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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%

glycerol, 5 mg/ml bovine serum albumin and 50 μg/ml poly(dI-dC)] in a final volume of 15 μl. Labeled probe (approximately 60,000 cpm) was added and the reaction was incubated for 20 min at room temperature.

2.10. Coimmunoprecipitation

Coimmunoprecipitation assays were done with 50 μg of nuclear extracts exactly as described previously [18,19]. Immunocomplexes were captured by incubating the samples with protein A-agarose suspension (Santa Cruz Biotechnology) overnight at 4°C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with phosphate-buffered saline-containing protease inhibitors. After microcentrifugation, the pellet was washed with 60 μl of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min at 100°C. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody against PPARα.

2.11. Statistics

The results are expressed as the mean of n values±S.D. Plasma samples were assayed in duplicate. Significant differences were established by the unpaired t test or the one-way analysis of variance test (with analysis *a posteriori*), using the computer program GraphPad InStat (GraphPad Software V2.03). When the number of samples was too small or the variance was not homogeneous, a nonparametric test was performed (Mann–Whitney U for comparing two groups or Kruskal-Wallis for comparing more than two groups). The level of statistical significance was set at $P \leq .05$.

3. Results

3.1. Ingestion of a 10% w/v fructose solution induced hypertriglyceridemia and hepatic steatosis in male and female rats, but hyperinsulinemia only in female rats

Fructose-supplemented rats showed a marked increase in the ingestion of liquids (1.9- and 3.0-fold, for male and female rats), and a reduction in the amount of ingested solid food (0.93- and 0.75-fold, for male and female rats), with no significant change in body weight by the end of the 14 days of fructose administration (Table 1). The increase in the total amount of ingested energy did not differ much between fructose-supplemented and control rats (2093 vs. 2239 kcal/14 days/2 rats for control and fructose-fed males, 7% increase; 1389 vs. 1633 kcal/14 days/2 rats for control and fructose-fed females, 17% increase); nevertheless, while fructose-supplemented males acquired 25% of their total amount of energy from fructose, the same figure for fructose-supplemented female rats rose to 47%, almost doubling the contribution of fructose for the total energy intake of supplemented females. Similar to previous results for male rats [10–12], fructose-fed rats were hypertriglyceridemic (1.39- and 1.45-fold, for male and female rats) and presented hepatic steatosis (1.75- and 1.50-fold, for male and female rats), in comparison with control rats. Despite these similarities, female rats showed differences to males in their response to fructose ingestion as a liquid solution. First, fructose-supplemented female rats showed a slight nonsignificant increase in their plasma leptin concentration (see Table 1), while fructose-supplemented male rats presented a significant increase in plasma leptin concentrations (1.84-fold), similar to what we have previously reported [11,12]. Further, in fructose-supplemented female rats, no modification in the liver expression of signal transducer and activator of transcription-3 phosphorylated on Tyr^{705}, the long form of the leptin receptor phosphorylated on Tyr^{1138}, or the suppressor of cytokine signaling-3 proteins was found (data not shown), in contrast to previous findings for fructose-supplemented male rats [11,12]. Second, fructose-supplemented female rats showed slight hyperglycemia (1.08-fold), and marked hyperinsulinemia (1.7-fold), together with a reduction in the insulin sensitivity (ISI) index (0.78-fold), indicating a disturbance in the insulin signal transduction pathway which was not present in fructose-supplemented male rats [10,11].

Table 1
Body weight, food and liquid ingestion, plasma analytes and hepatic triglyceride content in fructose-supplemented Sprague-Dawley rats

	Male		Female	
	Control	Fructose	Control	Fructose
AUC body weight (g/14 days per rat)	3194±177	3237±129	3098±253	3246±175
AUC ingested liquid (ml/14 days per 2 rats)	793±74	1475±184***	635±74	1942±45***
AUC consumed diet (g/14 days per 2 rats)	573±32	532±21*	366±21	276±24***
Liver triglycerides (mg/g liver)	5.3±0.9	9.3±2.5**	3.6±1.2	5.4±3.1‡
Plasma triglycerides (mg/dl)	88±18	122±29*	56±12	81±13***
NEFA (μmol/L)	349±81	426±103	315±87	317±95
Plasma glucose (mg/dl)	139±10	156±24	117±5	127±12**
Plasma insulin (ng/ml)	2.1±1.0	1.7±0.7	0.6±0.1	1.0±0.3**
Insulin sensitivity index	0.60±0.13	0.65±0.18	1.21±0.09	0.94±0.13***
Plasma leptin (ng/ml)	1.9±0.3	3.5±1.2***	1.7±0.3	2.4±1.1†
Plasma adiponectin (μg/ml)	5.2±0.8	7.6±1.1***	4.7±0.6	7.1±3.0**

Individual AUC values for ingested liquid and solid diet are the mean corresponding to two animals housed in the same cage.
* $P \leq .05$.
** $P \leq .01$.
*** $P \leq .001$.
† $P = .09$ vs. control.
‡ $P = .06$ vs. control.

3.2. Ingestion of a 10% w/v fructose solution for 14 days induces glucose intolerance in female, but not in male, Sprague-Dawley rats

To confirm a possible disturbance in glucose homeostasis in female, but not in male, Sprague-Dawley rats after fructose ingestion in liquid form, we supplemented two groups of male and female rats with a fructose solution (10 % w/v), and on the morning of the 14th day, we performed a glucose tolerance test, as described in the Experimental Procedures section. At time zero, the ISI index was unchanged in fructose-fed male rats vs. controls, whilst in females it was significantly decreased (0.74-fold) (Fig. 1A). The evolution of plasma glucose concentrations during the time course of the glucose tolerance-test was practically identical in fructose-fed male rats vs. controls, while in female fructose-fed rats, glucose concentrations at 30, 90 and 120 min were significantly higher than in controls (Fig. 1B). As a consequence, the area under the curve of plasma glucose concentration for the complete time course of the glucose tolerance test was significantly increased (1.34-fold) in female, but not in male, fructose-fed rats (Fig. 1C). Insulin plasma concentrations were unchanged during the different time points of the experiment in both sexes, although fructose-fed female rats showed a non significant increase in the area under the curve of plasma insulin concentration (4021 ± 962 vs. 3533 ± 675 and 2983 ± 846 vs. 3967 ± 564 ng of insulin/dl/120 min for control and fructose-fed male and female rats, respectively). Finally, only skeletal muscle from fructose-fed female rats showed a marked increase in triglyceride content (1.41-fold) (Fig. 1D). Overall, these results clearly show that 14 days of fructose ingestion as a 10 % w/v solution suffice to develop a state of glucose intolerance in female, but not in male, rats.

