High dietary fructose load aggravates lipid metabolism in the liver of Wistar rats through imbalance between lipogenesis and fatty acid oxidation

Ana TEOFILOVIĆ, Biljana BURSAČ, Ana DJORDJEVIC, Danijela VOJNOVIĆ MILUTINOVIĆ, Gordana MATIĆ, Nataša VELIČKOVIĆ*
Department of Biochemistry, Institute for Biological Research “Siniša Stanković”, University of Belgrade, Belgrade, Serbia

Abstract: Fructose ingestion is often associated with hepatic steatosis and hypertriglyceridemia. The homeostasis of hepatic lipids is mainly determined by the interplay of lipogenesis and fatty acid β-oxidation. In this study, we hypothesized that high fructose intake disturbs hepatic lipid metabolism through an imbalance between these processes. Therefore, we analyzed the effects of a 9-week-long consumption of a 60% fructose solution on physiological parameters, glycemia, and blood lipid profiles in male Wistar rats. The expression of key regulators of fatty acid oxidation (FAO) and lipogenesis in the liver were assessed by western blot and quantitative polymerase chain reaction. The results showed that fructose-fed rats were normoglycemic and hypertriglyceridemic with visceral adiposity, but without hepatic lipid deposition. A high-fructose diet is associated with increased nuclear levels of the lipogenic regulator sterol regulatory element binding protein 1c (SREBP-1c), which was followed by increased acetyl-CoA carboxylase and fatty acid synthase mRNAs. The nuclear level of the FAO transcriptional regulators peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1) and lipin-1 were unaltered, while carnitine palmityltransferase 1 (CPT1) mRNA was significantly decreased. Overall, our findings showed that liquid fructose overconsumption is associated with perturbation of hepatic lipid metabolism through predominance of lipogenesis over β-oxidation, resulting in spillover of triglycerides and visceral adiposity.

Key words: Dietary fructose, fatty acid oxidation, lipogenesis, liver, rat

1. Introduction
Numerous studies have reported that high dietary fructose intake is associated with various adverse metabolic disturbances in different animal models, including obesity, hypertriglyceridemia, and insulin resistance (Rutledge and Adeli, 2007; Dekker et al., 2010; Collison et al., 2011; Newell-Fugate et al., 2014), which successfully mimic nutrition-related disorders in the human population. However, over the last decade a series of questions have arisen regarding the role of fructose in the development of the current epidemic of metabolic disorders (Tappy and Mittendorfer, 2012). In addition, the mechanism underlying the hyperlipidemic effects of fructose remains controversial, since it appears that only a small percentage of the ingested fructose in the human diet (<1%) is directly converted to plasma triglycerides (Sun and Empie, 2012). Whether fructose overconsumption will lead to hypertriglyceridemia and hepatic steatosis depends primarily on the balance between de novo lipogenesis and fatty acid oxidation (Rebollo et al., 2014b). Rodent models provide the most convenient way to investigate the underlying molecular mechanism and to test potential therapeutic interventions in the human population (Panchal and Brown, 2011).

One of the master transcriptional regulators of fatty acid oxidation is peroxisome proliferator-activated receptor α (PPARα), which acts as a cellular fatty acid sensor (Moreno et al., 2010). Upon activation, PPARα induces the expression of multiple genes encoding proteins involved in the mitochondrial β-oxidation of fatty acids (Minnich et al., 2001). The transcriptional activation of target genes by PPARα requires the recruitment of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and lipin-1, which acts as an amplifier of the PPARα/PGC-1α signaling system (Finck et al., 2006). The nuclear PPARα/PGC-1α/lipin-1 complex stimulates the expression of genes involved in fatty acid oxidation, including carnitine palmityltransferase 1 (CPT1) and medium chain acyl-CoA dehydrogenase (Leone et al., 1999). CPT1 represents a rate-limiting enzyme for β-oxidation, a process that begins with the transport of free fatty acids delivered from circulation...
into the mitochondria by a CPT1/CPT2 shuttle system (Bonnefont et al., 2004). Interestingly, CPT1 activity is inhibited by malonyl-CoA, which is the product of the enzyme acetyl-CoA carboxylase (ACC) that catalyzes the first reaction of de novo lipogenesis. Therefore, reciprocal regulatory mechanisms have a crucial role in maintaining a balance between the opposite processes of lipid metabolism.

