Advanced glycation end products promote hepatosteatosis by interfering with SCAP-SREBP pathway in fructose-drinking mice

Raffaella Mastrocola,1 Massimo Collino,2 Mara Rogazzo,2 Claudio Medana,3 Debora Nigro,1 Giuseppe Boccuzzi,4 and Manuela Aragno1

1Department of Clinical and Biological Sciences, Experimental Medicine and Clinical Pathology Unit, University of Turin, Turin, Italy; 2Department of Drug Science and Technology, University of Turin, Turin, Italy; 3Department of Molecular Biotechnology and Life Sciences, University of Turin, Turin, Italy; and 4Department of Medical Sciences, University of Turin, Turin, Italy

Submitted 19 November 2012; accepted in final form 11 July 2013

Mastrocola R, Collino M, Rogazzo M, Medana C, Nigro D, Boccuzzi G, Aragno M. Advanced glycation end products promote hepatosteatosis by interfering with SCAP-SREBP pathway in fructose-drinking mice. Am J Physiol Gastrointest Liver Physiol 305: G398–G407, 2013. First published July 18, 2013; doi:10.1152/ajpgi.00450.2012.—Clinical studies have linked the increased consumption of fructose to the development of obesity, dyslipidemia, and impaired glucose tolerance, and a role in hepatosteatosis development is presumed. Fructose can undergo a nonenzymatic reaction from which advanced glycation end products (AGEs) are derived, leading to the formation of dysfunctional, fructosylated proteins; however, the in vivo formation of AGEs from fructose is still less known than that from glucose. In the present study C57Bl/6J mice received 15% (wt/vol) fructose (FRT) or 15% (wt/vol) glucose (GLC) in water to drink for 30 wk, resembling human habit to consume sugary drinks. At the end of the protocol both FRT- and GLC-drinking mice had increased fasting glycemia, glucose intolerance, altered plasma lipid profile, and marked hepatosteatosis. FRT mice had higher hepatic triglycerides deposition than GLC, paralleled by a greater increased expression and activity of the sterol regulatory element-binding protein 1 (SREBP1), the transcription factor responsible for the de novo lipogenesis, and of its activating protein SCAP. LC-MS analysis showed a different pattern of AGE production in liver tissue between FRT and GLC mice, with larger amount of carboxymethyl lysine (CML) generated by fructose. Double immunofluorescence and coimmunoprecipitation analysis revealed an interaction between CML and SCAP that could lead to prolonged activation of SREBP1. Overall, the high levels of CML and activation of SCAP/SREBP pathway associated to high fructose exposure here reported may suggest a key role of this signaling pathway in mediating fructose-induced lipogenesis.

AGEs; SREBP; triglyceride synthesis; soft drink; hepatosteatosis; fructose; glucose; carboxymethyl lysine

Many clinical studies have linked the rising consumption of soft drinks with added fructose to the development of obesity, dyslipidemia, insulin resistance, impaired glucose tolerance, and hypertension in adults (6, 9, 19, 26). Interestingly, clinical data show that inclusion of fructose in the diet for 10 wk leads to a greater increase in hepatic lipid synthesis than that occurring with an equal amount of glucose (42).

Lipid metabolism is regulated by the sterol regulatory element-binding proteins (SREBP) family, comprising three subtypes: SREBP-1a and SREBP-1c, which are generated by alternative splicing, and SREBP-2. SREBP-1c, expressed in most tissues with a greater prevalence than SREBP1a in liver and adrenal glands, is in charge of governing fatty acid and triacylglyceride metabolism, whereas SREBP-2, ubiquitously expressed, is involved in the regulation of cholesterol metabolism. Both SREBP-1 and SREBP-2 are synthesized as membrane proteins in the endoplasmic reticulum (ER), forming a complex with the SREBP-cleavage activating protein (SCAP). Despite the distinct roles of the SREBPs in lipid metabolism, they are both subjected to the identical processing pathway (37): when triglycerides (TG) or cholesterol synthesis are required, SCAP shuttles SREBPs from the ER to the Golgi, where they are cleaved by two proteases and enter the nucleus, bind to the sterol-regulatory elements in the promoters of target genes, and increase transcription of lipogenic or cholesterologenic enzymes (17). Interestingly, SREBP-1 and SREBP-2 processing is triggered by different types of stimuli: whereas SREBP-1 activation depends primarily on insulin signaling and nutritional status, SREBP-2 is sensitive to membrane sterol level (37).