3.3. Fructose reduced the expression of IRS-2 protein and impaired insulin signal transduction in livers of fructose-supplemented female rats

In order to confirm at the molecular level an alteration in the insulin signaling pathway in the livers of fructose-fed female rats, we determined several components of the insulin signal transduction pathway. Fructose ingestion in liquid form produced a marked decrease (0.27-fold) in the amount of liver IRS-2 protein (Fig. 2A), implying a significant reduction in insulin signal transduction in the

livers of fructose-fed animals. Consequently, the livers of fructose-fed female rats showed significant decreases in the ratio between the active, phosphorylated form of AKT (0.51-fold) and the total AKT protein, and the inactive, phosphorylated form of glycogen synthase kinase 3β (GSK3β, 0.63-fold) (Fig. 2B and C, respectively). Both phosphorylated forms should be increased by the existing hyperinsulinemia, if we assume a normal, unimpaired functioning of the insulin signaling pathway [20]. Similarly, the activity of protein kinase λ (PKC λ), another downstream effector of insulin action [21], was not increased in livers of fructose-fed female rats (Fig. 2D). Finally, the levels of the specific mRNAs for insulin sensitive gene-2, glucose-6-phosphatase and phosphoenol pyruvate kinase, genes whose expression is under negative control by insulin [20], were not reduced in livers of fructose-fed animals (data not shown). Overall, these results confirm impairment of the insulin transduction pathway in the livers of fructose-fed female rats.

3.4. Fructose reduced the expression of the mature form of the transcription factor sterol response element binding protein-1 (SREBP-1) only in the livers of fructose-supplemented female rats

Similar to previous findings for male rats [11], fructose ingestion induced a lipogenic effect in the livers of fructose-supplemented rats, showing an increased expression of the specific mRNA for lipogenic enzymes (Table 2), such as fatty acid synthase (3.8- and 1.4-fold, for male and female rats) and stearoyl-CoA desaturase-1 (3.1- and 3.7-fold, for male and female rats) and an increased amount of carbohydrate response element binding protein (ChREBP) (2.0- and 2.8-fold, for male and female rats, Fig. 3A). Liver pyruvate kinase specific mRNA was similarly increased in livers of fructose-fed rats (1.9- and 2.9-fold, for male and female rats, Table 2). The expression of the liver pyruvate kinase gene is directly under the control of ChREBP transcriptional activity [22].

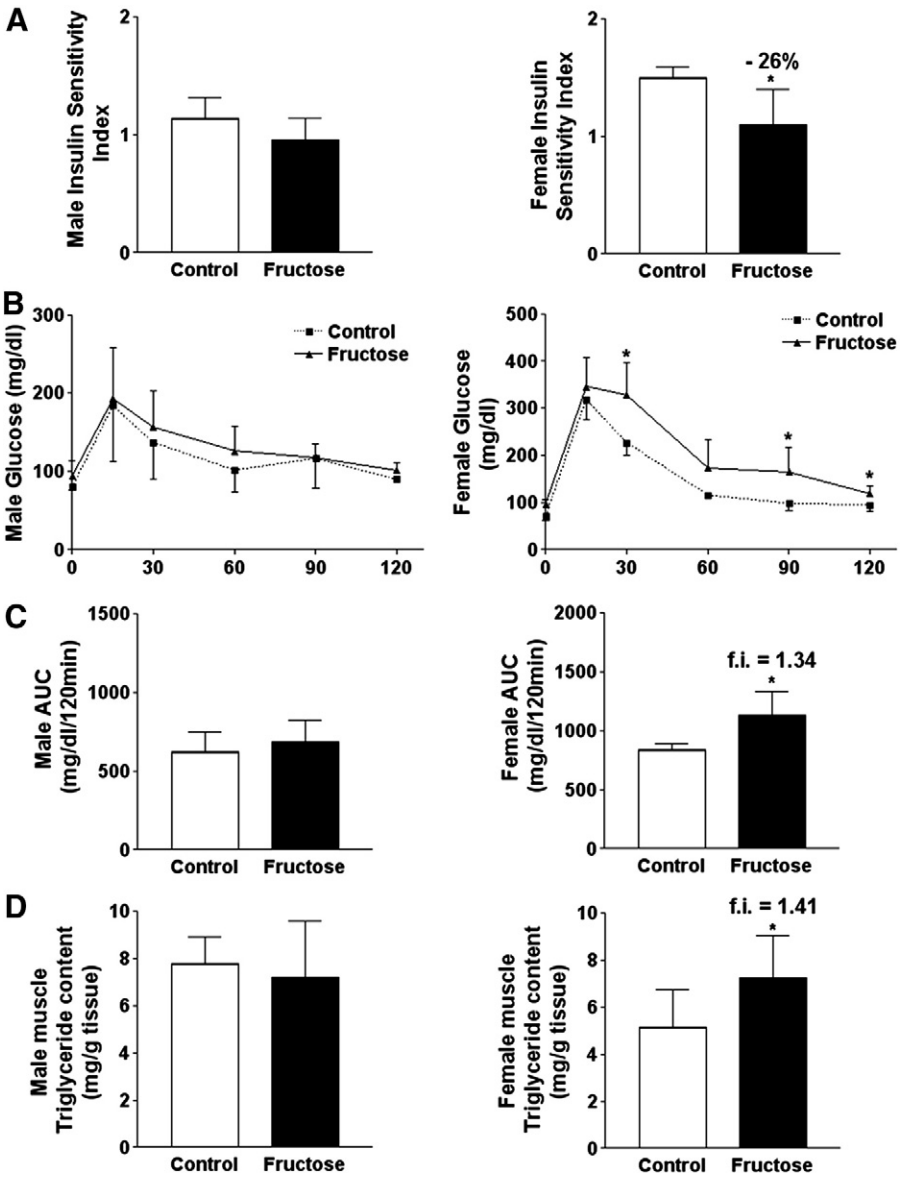

Fig. 1. Fructose administration as a liquid solution (10 % w/v) induces glucose intolerance only in female Sprague-Dawley rats. (A) Bar plot showing the Insulin Sensitivity Index values for control (empty bar) and fructose-fed male and female rats (black bar). (B) Plasma glucose values at different times after the administration of a glucose solution (2 g/kg body weight) into the peritoneal cavity of control and fructose-fed male and female rats. (C) Area under the curve (AUC) values for glucose plasma concentrations represented in Fig. 1B. (D) Bar plot showing soleus muscle triglyceride content in control (empty bar), and fructose-fed male and female rats (black bar). Results are the mean±S.D. of values from five animals. * $P \leq .05$ vs. control.