De novo lipogenesis in the liver is controlled by several transcription factors, including sterol regulatory element binding proteins (SREBPs) (Horton et al., 2002). SREBP-1c regulates triglyceride synthesis through the expression of several downstream target genes, such as ACC, fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1) (Horton et al., 2002). Subsequent studies implied that fructose-induced triglyceride accumulation is a result of de novo lipogenesis and that it is not driven by dietary lipid uptake (Nunes et al., 2014). However, the development of hepatic steatosis and hypertriglyceridemia depends not only on lipogenesis but also on the fatty acid oxidation in the liver. The importance of these processes in fructose-induced metabolic disturbances is still unclear; therefore, the aim of this study was to examine the effect of a high fructose diet (60% fructose solution) on both β-oxidation and de novo lipogenesis in the livers of male Wistar rats. Towards that end, we investigated hepatic lipid metabolism by examining the expression of key proteins of fatty acid β-oxidation (PPARα, PGC-1, nuclear lipin-1, CPT1) and de novo lipogenesis (SREBP-1c, ACC, FAS). The blood levels of free fatty acids (FFAs), triglycerides, and glucose, as well as liver triglyceride levels, were also compared between fructose-fed rats and animals on a control diet.

2. Materials and methods

2.1. Animals and diet
Male Wistar rats aged 21 days, bred in our laboratory, were randomly divided into two experimental groups (n = 9 animals per group) according to diet regime for 9 weeks. The control group had standard laboratory chow (Veterinary Institute, Subotica, Serbia) and drinking water available ad libitum, while the fructose-fed group was given the same food with both a 60% fructose (API-Pek, Bece, Serbia) solution and drinking water ad libitum. The detailed composition of the diet was published previously (Velickovic et al., 2013). Drinking water was provided for the fructose-fed group in order to avoid possible disturbances of the rats’ electrolyte–water balance. The animals were kept in a temperature-controlled room (22 ± 2 °C) with a 12 h light/dark cycle (lights on at 0700 hours) and constant humidity. Daily food and fluid intake were measured throughout the treatment. Total energy intake was expressed as kJ per animal per day and was calculated as previously reported (Vasiljevic et al., 2014).

The protocols were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethics Committee for the Use of Laboratory Animals of the Institute for Biological Research “Siniša Stanković”, University of Belgrade (reference number 3-12/12).

2.2. Blood plasma preparation, tissue collection, and determination of biochemical parameters
After overnight fasting, the animals were sacrificed by decapitation with a guillotine (Harvard-Apparatus, USA), always at the same time (0900–1000 hours). Trunk blood was rapidly collected into EDTA-containing tubes and the glucose and triglyceride concentrations were measured using a MultiCare apparatus with MultiCare glucose strips (product code 23955) and MultiCare triglyceride strips (product code 23960) (Biochemical Systems International, Italy). Blood plasma was isolated by centrifugation at 1600 × g for 10 min at room temperature and stored at −70 °C. Livers, perfused with cold 0.9% NaCl, and visceral adipose tissue (retroperitoneal and omental depots) were both carefully excised and kept in liquid nitrogen for later RNA and protein isolation. Liver triglycerides were isolated from 100 mg of liver tissue using a modified Folch method (Folch et al., 1957) and analyzed with the modified colorimetric method of Fletcher (Fletcher, 1968). FFA plasma level was determined by using a modified version of Duncombe’s (1964) method.

2.3. Preparation of cytoplasmic, nuclear, and whole-cell extracts of the liver
Frozen livers from individual animals were weighed and homogenized with a Janke–Kunkel Ultra-Turrax in 4 volumes (w/v) of ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.2, 10% glycerol, 50 mM NaCl, 1 mM EDTA-Na₂, 1 mM EGTA-Na₂, 2 mM DTT, and protease and phosphatase inhibitors). The homogenates were further processed to obtain cytoplasmic and nuclear fractions, as described previously (Vasiljevic et al., 2013). All steps were conducted at 4 °C and all samples were divided into aliquots and stored at −70 °C.

For preparation of whole-cell extracts, tissues were homogenized with a glass–Teflon homogenizer in 5 volumes (w/v) of ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.2, 1 mM EDTA-Na₂, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM DTT, and protease and phosphatase inhibitors) and the homogenates were sonicated (3 × 30 s, 1 A, 50/60 Hz). After 60 min of incubation on ice with continuous agitation and frequent vortexing, the suspensions were centrifuged (16000 × g, 20 min, 4 °C) and the resulting supernatants were divided into aliquots, stored at −70 °C, and used as whole-cell extracts.

