptin with respect to the control ones, none of the above mentioned effects attributed to leptin were found in the fetuses from

fructose-fed rats. A similar leptin resistance state had been previously reported in fructose-fed male rats [12]. That failure in the leptin response was confirmed, since fetuses from fructose-fed pregnant rats showed an increase in P-Tyr-STAT3 without a parallel increase in serine phosporylation of STAT3, necessary for the full transcriptional capability of this key factor in the leptin signal transduction. Consequently, SOCS-3 protein, whose expression is regulated by leptin through STAT3 activation [47], also remained unchanged.

In a previous work [48], fructose-induced state of hepatic leptin resistance has been related to an impairment of the leptin-signal transduction mediated by both janus-activated kinase-2 (JAK-2) and the mitogen-activated protein kinase pathway. These events were linked to an increase in the hepatic expression of the suppressor of cytokine signaling-3 protein and a deficit of Ser/Thr phosphoproteins involved in leptin transduction. In the present study, although fetuses from fructose-fed mothers did not show an increase in SOCS-3 protein, a deficit of phosphorylation in serine residue of STAT-3 protein and a lack of phosphorylation and activation of JAK-2 (data not shown) were found, suggesting a leptin resistance state.

The rat placenta appears to be permeable to leptin, therefore, leptin in the fetal circulation may have originated from the mother and/or the placenta [49]. A diminished maternal leptin-response to fasting and re-feeding such as the one observed in the current work, might be keeping maternal leptin at almost constant high levels, and consistently provoking a continuous transfer of leptin to the fetus. This hypothetical situation in the fructose-fed mothers could be producing an impaired hepatic transduction of the leptin signal in their fetuses which could be responsible for their hepatic steatosis.

Lifetime nutrition seems to be less important than the nutrition during pregnancy in producing changes in the offspring phenotype, suggesting that the postnatal sequels of maternal nutrition occur independently of preconceptional diet [1, 33]. Furthermore, the importance of maternal nutrition during critical periods of development is clear, since

maternal feeding can induce aberrant phenotype in offspring irrespective of postnatal nutrition [1]. The most prominent result found here is that the intake of just a small amount of fructose (10%) throughout gestation produces a clear impairment in the leptin signaling not only in the mothers but, more interestingly, in their fetuses. Thus, the common and worldwide frequent ingestion of fructose containing beverages by young adults, including reproductively active individuals, reinforces the notable clinical relevance of the observations obtained in the present study.

Acknowledgements

The authors would like to thank Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+i), Instituto de Salud Carlos III-Subdirección General de Evaluación y Fomento de la Investigación (PI-09/02192), European Community FEDER funds, and MCYT (SAF2010-15664), as well as the Fundación Universitaria San Pablo-CEU (PC 7/2010 and PC 9/2011) for financial support. Lourdes Rodríguez was a recipient of a fellowship from the CQS-Lab, Madrid, Spain. The authors thank Jose M. Garrido for his help in handling the rats, and Brian Crilly for his editorial help. The authors very especially thank Estrella Relaño for her excellent work with the lipid extracts and homogenates.

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Legends

Fig. 1. Ingestion of a 10% w/v fructose solution throughout gestation does affect maternal leptin response. (A) Plasma leptin values at different times after the oral administration of a bolus of glucose solution (2 g/kg body weight) to control (squares), fructose-fed (triangles), and glucose-fed (circles) 20d-pregnant rats. Results are the mean ± S.E. of values from five animals. Small letters correspond to the statistical comparisons between rats receiving different supplementation into the drinking water. Values not sharing a common letter are significantly different at $p < 0.05$. Broken lines and equations correspond to linear regressions made with the corresponding values of each experimental group. (B) Plasma leptin levels before (t0') and sixty minutes (t60') after receiving a bolus of glucose, from control (empty bar), fructose- (black bar), and glucose-supplemented (grey bar) 20d-pregnant rats. Each bar represents the mean ± S.E. of values from 5 animals. f.i., fold induction.