Fructose ingestion as a liquid solution by male Sprague-Dawley rats did not modify the liver expression and activity of SREBP-1 (see Table 2, Fig. 3B, and our previous results [11]). Increased plasma insulin concentration should be associated with increased SREBP-1 expression and activity in livers of fructose-fed female rats [19]. Surprisingly, the ingestion of liquid fructose by female rats resulted in a reduction in the liver content of the specific mRNA for SREBP-1 (0.71-fold, Table 2), and in the amount of the immature (0.71-fold) and mature (0.67-fold) forms of the SREBP-1 protein (Fig. 3B). Liver glucokinase specific mRNA was similarly significantly decreased only in livers of fructose-fed female rats (0.47-fold, Table 2). The expression of the glucokinase gene is directly under the control of SREBP-1 transcriptional activity [23].

3.5. Fructose reduced hepatic fatty acid β-oxidation activity in male and female rats but probably by different molecular mechanisms

Fructose-supplemented rats showed reduced hepatic expression of liver-carnitinepalmitoyltransferase-1 (L-CPT-1) (0.67- and 0.55-fold for male and female rats) (Fig. 4A) and fatty acid β-oxidation activity (0.69- and 0.57-fold for male and female rats) (Fig. 4B).

We have previously shown that, in fructose-supplemented male rats, the reduction in the hepatic fatty acid β-oxidation activity was related to reduced peroxisome proliferator-activated receptor α (PPARα) binding activity [11], as a consequence of physical sequestration of PPARα by the activated, unphosphorylated form of the forkhead box O1 (FoxO1) transcription factor [12]. Although

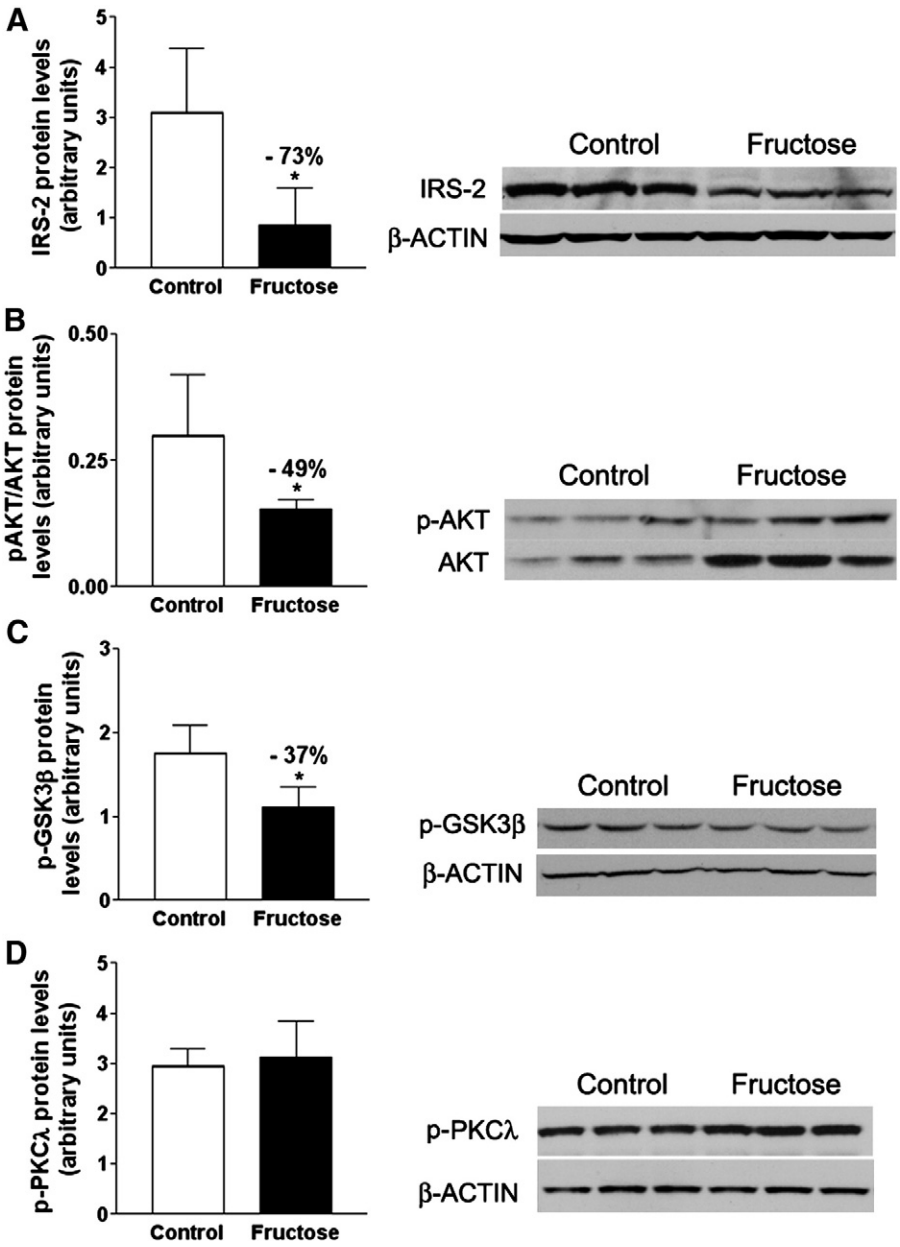

Fig. 2. Expression of proteins involved in the insulin signaling pathway in livers of fructose-supplemented female rats. Western blot of IRS-2 (A), p-GSK3β (C) and p-PKC λ (D) proteins in liver samples from control and fructose-supplemented female rats. On the left of each figure, a bar plot shows the levels of each protein, expressed as the mean arbitrary units (a.u.)±S.D. of values from six animals, in hepatic samples from control (empty bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed by determining the signal of β-actin as a control-loading protein. (B) Bar plot showing the ratio between the active, phosphorylated form, and the total AKT protein in hepatic samples from control (empty bar) and fructose-fed rats (black bar). Each bar represents the mean±S.D. of values from six animals. On the right of the figure, a representative Western blot shows the p-AKT and total AKT bands corresponding to three different control and fructose-fed female rats. *$P \leq .05$.