The protein content of all cellular fractions was determined using Spector’s (1978) method with bovine serum albumin as the standard.
2.4. RNA isolation, reverse transcription, and real-time PCR
Total RNA was extracted from the liver of each animal using TRIreagent (AmBion Inc., Austin, TX, USA). Quantitative and qualitative evaluation of the isolated RNA was performed spectrophotometrically (OD 260/280 > 1.8 was considered satisfactory) and on 2% agarose gel. Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. The cDNAs were stored at ~70 °C for later use.

Quantification of target gene expression in the liver was performed using TaqMan real-time polymerase chain reaction (PCR) following a previously published protocol (Djordjevic et al., 2012). The probe set used for the quantification was ACC (Rn00573474_m1), FAS (Rn 01463550_m1), and hypoxanthine-guanine phosphoribosyltransferase (HPRT1; Rn01527840_m1*) (Applied Biosystems Assay-On-Demand Gene Expression Products). HPRT1 was used as an internal control for quantitative normalization of cDNA.

2.5. Western blot analysis
After being boiled in Laemmli's sample buffer, 40 µg of liver protein was resolved on 7.5% or 10% SDS-polyacrylamide gels (samples to be compared were always run on the same gel). The transfer of proteins from the gels to polyvinylidene fluoride membranes (Immobilon-FL, Millipore) was done overnight at 135 mA and 4 °C in a transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol). Membranes were incubated overnight at 4 °C with the following rabbit polyclonal antibodies (all from Santa Cruz Biotechnology): anti-PPARα (sc-9000, 1:250), anti-PGC-1α (sc-13067, 1:250), anti-lipin-1 (sc-98450, 1:500), anti-CPT1 (sc-139482, 1:500), and anti-SREBP-1c (sc-366, 1:500). Mouse monoclonal anti-β-actin antibody (AC-15, Sigma-Aldrich, 1:10000) was used for equal load control. The membranes were incubated for 90 min with alkaline phosphatase conjugated secondary antibodies (Amersham Pharmacia Biotech, 1:20,000). Immunopositive bands were visualized using an enhanced chemiluminescent method on the STORM 840 Gel and Blot Imaging System (GE Healthcare) and quantified using ImageQuant (GE Healthcare).

2.6. Statistical analyses
The data for energy intake as well as morphological and biochemical parameters are given as mean ± SD (n = 9). Real-time PCR and western blot data are presented as mean ± SEM (n = 9). The normality of data distribution was tested using the Shapiro–Wilk test. Between-group comparisons were performed using the Student unpaired t-test. Statistical analyses were performed using GraphPad Prism v.5. Results were considered statistically significant at P < 0.05.

3. Results
3.1. Effect of dietary fructose on energy intake; body, liver, and adipose tissue masses; and biochemical parameters
Food intake was significantly lower (Table, P < 0.05) while energy intake was higher for the fructose-fed rats as compared to the controls (Table, P < 0.01). No significant between-group differences in body and liver masses were observed, but liver-to-body ratio was augmented in the fructose-fed rats. Moreover, rats consuming the fructose solution showed increased absolute (Table, P < 0.05) and relative (P < 0.05) visceral adipose tissue masses compared to the controls. Long-term consumption of the 60% fructose

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control</th>
<th>Fructose</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily food intake (g/animal)</td>
<td>21.73 ± 0.73</td>
<td>16.99 ± 2.71*</td>
<td>0.034</td>
</tr>
<tr>
<td>Daily caloric intake (kJ/animal)</td>
<td>239.05 ± 8.05</td>
<td>324.4 ± 23.8**</td>
<td>0.003</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>333.50 ± 32.34</td>
<td>345.64 ± 32.95</td>
<td>0.435</td>
</tr>
<tr>
<td>Liver mass (g)</td>
<td>11.37 ± 1.57</td>
<td>12.80 ± 1.40</td>
<td>0.064</td>
</tr>
<tr>
<td>Liver-to-body ratio (×1000)</td>
<td>34.00 ± 1.98</td>
<td>37.05 ± 3.21*</td>
<td>0.021</td>
</tr>
<tr>
<td>VAT mass (g)</td>
<td>4.37 ± 1.73</td>
<td>6.26 ± 1.62*</td>
<td>0.039</td>
</tr>
<tr>
<td>VAT-to-body ratio (×1000)</td>
<td>13.26 ± 5.10</td>
<td>18.21 ± 4.64*</td>
<td>0.049</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.08 ± 0.30</td>
<td>3.88 ± 0.24</td>
<td>0.157</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.13 ± 0.25</td>
<td>1.55 ± 0.32**</td>
<td>0.006</td>
</tr>
<tr>
<td>FFAs (mmol/L)</td>
<td>0.69 ± 0.18</td>
<td>0.70 ± 0.32</td>
<td>0.901</td>
</tr>
<tr>
<td>Triglycerides in the liver (mmol/L)</td>
<td>1.37 ± 0.70</td>
<td>1.20 ± 0.31</td>
<td>0.392</td>
</tr>
</tbody>
</table>
solution led to a significant increase in triglyceride levels in the blood (Table, \( P < 0.01 \)), while plasma glucose and FFA levels remained unchanged after the high-fructose diet. In addition, the liver triglycerides were not different between the two groups (Table).