The liver is the main organ in which fructose metabolism takes place rapidly leading to increased hepatic synthesis of glycogen, fatty acids, and TG (45). Nonalcoholic fatty liver disease (NAFLD) is the most common disorder in industrialized countries, affecting 15–20% of the general population (49), and epidemiological studies have indicated that the development of NAFLD may be associated with excessive fructose consumption (35, 50).

Among the chemical properties of fructose, a nonenzymatic pathway known as the Maillard reaction is reported, in which fructose reacts with the aminic groups of proteins. After this reaction, the anomerization equilibrium of fructose is displaced toward the open form of the sugar, which is highly reactive, especially compared with the forms derived from glucose (43). The Maillard reaction is also one of the “classic” pathways from which the advanced glycation end products (AGEs) are derived. It is known that a mixed class of toxic AGEs can be produced from the reduction of glucose (49): CML (carboxymethyl lysine) and pentosidine are obtained through an oxidative process, and MGO (methylglyoxal) and GLAP (glyceraldehyde- derived pyridinium compound) through a nonoxidative process. Additionally, glucose is known to form AGE α-oxoaldehydes, including GOLD (glyoxal-lysine dimer) and MOLD (methylglyoxal-lysine dimer), through the polyol pathway (41). AGEs can exert a direct interference with cellular proteins function or a receptor-mediated action, the latter being chiefly attributed to bonding with RAGE (receptor for AGE) (4). The interaction...
between AGEs and RAGE leads to intracellular signals responsible for activation of proinflammatory transcription factors, such as NF-κB (3).

The Maillard reaction undertaken by fructose leads to the formation of altered, fructosylated proteins, which are potentially toxic, indicating that fructose, together with glucose, plays an important role in the formation of AGEs. So far, the in vivo formation of fructose-derived AGEs has only been demonstrated in one study, and only through immunochemical analysis, without reporting a description of their chemical structure (44). Thus the chemical structure and toxicity of AGE molecules specifically derived from fructose are less well known than those derived from glucose.

It might be hypothesized that the entrance of fructose in hepatocytes, where fructose is metabolized, leads to the fructosylation of cytoplasmic proteins, causing a loss of their functionality and regulation, thus contributing to liver alterations.

This study is aimed to characterized fructose-derived AGEs and to investigate their target proteins in liver by a comparative analysis between fructose- and glucose-drinking mice.

**MATERIALS AND METHODS**

All compounds were purchased from Sigma Chemical (St. Louis, MO) and all primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), unless otherwise stated.

Animals and treatments. Male C57Bl6/N mice (Charles River Laboratories, Calco, Italy) aged 5 wk were cared for in compliance with the European Council directives (No. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH no. 85–23, revised 1985). The scientific project was approved by the local ethical committee. The animals were divided into three groups of 8–10 mice: CTRL group, drinking tap water; FRT group, drinking a 15% fructose solution; GLC group, drinking a 15% glucose solution. All groups were fed with a standard lab chow and received drink and food ad libitum.

Body weight, drink, and food intake were recorded weekly. Fasting glycemia was measured at the start of the protocol and every 8 wk by saphenous vein puncture using a glucometer (GlucoGmeter, Menarini Italy) and frozen in N2 for cryostatic preparations. Other portions were frozen in OCT (Optimal Cutting Temperature) compound (VWR, Milan, Italy) and frozen in N2 for cryostatic preparations. Other portions were frozen in N2 and stored at −80°C for protein analysis.