Table 2
Liver mRNA expression (a.u.) of several genes in fructose-supplemented Sprague-Dawley rats

Gene	Male		Female	
	Control	Fructose	Control	Fructose
Fas	0.83±0.38	3.15±1.07 **	0.90±0.19	1.23±0.33 *
Gk	1.69±0.71	1.25±0.81	0.99±0.53	0.46±0.19 *
Lpk	1.72±0.51	3.31±0.61 ***	0.42±0.86	1.21±0.17 ***
Scd-1	0.45±0.27	1.41±0.19 ***	0.48±0.24	1.76±0.3 ***
Srebp-1	3.64±2.5	4.90±3.45	2.36±0.32	1.68±0.32 **

* $P \leq .05$.
** $P \leq .01$.
*** $P \leq .001$ vs. control.

fructose ingestion reduced the liver expression of the specific mRNA for L-CPT-1 in both male and female rats (Fig. 4A), the binding activity of PPARα (Fig. 5A) and the physical interaction between PPARα and FoxO1 (data not shown) were only reduced in livers of fructose-supplemented male rats. On the contrary, fructose ingestion significantly reduced the expression of HNF4 only in the livers of female rats (0.55-fold, Fig. 5B). HNF4 is another transcription factor controlling the expression of the *l-cpt-I* gene [24].

3.6. Fructose increased AMPK activity in livers of female rats

We have previously reported that AMPK activity was not modified by fructose supplementation in male rats [11,12]. Activation of liver

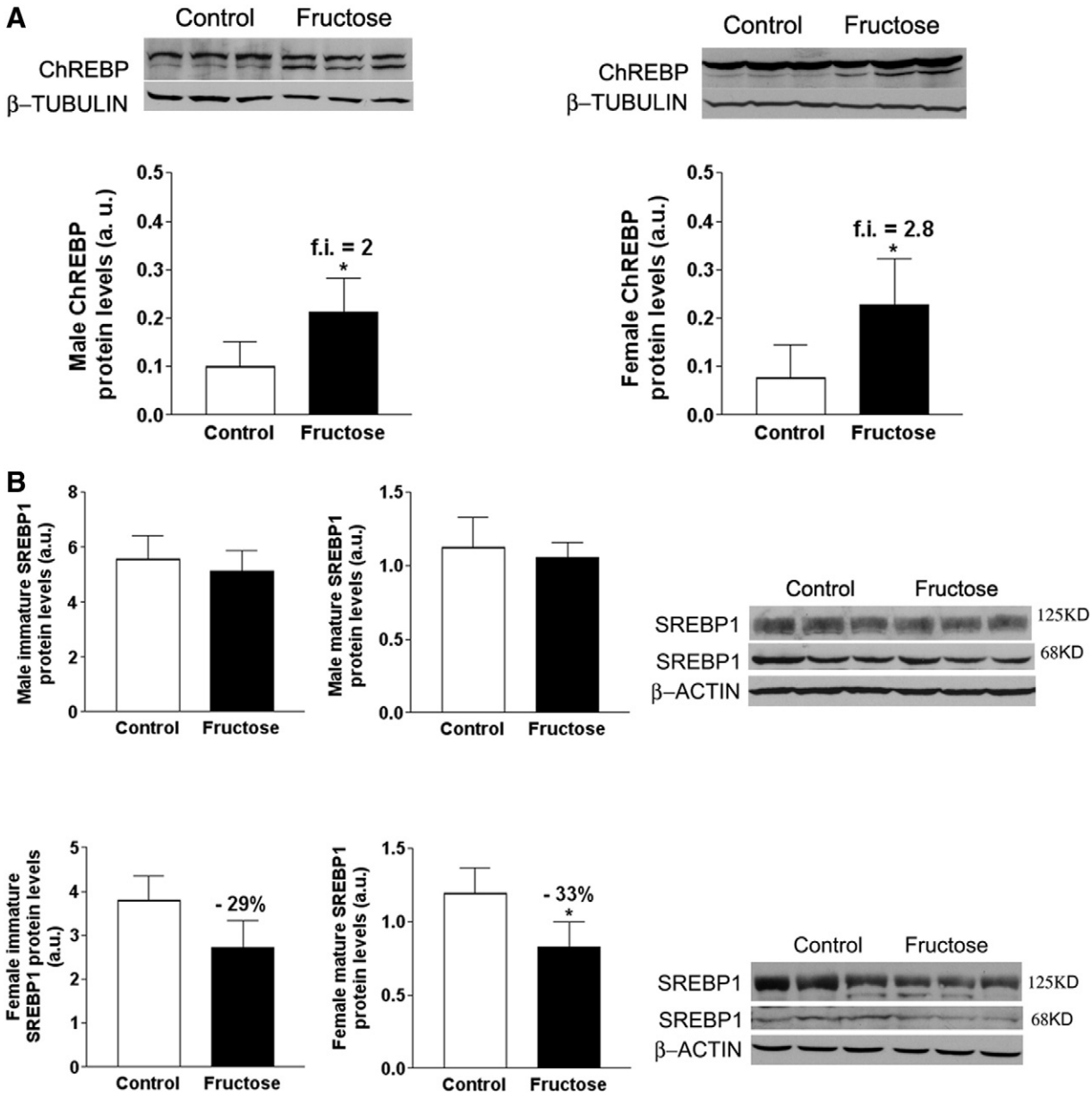

Fig. 3. Expression of ChREBP and SREBP1 in male and female fructose-supplemented rats. Western-blot of ChREBP (A) and immature and mature SREBP1 (B) protein in liver samples from control and fructose-supplemented male and female rats. Bar plot shows the levels of each protein, expressed as the mean±S.D. of values from six animals, in hepatic samples from control (empty bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed by determining the signal of β-tubulin and β-actin, respectively, as a control-loading protein. A representative autoradiography from Western blot determination of three animals per group is shown for each. *$P \leq .05$.

