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Liver AMP/ATP ratio and fructokinase expression are related to gender differences in AMPK activity and glucose intolerance in rats ingesting liquid fructose☆

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Abstract

Women, but not men, show an association between fructose consumption and an increased risk of Type 2 diabetes mellitus. As rats are considered a model for human fructose metabolism, we sought to determine whether such a gender-related difference is present in Sprague-Dawley rats and to analyze the molecular mechanism behind. Male and female Sprague-Dawley rats had free access to water or to a 10% w/v fructose solution for 14 days. Plasma analytes, liver triglycerides and enzyme activities and the expression of enzymes and transcription factors related to fatty acid metabolism, insulin signaling and glucose tolerance were determined. Fructose-fed rats had hypertriglyceridemia, steatosis and reduced fatty acid oxidation activity, although the metabolic pattern of fructose-fed female rats was different to that observed for male rats. Fructose-fed female, but not male rats, showed no change in plasma leptin; they had hyperinsulinemia, an altered glucose tolerance test and less liver insulin receptor substrate-2. Further, only fructose-fed female rats had increased adenosine 5'-monophosphate (AMP)-activated protein kinase activity, resulting in a decreased expression of hepatic nuclear factor 4 and sterol response element binding protein 1. These differences were related to the fact that liver expression of the enzyme fructokinase, controlling fructose metabolism, was markedly induced by fructose ingestion in female, but not in male rats, resulting in a significant increase in the AMP/adenosine 5'-triphosphate (ATP) ratio and, thus, AMP-activated protein kinase activation, in female rats only. The difference in fructokinase induction could explain the higher metabolic burden produced by fructose ingestion in the livers of female Sprague-Dawley rats.

Keywords: HNF4; IRS-2; Fatty acid oxidation; SREBP-1

1. Introduction

In the last few decades, obesity, metabolic syndrome, insulin resistance and diabetes have escalated to epidemic proportions in many countries worldwide. For example, diabetes is estimated to affect more than 150 million people worldwide, reaching 5.4% of the total world population in the next 25 years. Together with smoking, dyslipemia and hypertension, these disorders constitute major risk factors for atherosclerosis, contributing to keep cardiovascular diseases being the leading cause of mortality in the western world [1–3]. Although this trend affects the global human population, some disturbances, such as diabetic dyslipemia and cardiovascular disease associated with diabetes mellitus Type 2, seem to be more prevalent in females [4].

Environmental factors (diet, physical activity, etc.), in tandem with predisposing genetic factors, may be responsible for this trend. Along with an increase in total energy consumption during recent decades, there has also been a shift in the types of nutrients ingested. In particular, fructose consumption has increased, largely due to a rise in the intake of sugar-sweetened beverages containing high levels of fructose [5]. Very recently, in a study of 91 249 women followed up over 8 years, Schulze et al. showed that those who consumed one or more servings of soft drinks containing fructose per day were at twice the risk of developing diabetes as those who consumed less than one serving per month [6].

The rat is an effective model of human fructose metabolism [7]. A high fructose (50–60%) solid diet in male rats induces metabolic alterations similar to those found in metabolic syndrome, including insulin resistance [8]. However, feeding diets incorporating fructose in drinking water (10 % w/v) for 2 weeks to male rats induce hypertriglyceridemia and fatty liver without modifying plasma glucose and insulin levels [9,10]. By using the latter experimental design, which provides a fructose intake similar to the upper limit of

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human consumption, we have shown that the ingestion of fructose as a liquid solution by male rats induces a state of hepatic leptin resistance that finally blocks fatty acid oxidation, resulting in hypertriglyceridemia and fatty liver [11,12].