Fig. 2. Maternal ingestion of a 10% w/v fructose solution throughout gestation does affect fetal plasma and liver triglycerides. (A) Triglyceridemia values of fetuses from control, fructose-fed and glucose-fed pregnant rats. (B) Hepatic content of triglycerides of fetuses from control, fructose-fed and glucose-fed pregnant rats. Results are the mean ± S.E. of values from 6-7 plasma pools of fetuses of the same litter. Small letters correspond to the statistical comparisons between rats receiving different supplementation into the drinking water. Values not sharing a common letter are significantly different at $p < 0.05$.

Fig. 3. Maternal intake of a 10% w/v fructose solution throughout gestation does affect fetal liver gene expression. (A) Relative hepatic levels of specific mRNA for the fatty acid synthesis enzyme SCD1 of fetuses from control (empty bar), fructose- (black bar), and glucose-fed (grey bar) pregnant rats. (B) Relative hepatic levels of specific mRNA for the

transcription factor SREBP1c of fetuses from control (empty bar), fructose- (black bar), and glucose-fed (grey bar) pregnant rats. (C) Relative hepatic levels of specific mRNA for UCP2 of fetuses from control (empty bar), fructose- (black bar), and glucose-fed (grey bar) pregnant rats. Relative target gene mRNA levels were measured by Real Time PCR, as explained in Materials and Methods, and normalized to Rps29 levels and expressed in arbitrary units (a.u.). Each bar represents the mean ± S.E. of values from 6-7 animals. RNA was prepared from liver pools of fetuses of the same litter. Small letters correspond to the statistical comparisons between fetuses from mothers receiving different supplementation into the drinking water. Values not sharing a common letter are significantly different at $p < 0.05$.

Fig. 4. Maternal ingestion of a 10% w/v fructose solution throughout gestation produces defective STAT3 activation in livers of their fetuses. Bar plot showing the levels of P-Tyr-STAT3 (A), P-Ser-STAT3 protein (B) and SOCS-3 protein levels in hepatic samples from fetuses from control (empty bar), fructose- (black bar), and glucose-fed (grey bar) pregnant rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed by staining the blots with Ponceau S. Values were normalized to β-actin levels and expressed in arbitrary units (a.u.). Each bar represents the mean ± SD of values from 3 pools of two fetal hepatic samples in each one. Small letters correspond to the statistical comparisons between fetuses from mothers receiving different supplementation into the drinking water. Values not sharing a common letter are significantly different at $p < 0.05$.

Table 1

Table 1

Body weight, food and liquid ingestion in fructose- or glucose- supplemented pregnant rats.

	Control	Fructose	Glucose
Maternal body weight at day 0 (g)	225.0 ± 2.3	219.5 ± 4.4	217.5 ± 6.3
Maternal body weight at day 21 (g)	358.5 ± 5.6	352.6 ± 5.4	373.6 ± 13.5
AUC consumed diet (g/21 days per 2 rats)	970.8 ± 36.8	807.3 ± 48.3	797.4 ± 74.9
AUC ingested liquid (ml/21 days per 2 rats)	1648.0 ± 18.4[a]	2827.2 ± 257.8[ab]	4289.6 ± 734.1[b]
Number fetus/litter	12.7 ± 0.7	12.1 ± 0.5	13.2 ± 1.0
Fetal body weight (g)	6.3 ± 0.1	6.5 ± 0.2	6.3 ± 0.2
Conceptus weight (g)	90.0 ± 4.4	88.4 ± 3.8	97.4 ± 6.5
Maternal body weight increase (conceptus-free) (g)	43.5 ± 3.2	44.7 ± 4.6	58.6 ± 7.4

Data are expressed as M ± SE, n = 6-7 rats. Individual AUC values for ingested liquid and solid diet are the mean corresponding to two animals housed in the same cage. Different letters indicate significant differences between the groups (*P*< 0.05).

Table 2

Table 2
Plasma analytes, hepatic triglyceride content and liver gene expression in fructose- or glucose- supplemented pregnant rats.