Oral glucose tolerance test. Before killing, a glucose solution was administered orally at 2 g/kg body wt after a fasting period of 6 h. Plasma glucose levels were measured every 30 min for 2 h after glucose loading.

Biochemical parameters. Plasma lipid profile was determined by standard enzymatic procedures using reagent kits (TG, cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL); Hospitex Diagnostics, Florence, Italy; nonesterified fatty acid (NEFA): Wako Chemicals, Neuss, Germany).

Plasma insulin level was measured by using an ELISA kit (Merodia, Uppsala, Sweden).

For tissue TG and cholesterol content determination, colorimetric assay kits were used after lipid extraction (TG: Triglyceride Quantification Kit, Abnova, Aachen, Germany; cholesterol: Hospitex Diagnostics).

Oil Red O staining. Liver lipid accumulation was evaluated by Oil Red O staining on 4-μm cryostatic sections. Stained tissues were viewed under an Olympus Bx41 microscope (×10 magnification) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany).

The sections were analyzed on six fields/slide and scored by a blinded pathologist using the NAFLD activity score (NAS) system (21).

Preparation of tissue extracts. Liver cytosolic, nuclear, and total proteins were extracted as previously described (28). Protein content was determined by the Bradford assay, and samples were stored at −80°C until use.

AGE analysis with LC-MS. Pentosidine, GOLD, MOLD, CML, and GLAP were evaluated on total liver extracts after hydrolysis with 0.6 M trichloroacetic acid and 50 μl of hydrochloric acid 6 M for 12 h at 60°C.

The chromatographic separations were run on an Ultimate 3000 HPLC (Dionex, Milan, Italy) coupled to a high-resolution-power mass spectrometer LTQ Orbitrap (Thermo Scientific, Rodano, Italy), equipped with an atmospheric pressure interface and an ESI ion source. The samples were analyzed by using an Reverse Phase C18 column (Phenomenex Synergi 150 × 2.1 mm, 3 μm particle size) at a flow rate of 200 μl/min. A gradient mobile phase composition was adopted: 95/5 to 40/60 in 25 min, 5 mM heptafluorobutanoic acid/acetonitrile. The monitored protonated molecular ions were 205.1188 m/z for CML, 255.1344 m/z for GLAP, 341.2199 m/z for MOLD, 327.0232 m/z for GOLD, and 379.2094 m/z for pentosidine. Quantitative determination of all the analytes was done by using pentosidine calibration data.

Western blotting. Equal amounts of total, cytosolic, or nuclear proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membrane (GE-Healthcare Europe, Milan, Italy, Mouse). Antibody (anti-NF-κB-p65, rabbit anti-SREBP1, and mouse anti-SREBP2) antibodies were used in different concentrations for probing both cytosolic and nuclear extracts. Goat anti-ICAM-1 (intercellular adhesion molecule-1) and anti-CTGF (connective tissue growth factor), rabbit anti-SCAP, anti-ACC (acetyl coenzyme A carboxylase; Cell Signaling Technology, Danvers, MA), anti-HMG (hydroxymethyl coenzyme A reductase; Millipore, Temecula, CA), anti-apoB, anti-CPT1-L, and anti-AGE were probed on total extracts. Rabbit anti-apoB was also probed on 10 μl of plasma samples.

Proteins were detected with ECL chemiluminescence substrate (GE-Healthcare) and quantified by densitometry using analytic software (Quantity-One, Bio-Rad). β-Actin served as loading control for total and cytosolic protein extracts and lamin-B1 for nuclear extracts.

