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Liquid fructose down-regulates liver insulin receptor substrate 2 and gluconeogenic enzymes by modifying nutrient sensing factors in rats☆

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Abstract

High consumption of fructose-sweetened beverages has been linked to a high prevalence of chronic metabolic diseases. We have previously shown that a short course of fructose supplementation as a liquid solution induces glucose intolerance in female rats. In the present work, we characterized the fructose-driven changes in the liver and the molecular pathways involved. To this end, female rats were supplemented or not with liquid fructose (10%, w/v) for 7 or 14 days. Glucose and pyruvate tolerance tests were performed, and the expression of genes related to insulin signaling, gluconeogenesis and nutrient sensing pathways was evaluated.

Fructose-supplemented rats showed increased plasma glucose excursions in glucose and pyruvate tolerance tests and reduced hepatic expression of several genes related to insulin signaling, including insulin receptor substrate 2 (IRS-2). However, the expression of key gluconeogenic enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, was reduced. These effects were caused by an inactivation of hepatic forkhead box O1 (FoxO1) due to an increase in its acetylation state driven by a reduced expression and activity of sirtuin 1 (SIRT1). Further contributing to FoxO1 inactivation, fructose consumption elevated liver expression of the spliced form of X-box-binding-protein-1 as a consequence of an increase in the activity of the mammalian target of rapamycin 1 and protein 38-mitogen activated protein kinase (p38-MAPK). Liquid fructose affects both insulin signaling (IRS-2 and FoxO1) and nutrient sensing pathways (p38-MAPK, mTOR and SIRT1), thus disrupting hepatic insulin signaling without increasing the expression of key gluconeogenic enzymes.

Keywords: Insulin resistance; FoxO1; XBP-1; SIRT1; p38-MAPK; mTOR

1. Introduction

During the last decade, there has been an increasing number of published epidemiological and interventional studies on human populations linking the high consumption of sugar-sweetened beverages, enriched in simple sugars such as fructose and glucose, to the high prevalence of chronic metabolic and related cardiovascular diseases [1–4]. These diseases include dyslipidemia [5,6], gout [7], hypertension [8,9], obesity [10,11], insulin resistance [12] and type 2 diabetes mellitus [10,13–15]. High energy intake, lack of adequate energy compensation through a proportional decrease in the amount of energy ingested as solid foods and the special metabolism of fructose have been reported to contribute to this possible causal association [2,3].

Among the experimental animal models used, fructose-fed rats can reproduce metabolic alterations induced by fructose consumption in humans. Rats, like humans, cannot transform ingested fructose into glucose [16]. Moreover, in both rats and humans, continuous fructose ingestion induces the expression of fructokinase, an enzyme that drives fructose into liver metabolic pathways [17–19]. The changes associated with the human metabolic syndrome can be reproduced in rats by providing them fructose as a 10% (w/v) solution [20–22]. This mimics the level of dietary consumption of sweetened beverages (as a percentage of the daily caloric intake about 30%) that leads to clear metabolic disturbances in humans [23,24]. Using this model, we recently discovered a gender difference in the response to fructose consumption, with clear signs of glucose intolerance after a short 14-day course of fructose supplementation in female, but not male, rats

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[17]. We also found that fructose-fed female rats displayed a significantly reduced expression of liver insulin receptor substrate 2 (IRS-2) [17], one of the key insulin-signaling proteins in the liver [25].

We therefore pursued the search for the molecular mechanism underlying the glucose intolerance observed after fructose supplementation in female rats. In the present work, we show that fructose induces a set of complex molecular alterations in the liver expression of nutrient sensing factors, which reduces the expression of both liver IRS-2 and gluconeogenic enzymes in fructose-supplemented female rats.

2. Methods

2.1. Animals and experimental design

Female Sprague–Dawley rats purchased from Charles River (Barcelona, Spain) were maintained with water and food *ad libitum* at constant humidity and temperature with a light/dark cycle of 12 h. The animals were randomly separated into a control and a fructose-supplemented group (8 and 12 rats per group, respectively). Fructose was supplied as a 10% (w/v) solution in drinking water for 7 and 14 days. Control animals received no supplementary sugar. At the end of this period, animals were killed by decapitation under isoflurane anesthesia at 10 a.m. Food and fructose solution were removed at 8 a.m. To reduce the variability in plasma estrogen concentrations, female rats were killed during the diestrus period.

Subgroups of female rats were randomly separated into control and fructose-supplemented groups, as described above, for glucose and pyruvate tolerance tests.

All procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee, as stated in the Autonomous Government of Catalonia's Law 5/1995 (21st of July).

2.2. Sample preparations

Blood and liver tissue samples were collected and stored as described previously [21]. Total and nuclear extracts were isolated using the Helenius method [26]. Protein concentrations were determined by the Bradford method [27].

2.3. Lipids, glucose, insulin, adiponectin and leptin analysis

Plasma triglyceride, glucose, insulin, leptin and adiponectin concentrations were measured as described previously [21].

2.4. Glucose tolerance test

After a 2-h fast, the rats were anesthetized, and following the collection of an unchallenged sample (*time 0*), a glucose solution of 2 g/kg body weight was administered into the peritoneal cavity. During the test, blood was collected from the saphenous vein at 15, 30, 60, 90 and 120 min after glucose administration. Glucose measurements were performed using a hand-held glucometer. Plasma insulin levels were measured at baseline, 15, 60 and 120 min post-glucose administration using a rat insulin enzyme-linked immunosorbent assay kit (Millipore, Billerica, MA).