	Control	Fructose	Glucose
Glucose (mg/dL)	88.2 ± 5.6	117.2 ± 10.7	101.6 ± 7.6
Insulin (μg/L)	0.36 ± 0.07	0.57 ± 0.15	0.71 ± 0.23
Triglycerides (mg/dL)	65.9 ± 14.9[a]	117.9 ± 12.6[b]	84.4 ± 10.0[ab]
NEFA (mM)	0.52 ± 0.11	0.45 ± 0.12	0.66 ± 0.09
Leptin (ng/ml)	1.80 ± 0.42[a]	2.17 ± 0.12[ab]	3.88 ± 0.90[b]
Adiponectin (μg/ml)	6.83 ± 0.49[a]	8.82 ± 0.73[ab]	11.19 ± 1.60[b]
LAR (ng/μg)	0.18 ± 0.02[a]	0.24 ± 0.01[ab]	0.36 ± 0.07[b]
Liver triglycerides (mg/g tissue)	11.1 ± 0.7[a]	17.0 ± 1.6[b]	15.5 ± 1.4[ab]
Liver mRNA expression			
ChREBP (a.u)	0.354 ± 0.063[a]	0.504 ± 0.040[ab]	0.606 ± 0.063[b]
FAS (a.u)	3.80 ± 1.12[a]	9.93 ± 1.19[b]	8.75 ± 0.95[b]
ACC (a.u)	1.23 ± 0.28[a]	2.46 ± 0.21[b]	2.85 ± 0.50[b]
CPTI (a.u.)	0.993 ± 0.193	0.647 ± 0.223	0.530 ± 0.192
UCP2 (a.u)	0.577 ± 0.108[a]	0.326 ± 0.061[ab]	0.288 ± 0.059[b]

Data are M ± SE, n = 6-7 rats. LAR: Leptin/adiponectin ratio. Different letters indicate significant differences between the groups (*P*< 0.05). (a.u., arbitrary units)

Table 3

Table 3
Insulin sensitivity indexes in fructose- or glucose- supplemented 20-d pregnant rats.

	Control	**Fructose**	**Glucose**
Plasma glucose (mg/dL)	90.8 ± 7.2	95.4 ± 9.3	82.7 ± 3.1
Plasma insulin (µg/L)	0.61 ± 0.07	0.50 ± 0.09	0.39 ± 0.05
HOMA-IR	3.88 ± 0.85	3.12 ± 0.57	3.40 ± 1.24
QUICKI	0.318 ± 0.009	0.330 ± 0.015	0.331 ± 0.014
FGIR (mg/10^{-4} U)	5.88 ± 0.82	8.66 ± 1.95	8.90 ± 2.17
WBISI	3.96 ± 0.68	4.71 ± 1.05	3.08 ± 0.70
AUC-Glucose (mg/dL/60 min)	1866 ± 352	2014 ± 464	2795 ± 568
AUC-Insulin (µg/L/60min)	76.4 ± 24.1	75.3 ± 27.9	113.6 ± 21.9

Data are M ± SE, n = 5 rats. AUC: Area under the curve for glucose or insulin during the OGTT. Insulin sensitivity indexes were calculated from short-term fasting plasma glucose and insulin values as described in Materials and Methods.

Table 4
Plasma analytes in fetuses from fructose- or glucose- supplemented pregnant rats.

	Control	**Fructose**	**Glucose**
Glucose (mg/dL)	89.8 ± 4.2	94.6 ± 4.4	122.3 ± 14.3
Insulin (µg/L)	1.00 ± 0.09	1.19 ± 0.24	1.35 ± 0.29
NEFA (µM)	83,9 ± 13,7	68,1 ± 18,0	82,2 ± 9,7
Leptin (ng/ml)	0.82 ± 0.26	1.33 ± 0.07	1.35 ± 0.25
Adiponectin (µg/ml)	0,83 ± 0,13[a]	0,83 ± 0,07[a]	1,44 ± 0,18[b]

Data are expressed as M ± SE, n = 6-7 plasma pools of the fetuses of the same litter. Different letters indicate significant differences between the groups (*P*< 0.05).