AMPK, a key enzyme involved in the control of energy homeostasis [25], decreases the expression of both HNF4 and SREBP-1 [26–28]; thus, its activation in livers of fructose-fed female rats could explain the observed changes in the expression of these transcription factors. In order to confirm a possible difference in the state of AMPK activation between male and female fructose-fed rats, we specifically studied the AMPK system in fructose-fed male and female rats. Livers of fructose-supplemented female rats showed a marked increase in AMPK activity (1.55-fold, Fig. 6A), in the expression of the phosphorylated and active form of AMPK (1.58-fold, Fig. 6B) and in the expression of the phosphorylated and inactive form of acetyl-CoA carboxylase (1.54-fold, Fig. 6C), a well-known substrate of AMPK activity [25]. These changes were not observed in livers from fructose-fed male rats, confirming that the AMPK system was specifically activated by fructose ingestion in liquid form only in the livers of female rats.

3.7. The ingestion of fructose as a liquid solution efficiently induced its own metabolism in livers of female Sprague-Dawley rats

We have previously shown that two hours of fasting suffice to eliminate any detectable increase in PP2A and ChREBP binding activity in the livers of fructose-fed male rats [12]. In contrast, the livers of fructose-fed female rats showed a marked increase in the expression of the active, catalytic subunit of PP2A (1.46-fold, Fig. 1A Supplemental Data) and in the binding activity of ChREBP after 2 h of fasting. As shown in Fig. 1B (Supplemental Data), the incubation of liver nuclear extracts with an oligonucleotide probe mimicking a ChREBP response element produced a single retardation band, the intensity of which was markedly increased (1.93-fold) in liver samples from fructose-fed animals.

Fructose ingestion increases the expression of FK, an enzyme whose activity controls the metabolic handling of fructose [29]. We were interested to determine whether the greater sensitivity to liquid fructose found in female rats was related to different liver expression of FK after fructose ingestion. Indeed, although the specific mRNA for FK increased similarly in liver samples from fructose-fed male (1.35-fold) and female (1.55-fold) rats (Fig. 7A), the increase in the amount of FK protein present in livers of fructose-fed female rats was markedly higher than in males (3.9 vs. 1.5-fold, for female and male rats, respectively, Fig. 7B). This highly increased metabolism of fructose in the livers of fructose-fed female rats produced a marked increase (1.67-fold) in the AMP/ATP ratio which was not observed in fructose-fed males (Fig. 7C), thus explaining the differential activation of the AMPK system in the livers of fructose-fed female rats.

4. Discussion

We have previously shown that ingestion of liquid fructose by male Sprague-Dawley rats for only 14 days alters hepatic lipid metabolism, inducing hepatic steatosis and plasma hypertriglyceridemia, without promoting a state of insulin resistance [10]. These

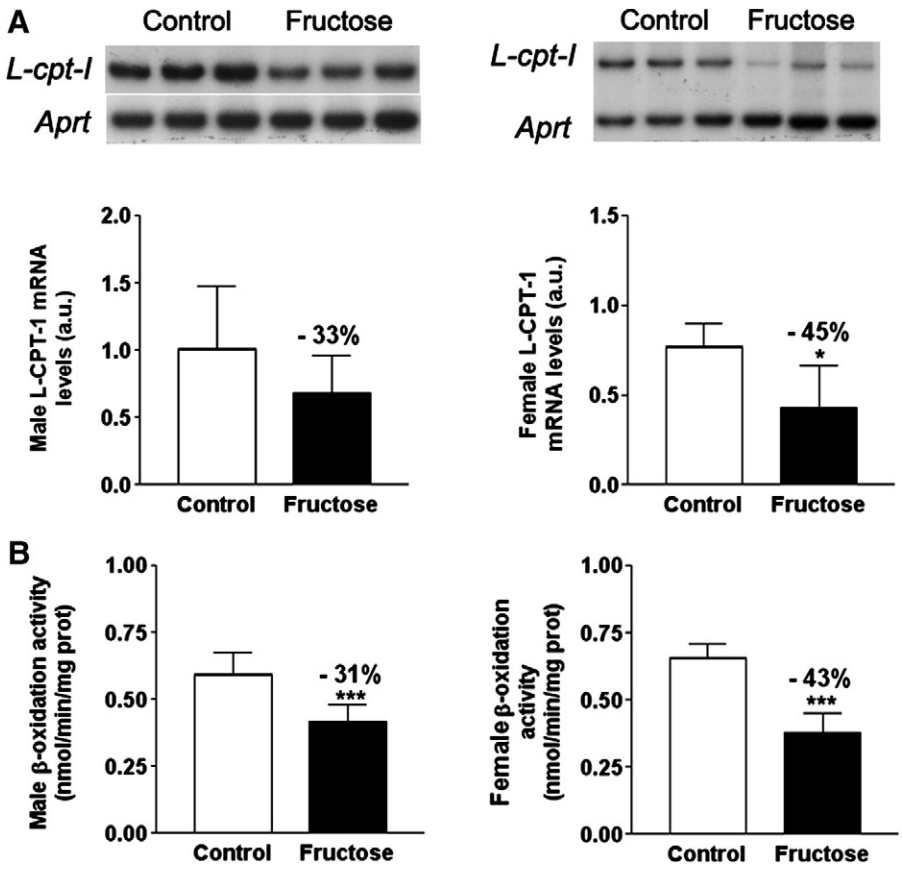

Fig. 4. Expression of L-CPT-1 gene and fatty acid β-oxidation activity in livers from male and female fructose-supplemented rats. (A) Bar plot showing the relative levels of L-CPT-1 specific mRNA in hepatic samples from male and female control (empty bar) and fructose-fed rats (black bar). Each bar represents the mean±S.D. of values from six animals. A representative autoradiography at the top of the figure shows the bands corresponding to L-CPT-1 mRNA and that of the *aprt* gene (used as an internal control in the PCR reaction to normalize the results) from liver samples of three animals from each treatment group. (B) Fatty acid β-oxidation activity, expressed as nanomoles of oxidized palmitoyl-CoA/min per milligram of protein of liver postnuclear supernatant, in male and female livers of control (empty bar) and fructose-fed (black bar) rats. Each bar represents the mean±S.D. of values from six animals, performed in duplicate. *$P \leq .05$; ***$P \leq .001$.

metabolic alterations are related to a fructose-induced state of hepatic leptin-resistance [11], 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 [12]. Our results show that Sprague-Dawley rats submitted to a 14-day liquid fructose challenge disclose hepatic metabolic alterations that differ by gender, namely, female rats have a more detrimental response, including glucose intolerance, than their male counterparts.