Immunofluorescence. Localization of SCAP, SREBP1, CML, and MGO was assessed by immunofluorescence. Sections were blocked for 1 h with 3% BSA for cytosolic sections by indirect immunofluorescence. Sections were blocked for 1 h with 3% BSA in PBS added with unconjugated goat anti-mouse IgG to prevent mouse-on-mouse interferences. Thus sections were incubated overnight with rabbit anti-SCAP, rabbit anti-SREBP1, mouse anti-CML (Trans-Genic, Kobe, Japan), or mouse anti-MGO (Trans-Genic) primary antibodies and for 1 h with fluorescent secondary antibodies (Dako, Glostrup, Denmark): TRITC-conjugated anti-rabbit IgG or biotin-conjugated anti-mouse IgG followed by FITC-conjugated streptavidin. Negative controls were prepared incubating sections with secondary antibodies. Sections were examined with a Leica Olympus epifluorescence microscope (Olympus Bx41) and digitized with a high-resolution camera (Zeiss).

| Table 1. General parameters of mice after 30 wk drinking water, fructose, or glucose |
|---------------------------------|--------|--------|--------|
|                                 | CTRL   | FRT    | GLC    |
| Drink intake, ml/day            | 3.7 ± 0.4 | 6.5 ± 0.5††† | 11.4 ± 3.8***††† |
| Food intake, g/day              | 3.8 ± 0.9 | 2.6 ± 0.6*  | 2.0 ± 0.9*** |
| Caloric intake, kcal/day        | 11.1 ± 2.8 | 11.3 ± 1.8 | 12.4 ± 2.1 |
| Body weight increase, g         | 12.4 ± 2.0 | 16.3 ± 2.5*** | 16.2 ± 2.4*** |
| Fasting glycemia, mmol/l        | 4.7 ± 1.0 | 7.2 ± 1.5**  | 7.1 ± 0.9*** |
| Insulinemia, μg/l               | 1.27 ± 0.07 | 2.13 ± 1.02 | 1.68 ± 1.03 |

Data are means ± SD of 8–10 mice per group, CTRL, tap water control group; FRT, fructose-drinking group; GLC, glucose-drinking group. *P < 0.05, **P < 0.01, ***P < 0.005 vs. CTRL; †††P < 0.005 vs. FRT.
Double immunofluorescence. Double immunofluorescence was performed for SCAP and CML on liver cryostatic sections. After blocking, sections were incubated with a mix of primary antibodies for 1 h. After washing, sections were incubated with a mix of labeled secondary antibodies. The images were color combined and assembled into photomontages by using Adobe Photoshop (Universal Imaging, West Chester, PA).

Coimmunoprecipitation. Equal amounts of total proteins (500 μg) were incubated overnight with SCAP rabbit-polyclonal antibody (2 μg). The antibody-antigen complexes were then incubated with fresh protein A-Sepharose beads for 3 h. SDS Laemmli buffer was added to the beads, and eluted proteins were subjected to SDS-PAGE and immunoblotted with mouse anti-CML monoclonal antibody and, after stripping, with rabbit anti-SCAP antibody.

Statistical analysis. All values are expressed as means ± SD and were analyzed by ANOVA test followed by Bonferroni’s posttest. A P value <0.05 was considered statistically significant.

RESULTS

Fructose and glucose drinking in mice induces alterations in body weight, glucose tolerance, and plasma lipid profile. The daily drink intake in the FRT- and GLC-drinking groups was markedly higher than in the CTRL group, drinking tap water. Moreover, glucose intake was also significantly higher than fructose, but despite that the total daily caloric intake was similar among the groups, being proportionally reduced the food intake (Table 1).

As shown in Table 1, mice drinking fructose or glucose for 30 wk showed a significant increase in body weight compared with CTRL mice (+31%).

Fasting glycemia was significantly higher both in FRT and in GLC groups compared with CTRL group (Table 1). During oral glucose tolerance test (Fig. 1A), the glycemic curves of
FRT and GLC mice were markedly moved away from CTRL curve at every time point after glucose charge. Plasma insulin level was slightly increased in the GLC group and to a greater extent in the FRT group, with respect to CTRL, without reaching any statistical significance (Table 1).