Figure 1
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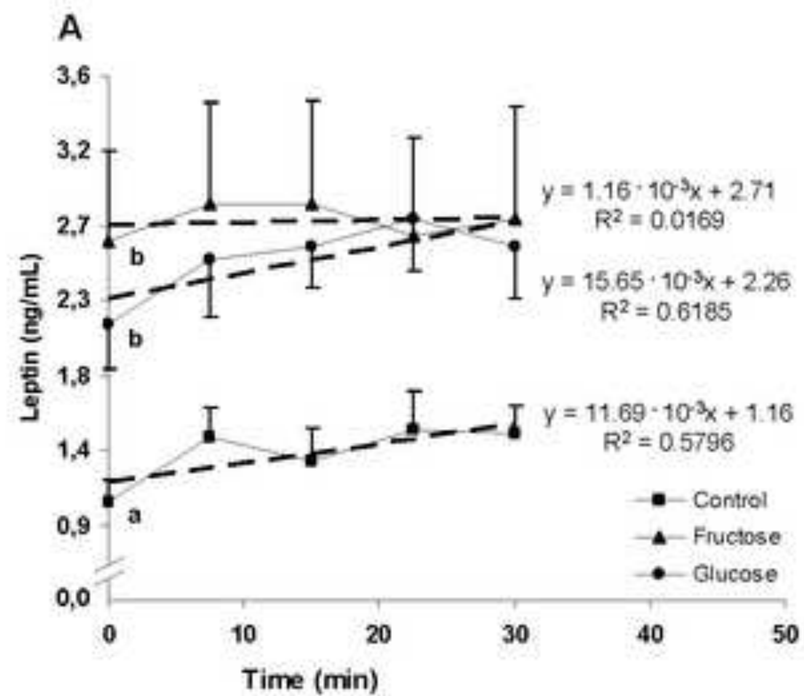

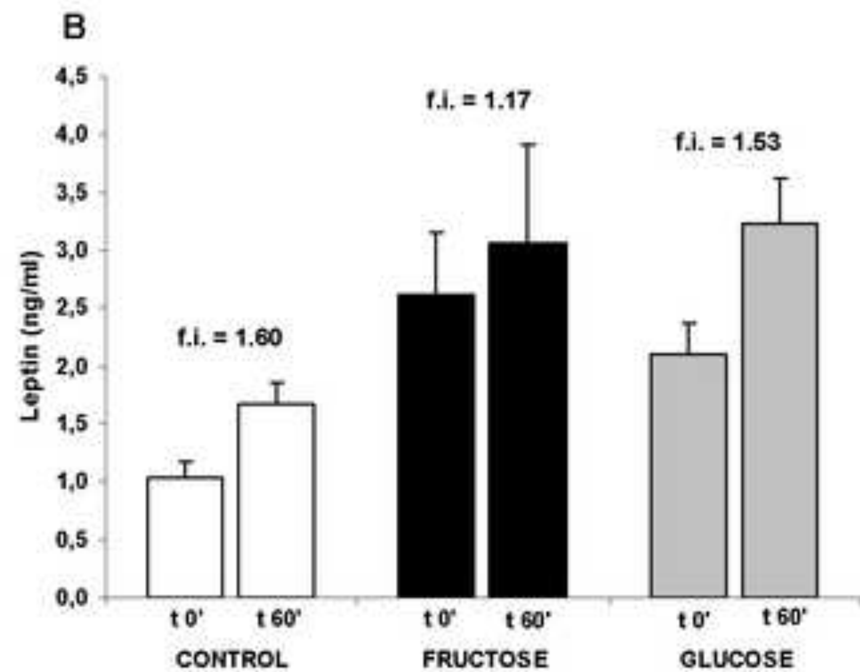

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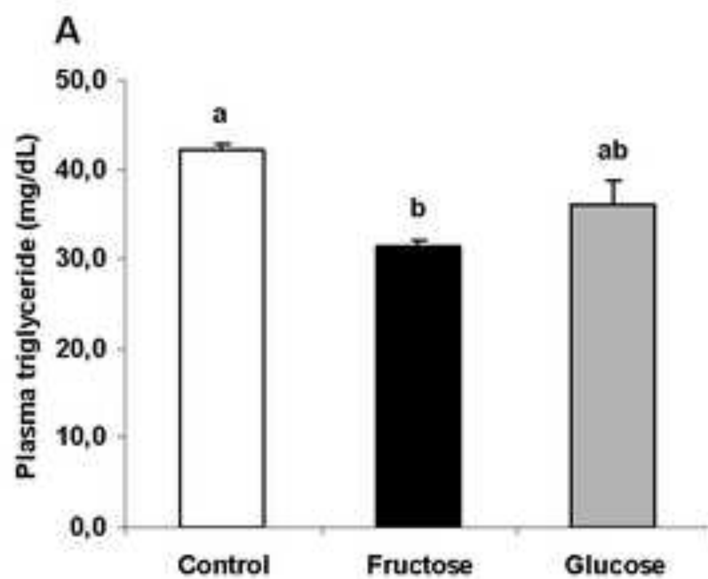