In order to elucidate possible molecular mechanisms involved in the development of diabetes in women as a consequence of consuming soft beverages containing fructose, we investigated fatty acid and glucose metabolism in livers of male and female Sprague-Dawley rats receiving fructose as a liquid solution (10 % w/v in drinking water) for 2 weeks. Such a short feeding period sufficed to induce a clear state of glucose intolerance in female, but not in male rats, with a marked reduction in the expression of liver insulin receptor substrate-2 (IRS-2) and a strong inhibition of fatty acid oxidation. This effect may be related to differences in the hepatic metabolic handling of fructose between males and females, given that in liver samples of 2-h-fasted, fructose-fed female rats we found a marked increase in the adenosine 5'-monophosphate (AMP)/adenosine 5'-triphosphate (ATP) ratio, and AMP-activated protein kinase (AMPK) activity, effects that were not observed in males. Key to this increased metabolic burden presented by female rats ingesting liquid fructose is the fact that fructose induced a much higher expression of fructokinase (FK), a key enzyme controlling fructose metabolic handling, in the livers of female rats than in those of male rats.

2. Methods and materials

2.1. Animals and experimental design

Female and male Sprague-Dawley rats purchased from Harlan Interfauna Ibérica (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 group 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 two weeks. Control animals received no supplementary sugar. At the end of this time, animals were killed by decapitation under isoflurane anesthesia at 10 a.m. Prior to sacrifice, food and fructose solution were removed at 8 a.m. In order to reduce the variability in plasma estrogen concentrations, female rats were killed during the diestrus period. Animal weight and intake of solid food and liquid per cage were daily recorded; daily data for each parameter was introduced in the program Graph Pad Prism (GraphPad Software V2.03) for calculation of "Area Under the Curve" values for the whole duration of the experimental procedure.

Subgroups of male and female rats were randomly separated into control and fructose-supplemented groups as described above, for glucose tolerance test and AMP/ATP ratio determination.

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 (July 21).

2.2. Sample preparations

Blood and liver tissue samples were collected and stored as described previously [11]. Total and nuclear extracts were isolated using the Helenius method [13]. Protein concentrations were determined by the Bradford method [14].

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

Plasma triglyceride, glucose, non-esterified free fatty acids (NEFA), insulin, leptin, and adiponectin concentrations, as well as liver and soleus muscle triglyceride content, were measured as described previously [11].

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 by using a rat insulin ELISA kit (Millipore, Billerica, MA). ISI (Insulin Sensitivity Index) was calculated as the ratio $2/[(\text{plasma insulin } \mu M \times \text{plasma glucose } \mu M) + 1]$ [15].

2.5. Nucleotide analysis

The nucleotide analysis was performed basically as described by Izquierdo et al. [16]. One gram of each liver sample was homogenized in 6 ml cold 0.6 M $HClO_4$. After centrifugation at 3000×*g* for 5 min, the precipitate was extracted again by the same method. The supernatants were combined and adjusted to pH 6.5 with 50% and 5% KOH. The pH adjusted sample was incubated for several minutes to precipitate most of the potassium perchlorate. The precipitate formed was removed by filtration and the supernatant was made up to 25 ml with 0.1 M potassium phosphate buffer at pH 6.5. Samples were stored at −80°C until analyzed. For AMP/ATP ratio determination, a Waters 2695 Alliance high-performance liquid chromatography was used equipped with a Waters 2696 DAD-UV detector and a Tracer Excel 120 ODSB column (25 cm×4.6 mm, 5 μm). The conditions were as follows: sample injection, 50 μl; flow rate, 0.9 ml/min; wavelength, 254 nm. A linear gradient consisting in 0.1M KH_2PO_4 adjusted to pH 6.4, containing 40% of 5 mM tetrabutylammonium hydroxide (TBA) as an initial eluant and increasing to 30% (v/v) methanol was used over a period 45 min.