In comparison with CTRL animals, FRT mice showed alterations in plasma lipid profile (Table 2) featured by increased levels of TG (+43%), cholesterol (+37%), and LDL (+80%), paralleled by a decrease in NEFA (−24%). GLC mice only showed a trend to dyslipidemia that didn’t reach statistical significance, excepting for HDL level (−16%) and for NEFA level, which, conversely to the FRT group, was increased (+20%) compared with CTRL.

Fructose and glucose drinking increases liver TG and cholesterol content and induces hepatosteatosis. Hepatic homogenates of FRT mice showed alterations in plasma lipid profile (Table 2) featured by increased levels of TG (+43%), cholesterol (+37%), and LDL (+80%), paralleled by a decrease in NEFA (−24%). GLC mice only showed a trend to dyslipidemia that didn’t reach statistical significance, excepting for HDL level (−16%) and for NEFA level, which, conversely to the FRT group, was increased (+20%) compared with CTRL.

Fructose and glucose drinking increases liver TG and cholesterol content and induces hepatosteatosis. Hepatic homogenates of FRT mice showed a marked increase in TG and cholesterol content compared with CTRL (+100 and +50%, respectively). In liver homogenates from GLC mice an increase in cholesterol content similar to FRT was found (+42% of the CTRL value), whereas the TG level tended to increase compared with CTRL but remained significantly lower than in the FRT group (−60%) (Fig. 1B).

Oil Red O staining of liver sections (Fig. 1, C–E) highlighted a marked lipid deposition both in FRT (D) and in

Table 2. Plasma lipid profile of mice after 30 wk of water, fructose, or glucose drinking

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>FRT</th>
<th>GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG, mmol/l</td>
<td>1.12 ± 0.18</td>
<td>1.60 ± 0.28**</td>
<td>1.34 ± 0.33</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>2.26 ± 0.20</td>
<td>3.10 ± 0.36***</td>
<td>2.68 ± 0.63</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>1.46 ± 0.23</td>
<td>1.31 ± 0.24</td>
<td>1.23 ± 0.12*</td>
</tr>
<tr>
<td>LDL, mmol/l</td>
<td>0.80 ± 0.17</td>
<td>1.44 ± 0.44**</td>
<td>0.95 ± 0.31</td>
</tr>
<tr>
<td>NEFA, mg/dl</td>
<td>12.76 ± 1.58</td>
<td>9.75 ± 2.08**</td>
<td>15.29 ± 3.36††</td>
</tr>
</tbody>
</table>

Data are means ± SD of 8–10 mice per group. TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-esterified fatty acid. *P < 0.05, **P < 0.01, ***P < 0.005 vs. CTRL; ††P < 0.01 vs. FRT.
GLC mice (E) compared with CTRL (C), resembling a condition of NAFLD, with different histopathological features. FRT mouse liver showed enlarged hepatocytes with periportal macrovesicular steatosis. In contrast, liver of GLC mice showed a microvesicular steatosis with a panlobular dissemination. A significantly higher steatosis grade was detected in FRT compared with GLC mice, conferring an overall NAS score of $5.2 \pm 1.3$ to FRT vs. $3.8 \pm 0.9$ to GLC mouse liver ($P < 0.05$) (Fig. 1F).

Fructose and glucose drinking enhances TG and cholesterol synthesis through activation of SCAP-SREBP signaling. To further investigate the greater lipogenic effect of fructose with respect to glucose, we assessed the expression and activation of SREBP1c, SREBP2, and their activating protein SCAP by Western blotting analysis (Fig. 2).