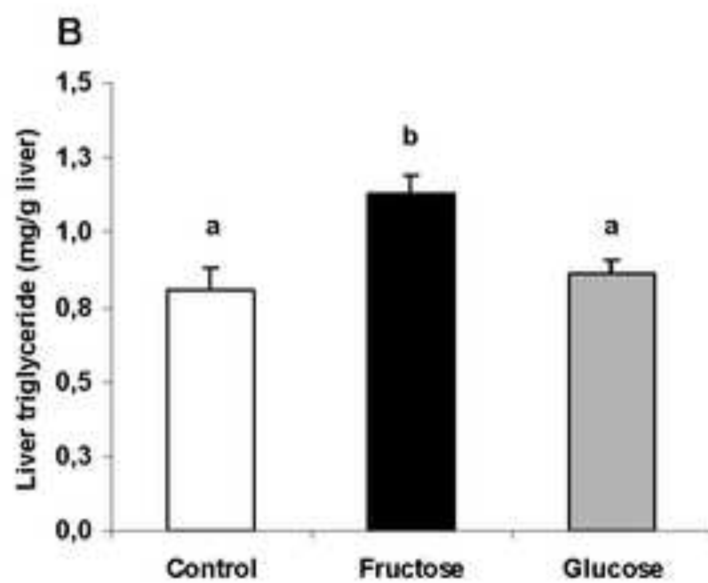

Figure 3

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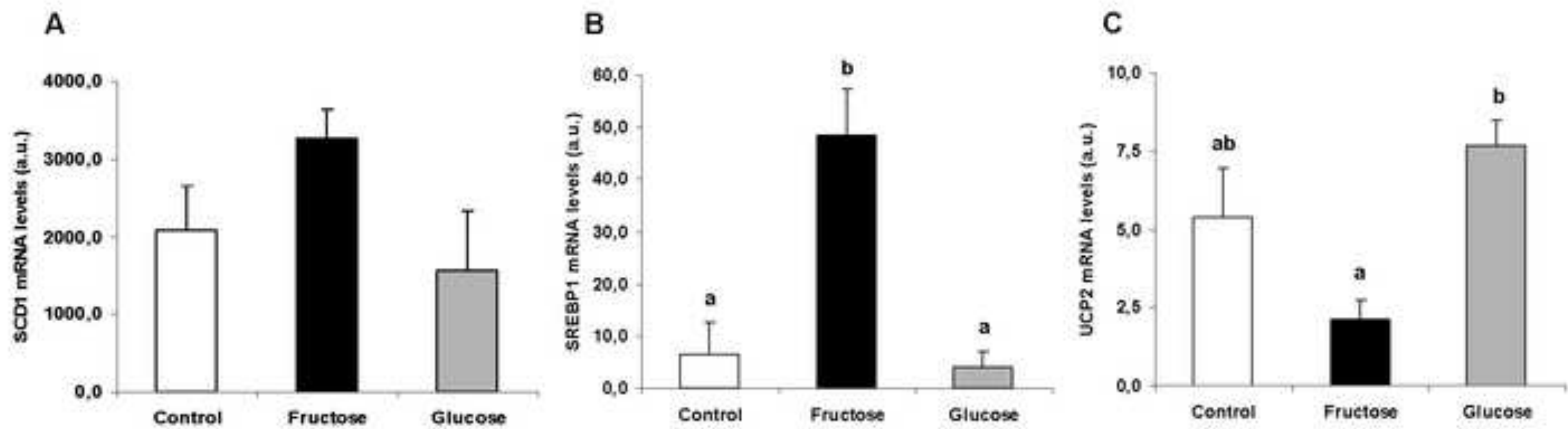

Figure 4
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