Although both male and female rats responded similarly to the ingestion of liquid fructose with the appearance of hepatic steatosis and hypertriglyceridemia, the molecular mechanisms involved were different. The reduction in PPARα activity due to its sequestration by FoxO1, leading to decreased expression of its rate-limiting enzyme, L-CPT-1, was determinant in the reduction of liver fatty acid oxidation in male rats ([12] and our present results). In contrast, the PPARα system in female rats remained unaltered; the reduction in the expression of L-CPT-1 and, hence, hepatic fatty acid oxidation, could be attributed to a marked decrease in the expression and binding activity of the transcription factor HNF4, which also controls the expression of L-CPT-1 [24]. Furthermore, although fructose increased lipogenesis mediated by ChREBP activation in both sexes (Ref. [12] and our present results), only female rats responded to fructose ingestion by decreasing the expression of the total and active form of SREBP-1 in liver.

Fructose-fed male rats were resistant to the hepatic effects of leptin [11,12], whereas fructose-fed females showed no sign of leptin resistance but an alteration in the liver insulin signal transduction pathway, with a marked reduction in the hepatic expression of IRS-2 protein, the main transducer of the insulin signal in liver [30]. Probably as a consequence of an inappropriate amount of IRS-2 signaling, the enhanced plasma concentration of insulin in fructose-fed females did not increase the hepatic amount of the inactive, phosphorylated form of GSK3β or the active form of PKC λ. This could facilitate the reduction of liver SREBP-1 by fructose in female rats. The inactivation of GSK3β [31] and the activation of PKC λ [21] by insulin are known to be transducing signals involved in the liver insulin-dependent activation of the SREBP-1 system. In most situations of hyperinsulinemia related to insulin resistance, the stimulating effect of insulin on hepatic SREBP-1 activity is preserved [20]; however, this is not the case for fructose-fed female rats. At this point of time, we do not know

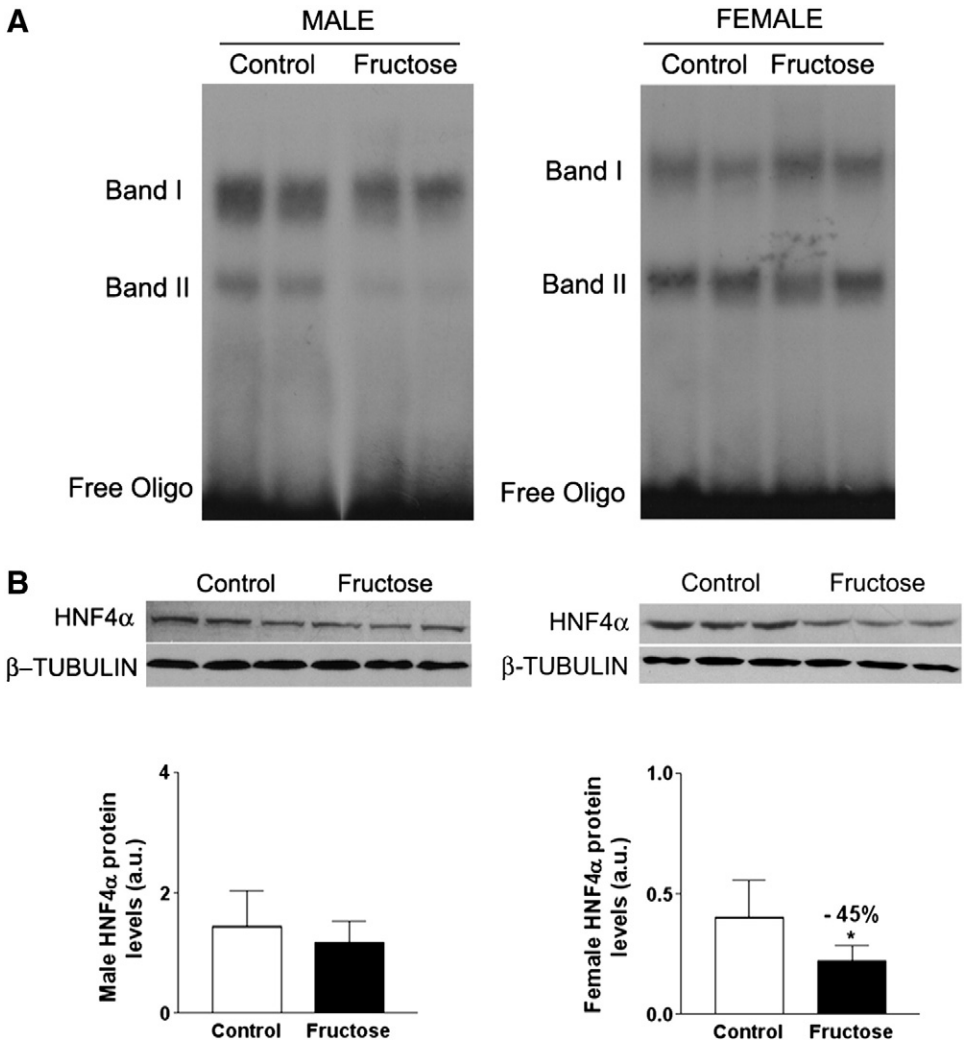

Fig. 5. PPARα binding activity and HNF4 protein levels in fructose-supplemented rats. (A) Representative EMSA autoradiography showing the binding to a PPAR response element oligonucleotide (two specific bands I–II formed) with hepatic nuclear extracts from two male and female control and fructose-fed rats. (B) Bar plot showing the levels of HNF4 protein in hepatic samples from control (empty bar) and fructose-supplemented (black bar) male and female rats. Each bar represents the mean±S.D. of values from six animals. On the upper part of the figure, a representative Western blot shows the HNF4 band corresponding to three different control and fructose-fed rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed by determining the signal of β-tubulin, as a control-loading protein. *$P \leq .05$.