SCAP was markedly upregulated in FRT and GLC mice compared with CTRL (Fig. 2, A and B), to a significantly greater extent in FRT than in GLC. Both SREBP1c and
SREBP2 were upregulated in FRT and GLC groups compared with CTRL (Fig. 2, C–H). Specifically, the 68-kDa active form (Fig. 2, C and E) and the 125-kDa inactive form (Fig. 2, F and H) of SREBP1c were significantly more expressed in liver of FRT mice than in GLC. In contrast, SREBP2 was equally activated in FRT and GLC liver (Fig. 2, G and H), whereas inactive form of SREBP2 was more expressed in GLC liver than in FRT, without reaching significant difference (Fig. 2, G and H). The activation of the SCAP/SREBP pathway is confirmed by the increased expression in FRT and GLC mouse liver of both the SREBP1c target gene encoding ACC, one of the enzymes that promote TG synthesis (Fig. 3, A and B), and the SREBP2 target gene encoding HMGR, the rate-limiting enzyme of the cholesterol synthesis (Fig. 3, C and D). Notably, the expression of ACC is ~35% greater in FRT than in GLC mouse liver (Fig. 3B) according to the higher activation of SREBP1c.

ApoB100 protein level was measured in plasma and liver as marker of VLDL secretion (Fig. 3E), whereas the expression of carnitine palmitoyl transferase 1 (CPT1-L) (Fig. 3G) indicates the efficiency of β-oxidation. Any significant differences were seen in ApoB100 plasma-to-liver protein level (Fig. 3F) and in liver expression of CPT1-L (Fig. 3H), among the three groups.

Table 3. Advanced glycated end-products evaluated by LC-MS in liver homogenates

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>FRT</th>
<th>GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentosidine, pg/mg protein</td>
<td>n.d.</td>
<td>7.02 ± 1.98*</td>
<td>8.69 ± 0.92**</td>
</tr>
<tr>
<td>GLAP, ng/mg protein</td>
<td>n.d.</td>
<td>0.36 ± 0.09*</td>
<td>2.20 ± 0.86†</td>
</tr>
<tr>
<td>GOLD, ng/mg protein</td>
<td>n.d.</td>
<td>148.1 ± 53.7**</td>
<td>98.5 ± 30.0**</td>
</tr>
<tr>
<td>MOLD, ng/mg protein</td>
<td>n.d.</td>
<td>0.37 ± 0.24*</td>
<td>1.08 ± 0.11***††</td>
</tr>
<tr>
<td>CML, ng/mg protein</td>
<td>0.57 ± 0.09</td>
<td>1.34 ± 0.14***</td>
<td>0.76 ± 0.03†††</td>
</tr>
</tbody>
</table>

Data are means ± SD of 8–10 mice per group. GLAP, glyceraldehyde-derived pyridinium compound; GOLD, glyoxal-lysine dimer; MOLD, methyglyoxal-lysine dimer; CML, carboxymethyl lysine; n.d., not determined. *P < 0.05, **P < 0.01, ***P < 0.005 vs. CTRL; †P < 0.05, ††P < 0.01, †††P < 0.005 vs. FRT.
although a trend to a reduction of CPT1-L level was seen in GLC mice.

*Fructose and glucose drinking enhances AGES generation and activates RAGE signaling.* As shown in Table 3, all AGES here measured were markedly increased in liver homogenates of FRT and GLC mice compared with CTRL. Most notably, GLAP and MOLD highest levels were detected in the liver of GLC-drinking group, whereas GOLD and CML were produced in the greatest amount in the liver from FRT group (Table 3). The receptor for AGES, RAGE, was upregulated both in FRT and GLC mice compared with CTRL (+100%) (Fig. 4, A and B) and the downstream signaling was activated as demonstrated by the nuclear translocation of NF-κB-p65 (Fig. 4, C and D). As consequence, we found increased levels of the NF-κB-dependent protein ICAM-1 in both sugar-drinking groups (Fig. 4, E and F), and a slight, but not significant, increase of an early marker of fibrosis, CTGF (Fig. 4, E and F), even if morphological signs, such as collagen I and IV deposition, were still not detectable (data not shown).