### 3.2. Lipid metabolism in the livers of fructose-fed rats

To evaluate the effects of the fructose solution on the key regulators of lipid metabolism in the liver, we analyzed the concentration and cytoplasmic/nuclear redistribution of proteins involved in the regulation of mitochondrial fatty acid \( \beta \)-oxidation and de novo lipogenesis. We observed that 60% fructose consumption led to a significant decrease of PPAR\( \alpha \) in the cytoplasmic fraction (Figure 1a, \( P < 0.001 \)) and a concomitant elevation in the nuclear fraction (Figure 1b, \( P < 0.01 \)). The analysis of PGC-1\( \alpha \) intracellular redistribution showed that its cytoplasmic level was decreased (Figure 2a, \( P < 0.05 \)), while its nuclear level remained unchanged (Figure 2b). Concomitantly, the levels of another transcription cofactor involved in \( \beta \)-oxidation, lipin-1, were unaltered in the cytoplasmic and nuclear fractions of the livers of fructose-fed animals (Figures 3a and 3b). Since nuclear accumulation of PGC-1\( \alpha \) and lipin-1 can enforce the transcription of the CPT1 gene, the observation that a fructose diet did not affect the nuclear levels of these proteins is in accordance with the finding of decreased CPT1 protein levels in the livers of fructose-fed animals (Figure 4, \( P < 0.05 \)).

When we examined the key hepatic lipogenic transcription factor SREBP-1c, we noticed a significant increase in its nuclear concentration (Figure 5a, \( P < 0.05 \)). Furthermore, the mRNA level of SREBP-1c target genes ACC and FAS were also increased in the livers of fructose-fed rats (Figures 5b and 5c, \( P < 0.05 \)).

### 4. Discussion

In the present study, we examined the effects of high dietary fructose (60% solution) on the development of metabolic syndrome features and on hepatic lipid metabolism in a rodent model. The results demonstrated that rats exposed to a fructose-enriched diet were hypertriglyceridemic and normoglycemic, with no significant differences in body and liver masses in comparison to the controls; however, these animals showed signs of increased visceral adiposity and augmented liver-to-body ratio. These metabolic alterations most likely resulted from the simultaneous induction of hepatic lipogenic factors SREBP-1c, ACC, and FAS, and the inhibition of mitochondrial \( \beta \)-oxidation. In spite of the decreased fatty acid oxidation nuclear PPAR\( \alpha \) was increased, implying that it has a role in hepatic lipogenesis. The alleviation of fructose-induced metabolic disturbances could be achieved by the use of pharmacological stimulators of fatty acid oxidation.

In our study, caloric intake was higher for the fructose-fed rats, but both body weight and liver mass were

**Figure 1.** Subcellular distribution of PPAR\( \alpha \) protein in the liver after a fructose-rich diet. Representative western blots and relative quantification of the PPAR\( \alpha \) protein are shown in the hepatic cytoplasmic (a) and nuclear (b) fractions of the control (C) and fructose-fed (F) rats. The lower parts of the blots were probed with antibodies against \( \beta \)-actin, which was used as a loading control. Values are means ± SEM and are presented as ratios of the control (n = 9). Comparisons between the groups were made using an unpaired Student t-test. Asterisks indicate significant differences, **\( P < 0.01 \) and ***\( P < 0.001 \).
unaffected by the applied diet regime, which is consistent with the results obtained from another animal study using a 60% fructose diet (Kelley et al., 2004). The fructose-fed group had significantly increased adipose tissue mass and a higher visceral adipose tissue-to-body ratio, pointing to visceral adiposity. The liver-to-body ratio was also
increased in the fructose-fed animals; however, this was not accompanied by an accumulation of triglycerides in the liver. The hypertriglyceridemia observed in the 60% fructose-fed animals represents a metabolic hallmark of fructose-enriched diets (Roglans et al., 2007; Rebollo et al., 2014a). This was not associated with increased plasma FFA levels, which is in accordance with some other studies (Roglans et al., 2002; Menard et al., 2010). It is well known that hepatic production of very-low-density lipoproteins (VLDLs) is primarily responsible for hypertriglyceridemia (Wang et al., 1984) and that the main regulator of hepatic lipogenesis is SREBP-1c, which acts through transcriptional control of ACC and FAS. In this study, we detected an increased level of active SREBP-1c followed by enhanced ACC and FAS gene expression. Taken together, these results point toward increased de novo lipogenesis, presumably due to the accumulation of lipogenic precursors upon high fructose consumption. On the other hand, mitochondrial fatty acid β-oxidation was inhibited, as judged by the significantly decreased CPT1 level in the whole-cell extract. This is probably due to higher ACC expression that could lead to increased production of malonyl-CoA, which is an inhibitor of CPT1 and consequently of fatty acid oxidation (Rodriguez-Calvo et al., 2009). However, in spite of the reduced β-oxidation, we observed increased levels of nuclear PPARα protein, which can be explained by the fact that PPARα is also involved in the regulation of hepatic lipid metabolism apart being a