2.5. Pyruvate tolerance test

Rats were injected with sodium pyruvate (2 g/kg ip). Plasma glucose levels were determined at 0, 15, 30, 60, 90, 120 and 150 min after injection with a hand-held glucometer as described above.

2.6. Cell culture

Rat hepatoma FaO cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were cultured in low glucose DMEM (Gibco, Life Technologies, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS Gold; PAA, Piscataway, NJ) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco, Life Technologies, Madrid, Spain). When cells reached 80% confluence, the proportion of serum was reduced to 1% and the cells were incubated in the absence or presence of fructose (Sigma-Aldrich, St. Louis, MO) at a concentration of 25 mM for 24 h.

2.7. RNA preparation and analysis

Total RNA was isolated using the TrizolR reagent (Invitrogen, Life Technologies, Madrid, Spain). The levels of specific mRNAs were assessed by real-time reverse transcription (RT)–polymerase chain reaction (PCR) using Sybergreen PCR Master Mix, specific primers and the Applied Biosystems One-Step Plus sequence detection system (Applied Biosystems, Foster City, CA). For G6Pc, phosphoenolpyruvate carboxykinase (PEPCK) and X-box-binding-protein-1 (XBP-1), we used conventional RT–PCR as described previously [34]. Adenosyl phosphoribosyl transferase or 18S was used as an internal control. The primer sequences, resulting PCR products and number of cycles are listed in Supporting Table 1.

2.8. Western-blot analysis

Ten to thirty micrograms of different protein fractions from the rat livers were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA), blocked for 1 h at room temperature with 5% non-fat milk solution in 0.1% Tween-20 Tris-buffered saline (TBS) and incubated as described previously [21]. Detection was performed with the ECL chemiluminescence kit for HRP (Amersham GE Healthcare Europe GmbH, Barcelona, Spain). To confirm the uniformity of protein loading, the blots were incubated with the β-actin or β-tubulin antibody (Sigma-Aldrich, St. Louis, MO) as a control. The size of the detected proteins was estimated using protein molecular mass standards (Invitrogen, Life Technologies, Madrid, Spain). Antibodies were obtained from Santa Cruz Technologies (Santa Cruz, CA), except those for phospho- and total protein kinase B or Akt, phospho- and total protein 38-mitogen activated protein kinase (p38-MAPK) and PP2Ac, which were obtained from Cell Signaling (Danvers, MA), and phospho- and total mTOR were purchased from Millipore (Billerica, MA).

2.9. Co-immunoprecipitation

Two hundred micrograms of protein extracts was precleared in preclearing matrix F (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C, and the resulting supernatant was immunoprecipitated for 4 h at 4°C with 4 μg of anti-acetyl-lysine (Cell Signaling, Danvers, MA) in a final volume of 0.5 ml made up with buffer containing 10 mM phosphate-buffered saline (PBS) and 2% bovine serum albumin. Immunocomplexes were captured by incubating the samples with protein A-agarose suspension (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS-containing protease inhibitors. After microcentrifugation, the pellet was re-suspended with 60 μl of SDS-PAGE sample buffer and boiled for 5 min at 100°C. The supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody against forkhead box O1 (FoxO1) (Santa Cruz Biotechnology, Santa Cruz, CA).

2.10. PCR arrays

Total RNA was isolated from control and fructose-fed rats as described above and then purified using RNeasy kit columns (Qiagen Iberia, Madrid, Spain). Single-stranded cDNA and PCR arrays were performed using the *Rat Insulin Signaling Pathway RT² Profiler PCR Array* (PARN-030E) from SABiosciences (Qiagen Iberia, Madrid, Spain), following the manufacturer's guidelines. Array data processing and analysis were performed using a Web portal from SABiosciences.

2.11. Statistics

The results are expressed as the mean of *n* values±standard deviation. Plasma samples were assayed in duplicate. Significant differences were established by the unpaired *t* test, one-way analysis of variance (ANOVA) test (with *a posteriori* analysis) for cell culture experiments or the two-way ANOVA test (with *a posteriori* analysis) for plasma analytes, using the computer program GraphPad InStat (GraphPad Software V2.03). When the number of animals was too small or the variance was not homogeneous, a non-parametric Mann–Whitney test was performed for comparing two groups. $^{*}P<.05$, $^{**}P<.01$; $^{\#}P<.001$ 14-day control vs. 7-day control, in tables and figures.

Table 1
Fructose effects on liquid and food ingestions, plasma analytes, insulin sensitivity index and glucose tolerance test

	7 days		14 days	
	Control	Fructose	Control	Fructose
AUC ingested liquid (ml/days/2 rats)	360±8	845±198*	706±91	1995±430*
AUC consumed diet (g/days/2 rats)	227±2	177±13**	503±12	392±44*
Body weight (g)	249±7	257±19	264±26	278±21
Adiponectin (μg/ml)	2.7±0.7	3.9±1.5	2.3±0.7	4.0±0.7*
Leptin (ng/ml)	2.0±1.0	3.2±2.3	2,8±2,7	4.7±2.2
Insulin (μg/ml)	0.46±0.07	0.65±0.26	0.51±0.12	0.94±0.43*
Glucose (mg/dl)	151±7	158±11	190±12#	210±16*
ISI	1.21±0.07	1.09±0.14	1.06±0.14#	0.76±0.21*
AUC glucose concentration for glucose tolerance test (% vs. control)	100±15	100±12	100±23	132±25*