2.6. Enzyme activity assays

AMPK was assayed in the 6% PEG 8000 fraction following the incorporation of [^{32}P] ATP into SAMS peptide (Upstate Biotechnology, Lake Placid, NY, USA), basically as described in our previous publication [12]. Briefly, 100 mg of each frozen tissue was homogenized in 0.4 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 0.25 M mannitol, 1 mM EGTA, 1 mM EDTA, 1 mM DDT, 50 mM NaF, 1 mM PMSF, 5 mM ortovanadate and 1 mM benzamidine. The homogenate was then centrifuged at 14000×*g* for 20 min at 4°C, and the supernatant made up to 2.5% (w/v) PEG 8000 using a stock 25% (w/v) PEG 8000 solution. The mix was stirred for 10 min, and after centrifugation at 10000×*g* for 10 min., the supernatant was collected and made up to 6% PEG 8000. After two centrifugations, the pellet was washed in a 6% PEG 8000-homogenizing buffer and suspended in 100 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM DDT, 50 mM NaF, 1 mM PMSF, 1 mM benzamidine, 0.02% sodium azide and 10% glycerol. The activity of hepatic fatty acid β-oxidation was determined as described elsewhere [17].

2.7. RNA preparation and analysis

Total RNA was isolated using the Trizol^R reagent (Invitrogen Biotechnologies). The relative levels of specific mRNAs were assessed by the reverse transcriptase (RT) polymerase chain reaction (PCR), as described previously [10]. Adenosyl phosphoribosyl transferase (APRT) was used as an internal control. Single-stranded cDNA was synthesized from 1 μg of liver total RNA using 125 ng random hexamers as primers and 200 U M-MLV-RT in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 U RnaseOut and 500 μM of each dNTP in a total volume of 20 μl. The RT reaction was performed for 60 minutes at 37°C. PCR was carried out using a 5 μl aliquot of the RT reaction mix, 0.5 μg of both sense and antisense primers, 200 μM dNTPs, 1 U Taq DNA polymerase and 1.25 μCi α-[^{32}P]-dATP in 20 mM Tris-HCl, pH 8.5, 2.5 mM Mg Cl_2 (final volume 50 μl). PCR was performed in an MJ Research Thermocycler equipped with a Peltier system and temperature probe. The number of cycles, primer sequences and resulting PCR products were as shown previously [10–12]. The mRNA levels were always expressed as the ratio to APRT mRNA levels.

2.8. Western blot analysis

Thirty micrograms of different protein fractions from rat livers were subjected to SDS–polyacrylamide gel electrophoresis as described previously [11]. Briefly, proteins were transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA, USA), blocked for 1 h at room temperature with 5% nonfat milk solution in Tris-buffered saline 0.1% Tween-20. Detection was achieved using the enhanced chemiluminiscence (ECL) kit for horseradish peroxidase (HRP) (Amersham Biosciences). To confirm the uniformity of protein loading, the blots were incubated with β-actin (Sigma-Aldrich) as a control. The size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Life Technologies). All antibodies were obtained from Santa Cruz Technologies, except those for acetyl-CoA carboxylase (ACC), p-ACC, phospho-protein kinase B (p-AKT), AMPK, p-AMPK, IRS2 and PP2Ac, which were obtained from Cell Signaling (Danvers, MA, USA).

2.9. Electrophoretic mobility shift assays

DNA sequences of double-stranded oligonucleotides were as follows: PPRE probe 5′-agtacgggcatggagcaaagagct-3′, and ChREBP probe 5-tcctgcatgtgcca-caggcgtgtcacc-3′. Electrophoretic mobility shift assays (EMSA) were performed exactly as described previously [18]. Oligonucleotides were end-labeled in the following reaction: 1 μl of oligonucleotide (20 ng/μl), 2 μl of 5× kinase buffer, 5 U of T4 polynucleotide kinase and 3 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml, Amersham) incubated at 37°C for 2 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia, Sant Cugat, Spain) according to the manufacturer's instructions. Eight micrograms of crude nuclear proteins were incubated for 10 min on ice in binding buffer [10 mM Tris-HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5%