Figure 4. The effect of a fructose-rich diet on CPT1 protein level in the liver. Representative western blots and relative quantification of the CPT1 protein are shown in the whole-cell extract of the livers of the control (C) and fructose-fed (F) rats. The lower parts of the blots were probed with antibody against β-actin, which was used as a loading control. Values are means ± SEM and are presented as folds of the control (n = 9). Comparisons between the groups were made using an unpaired Student t-test. Asterisks indicate a significant difference, *P < 0.05.

Figure 5. The effect of a fructose-rich diet on SREBP-1c protein level and gene expression of ACC and FAS in the liver. Representative western blots and relative quantification of the SREBP-1c protein are shown in the hepatic nuclear fractions of the control (C) and fructose-fed (F) rats. Lower parts of the blots were probed with antibody against β-actin, which was used as a loading control (a). Relative quantification of ACC (b) and FAS (c) mRNA levels in the livers were done relative to the amount of HPRT1 mRNA. Values are means ± SEM and are presented as folds of the control (n = 9). Comparisons between the groups were made using an unpaired Student t-test. Asterisks indicate a significant difference, *P < 0.05.
TEOFILOVIĆ et al. / Turk J Biol

regulator of fatty acid oxidation (Oosterveer et al., 2009). Namely, increased PPARα activity was shown to enhance the flux of fatty acids from the adipose tissue to the liver via stimulation of fibroblast growth factor 21 gene expression (Inagaki et al., 2007), thus maintaining circulating FFA concentrations at a constant level. Moreover, PPARα seems to be a transcriptional activator of SREBP-1c (Fernandez-Alvarez et al., 2011) and SCD1 (Miller and Ntambi, 1996), two main promoters of de novo lipogenesis. Since in this study we observed an increased expression of active SREBP-1c and FAS in the livers of the fructose-fed rats as well as an unchanged level of plasma FFAs, we suggest that the hepatic PPARα activation in these animals was predominantly associated with stimulated lipogenesis (Chakravarthy et al., 2005).

The expected outcome of enhanced de novo lipogenesis should be a high rate of triglyceride secretion in the form of VLDLs or the ectopic accumulation of lipids in the hepatocytes (Kawano and Cohen, 2013). In the present study, 60% fructose consumption was found to be associated with hypertriglyceridemia. On the other hand, we did not observe excessive triglyceride accumulation in the liver, which is in accordance with the results of other animal studies using a 60% fructose diet (Kelley et al., 2004; de Moura et al., 2009). According to these results, we presume that the hepatic overproduction of triglyceride-rich lipoproteins due to a liquid high-fructose diet is channeled into secretory pathways instead of being accumulated in the liver, resulting in excessive visceral adiposity (Sam et al., 2009) and an increased risk of metabolic complications (Lemieux et al., 2007). The observed hypertriglyceridemia without concomitant signs of hepatic steatosis may merely reflect an adaptation to a high-fructose diet (Tappy and Le, 2015) that still has not progressed to pathological status.

In summary, the results of the present study indicate that consumption of 60% fructose solution disturbs hepatic lipid metabolism by simultaneously inducing de novo lipogenesis and inhibiting fatty acid β-oxidation, thus resulting in hypertriglyceridemia and visceral adiposity, but without ectopic hepatic lipid deposition. Future longitudinal studies are required to elucidate the timing of hepatic mitochondrial dysfunction and lipogenesis in relation to fructose-induced metabolic syndrome development.

Acknowledgment
This study was supported by Project Grant No. III41009 from the Ministry of Education, Science, and Technological Development, Republic of Serbia.

References