*CML colocalizes with SCAP in liver of fructose-drinking mice.* Immunofluorescence analysis on liver sections from FRT mice showed a prevalent nuclear localization for SREBP1c (Fig. 5, A and B), consistent with its activation, and a cytosolic perinuclear localization for SCAP (Fig. 5, D and E). CML localized mainly in cytosol of hepatocytes (Fig. 5, G and H), with a perinuclear distribution similar to SCAP. Interestingly, MGO was detected mainly in the endothelium and at the plasma membrane of hepatocytes (Fig. 5, J and K).

**Fig. 5.** Liver localization of SREBP1c, SCAP, carboxymethyl lysine (CML), and methylglyoxal (MGO). Representative 40×100 magnification photomicrographs of immunofluorescence analysis for SREBP1c (A and B), SCAP (D and E), CML (G and I), and MGO (J and K) on 4-μm cryostatic liver sections. To assess aspecific staining, negative controls (C neg) were prepared by incubating sections only with secondary antibodies (anti-rabbit: C and F; anti-mouse: I and L).

Double immunofluorescence studies in liver of FRT group confirmed that CML colocalizes with SCAP in the perinuclear zone of the hepatocytes (Fig. 6, A–F).

*CML modifies SCAP in liver of fructose-drinking mice.* Finally, coimmunoprecipitation assay has been performed to evaluate CML glycosylation by CML (Fig. 6, G and H). SCAP was immunoprecipitated with protein A-Sepharose, electrophoresed, and blotted on nitrocellulose membrane. Membrane was then exposed to CML antibody, revealing a complex between SCAP and CML in liver of FRT mice.

**DISCUSSION**

This study clearly demonstrates a significant activation of SCAP/SREBP pathway and the following increase in de novo lipogenesis, which were associated to high levels of fructose-derived AGEs in the liver of mice chronically exposed to high fructose intake.

Reducing sugars, as fructose and glucose, react spontaneously with amino groups of proteins to AGES (27). Although glucose plays a primary role in the formation of AGES, it is now known that fructose undergoes the same nonenzymatic glycation reaction at a much faster rate. When fructose assumption with foods or beverages is remarkable, its high reactivity may substantially contribute to the tissue formation of AGES and lead to cellular alterations and dysfunction (38).

Our study shows for the first time a different pattern of hepatic AGEs between FRT and GLC detected by LC-MS. In
whereas both the liver expression of CPT-1, the rate limiting fatty acid synthetase enzyme of mitochondrial β-oxidation, and the ratio between plasma and liver levels of ApoB100, the structural component of VLDL, did not differ in FRT and GLC mice. These data indicate for the first time that the de novo synthesis is the main pathway responsible for the higher lipid accumulation in liver of FRT mice with respect to GLC, in which other mechanisms, such as reduction of β-oxidation and higher hydrolysis of adipose fat, may contribute to hepatic steatosis, as previously suggested by other authors (30).

Liver is the main tissue involved in fructose handling and de novo lipogenesis (18), and many studies have shown that fructose plays a specific role in the pathogenesis of hepatosteatosis and metabolic syndrome due to differential hepatic fructose metabolism (25, 32, 33). However, the molecular mechanisms by which high fructose diets induce abnormalities in liver TG metabolism are not fully understood.

It has been observed that a simultaneous induction of glycolytic and lipogenic genes is a salient feature when dietary glucose is replaced with fructose. Indeed, fructose ingestion at high doses increases expression of the genes encoding for lipogenic enzymes via the activation of SREBP1 in the liver (29). Thus we have analyzed the expression of SREBP1c, SREBP2, and their chaperone protein SCAP in the liver of FRT- and GLC-drinking mice.