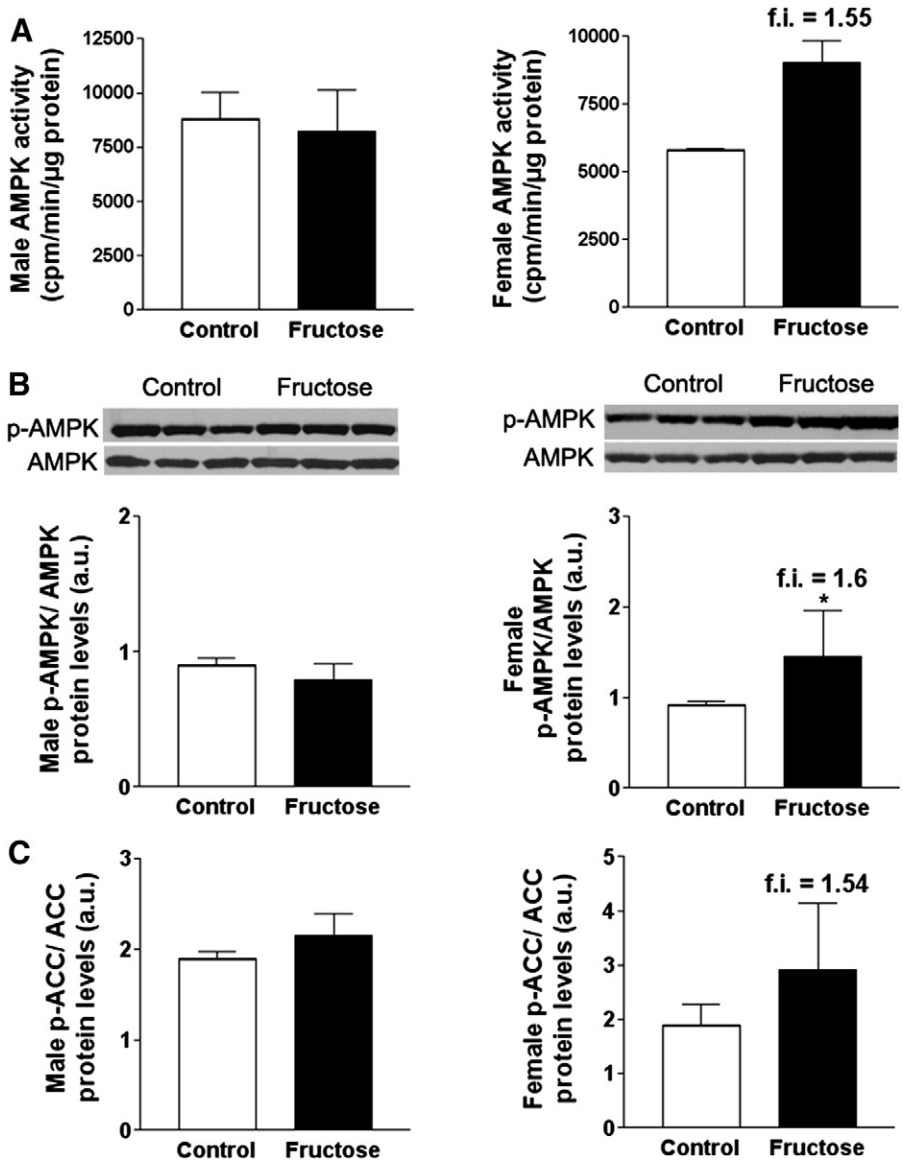

Fig. 6. AMPK activity in livers of fructose-supplemented male and female rats. (A) Bar plot showing the AMPK activity in liver pooled samples from six control (empty bar) and six fructose-fed (black bar) male and female rats. (B) Bar plot showing the ratio between the active, phosphorylated form and the total AMPK protein in hepatic samples from control (empty bar), and fructose-fed male and female rats (black bar). Each bar represents the mean±S.D. of values from six animals. On the upper part of the figure, a representative Western blot shows the p-AMPK and total AMPK bands corresponding to three different control and fructose-fed rats. (C) Bar plot showing the ratio between the inactive, phosphorylated form, and the total ACC protein in hepatic samples from control (empty bar), and fructose-fed (black bar) male and female rats. Each bar represents the mean±S.D. of values from 6 animals. *$P \leq .05$.

exactly what molecular mechanism is involved in the reduced expression of IRS-2 protein in the livers of fructose-fed female rats and the ensuing impairment in the hepatic insulin signalling.

Fructose ingestion by female rats increased liver AMPK activity, an effect which is not induced by fructose administration to male rats (Refs. [11,12] and our present results). Although increasing AMPK activity, at least in skeletal muscle, seems to help restore an appropriate glucose metabolism [32], our present data indicate that the activation of liver AMPK do not prevents the appearance of glucose intolerance in fructose-supplemented female rats. Nevertheless, the increased activity of liver AMPK could explain why livers of fructose-fed female rats did not show signs of leptin resistance, given that increased AMPK activity is one of the main results of leptin activity in liver [33,34]. Furthermore, activation of AMPK in hepatocytes greatly diminishes HNF4 protein levels [26,27] and reduces SREBP-1 mRNA and protein [28]. Thus, two of the differential molecular changes found in the livers of fructose-fed female rats, the reduced expression of HNF4 and SREBP-1, can both be related to the marked increase in AMPK activity, which was over 50% higher than in control female rats.

In liver cells, fructose is metabolized to fructose-1-phosphate by fructokinase, which consumes ATP. Unlike glucokinase, the activity of fructokinase is not regulated, and massive incorporation of fructose into liver metabolism can lead to ATP depletion and an increase in the AMP/ATP ratio [29,35]. Furthermore, one of the first recognized mechanisms of activation of AMPK was related to the metabolic stress associated with cellular ATP depletion and increased AMP/ATP ratios [25]. Thus, the reason for the different response to fructose in female and male rats is that females incorporate fructose into liver metabolism to a greater extent than males, reaching a sufficiently high AMP/ATP ratio as to activate AMPK. This situation is in agreement with the fact that fructose-supplemented rat acquired

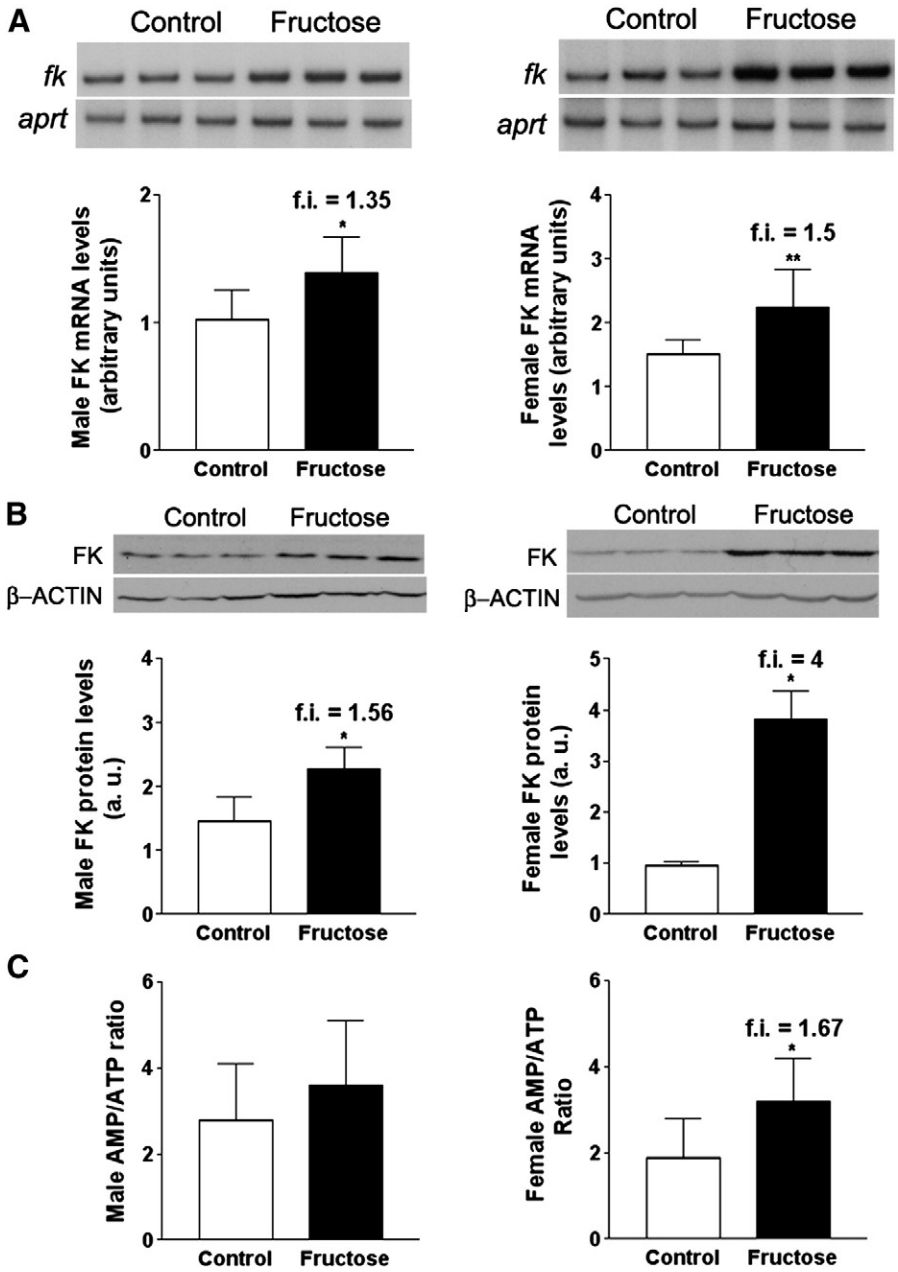

Fig. 7. Effect of fructose supplementation on the expression of liver FK and the AMP/ATP ratio in livers of male and female Sprague-Dawley rats. (A) Relative levels of FK mRNA in hepatic samples from control (empty bar), and fructose-supplemented male and female rats (black bar). Each bar represents the mean±S.D. of values from six animals. A representative autoradiography at the top of each figure shows the band corresponding to FK and that of the *aprt* gene (used as an internal control in the PCR reaction to normalize the results) from liver samples of three animals from each treatment group. (B) Western blot of FK protein in liver samples from control and fructose-supplemented male and female rats. At the bottom of each figure, a bar plot shows the levels of FK protein, expressed as the mean±S.D. of values from six animals, in hepatic samples from control (empty bar) and fructose-supplemented (black bar) male and female rats. At the bottom of each figure, a bar plot shows the levels of FK protein, expressed as the mean±S.D. of values from six animals, in hepatic samples from control (empty bar) and fructose-supplemented (black bar) male and female rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed by determining the signal of β-actin, as a control-loading protein. (C) Bar plot showing the AMP/ATP ratio, expressed as the mean±S.D. of values from six animals, in hepatic samples from control (empty bar) and fructose-supplemented (black bar) male and female rats. *$P \leq .05$; **$P \leq .01$.

almost half of their total energy intake from fructose. Furthermore, livers of 2-hour fasted fructose-fed female rats, in contrast to males [12], still showed a robust increase in the expression of PP2Ac and ChREBP binding activity. Both situations are increased in the presence of xylulose-5-P, which is a product of fructose metabolism [36]. In 1991, Korieh and Crouzoulos first reported that fructose induced its own metabolism in rats, by inducing the expression of liver fructokinase [37]. Although male and female rats showed similar increases in hepatic levels of fructokinase-specific mRNA after 14 days of fructose ingestion as a liquid solution, surprisingly, livers of female rats revealed a much higher increase in the amount of enzyme protein than males. Thus, although we do not know the cause of this difference, the stronger induction of fructokinase in livers of fructose-fed female rats is the key factor in determining the differential response between male and female rats to fructose ingestion.

The only published interventional study finding increased weight gain for women, but not men, during a high-fructose corn

syrup treatment, studied only nine normal-weight women [38]. Couchepin et al. have reported that short-term fructose overfeeding produces hypertriglyceridemia and hepatic insulin resistance in men, but not in healthy young women, although they only included eight women in the study [39]. Nevertheless, several follow-up studies including a larger number of women have associated increased dietary fructose intake with the incidence of Type 2 diabetes [6], especially in older women [40] and in African-American women [41]. Indirect data indicate that human liver fructokinase expression is also induced after fructose ingestion in liquid form [29]. Thus, our results provide a plausible molecular mechanism, related to the induction of human liver fructokinase and the appearance of glucose intolerance, which could help to explain why frequent ingestion of fructose containing beverages is associated with increased incidence of Type 2 diabetes in women.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.jnutbio.2010.06.005.

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