In our work, chronic exposure to low levels of both fructose and glucose induced the activation of the SCAP/SREBP system. Notably, there was a marked difference in SCAP expression between FRT and GLC mice, being higher in FRT, and this could be crucial for the greater induction of lipogenesis by fructose. Indeed, we observed a significantly higher expression of mitochondrial β-oxidation, and the ratio between plasma and liver levels of ApoB100, the structural component of VLDL, did not differ in FRT and GLC mice. These data indicate for the first time that the de novo synthesis is the main pathway responsible for the higher lipid accumulation in liver of FRT mice with respect to GLC, in which other mechanisms, such as reduction of β-oxidation and higher hydrolysis of adipose fat, may contribute to hepatic steatosis, as previously suggested by other authors (30).

Liver is the main tissue involved in fructose handling and de novo lipogenesis (18), and many studies have shown that fructose plays a specific role in the pathogenesis of hepatosteatosis and metabolic syndrome due to differential hepatic fructose metabolism (25, 32, 33). However, the molecular mechanisms by which high fructose diets induce abnormalities in liver TG metabolism are not fully understood.

It has been observed that a simultaneous induction of glycolytic and lipogenic genes is a salient feature when dietary glucose is replaced with fructose. Indeed, fructose ingestion at high doses increases expression of the genes encoding for lipogenic enzymes via the activation of SREBP1 in the liver (29). Thus we have analyzed the expression of SREBP1c, SREBP2, and their chaperone protein SCAP in the liver of FRT- and GLC-drinking mice.

In our work, chronic exposure to low levels of both fructose and glucose induced the activation of the SCAP/SREBP system. Notably, there was a marked difference in SCAP expression between FRT and GLC mice, being higher in FRT, and this could be crucial for the greater induction of lipogenesis by fructose. Indeed, we observed a significantly higher expression of mitochondrial β-oxidation, and the ratio between plasma and liver levels of ApoB100, the structural component of VLDL, did not differ in FRT and GLC mice. These data indicate for the first time that the de novo synthesis is the main pathway responsible for the higher lipid accumulation in liver of FRT mice with respect to GLC, in which other mechanisms, such as reduction of β-oxidation and higher hydrolysis of adipose fat, may contribute to hepatic steatosis, as previously suggested by other authors (30).

Liver is the main tissue involved in fructose handling and de novo lipogenesis (18), and many studies have shown that fructose plays a specific role in the pathogenesis of hepatosteatosis and metabolic syndrome due to differential hepatic fructose metabolism (25, 32, 33). However, the molecular mechanisms by which high fructose diets induce abnormalities in liver TG metabolism are not fully understood.

It has been observed that a simultaneous induction of glycolytic and lipogenic genes is a salient feature when dietary glucose is replaced with fructose. Indeed, fructose ingestion at high doses increases expression of the genes encoding for lipogenic enzymes via the activation of SREBP1 in the liver (29). Thus we have analyzed the expression of SREBP1c, SREBP2, and their chaperone protein SCAP in the liver of FRT- and GLC-drinking mice.

In our work, chronic exposure to low levels of both fructose and glucose induced the activation of the SCAP/SREBP system. Notably, there was a marked difference in SCAP expression between FRT and GLC mice, being higher in FRT, and this could be crucial for the greater induction of lipogenesis by fructose. Indeed, we observed a significantly higher expression
and activation of SREBP1c in liver of FRT vs. GLC mice, as confirmed by the resulting higher expression of ACC and by the greater hepatic TG accumulation. On the other hand, SREBP2 is equally hyperactivated in FRT and GLC mice, leading thus to similar expression of HMGR and thereby to similar level of cholesterol in liver. Although SCAP is the common activating protein of both SREBP1c and SREBP2, the existence of unidentified regulatory factors, such as nutritional status or food composition, that determine the fate of the SREBP-SCAP complex by distinguishing between SREBP-1c and SREBP-2 processing, has been supposed (17, 37).

Insulin is a well-known inducer of SREBP1c activity, and hyperinsulinemia may contribute in hyperactivation of lipogenic pathway (7, 8). However, in our experimental model FRT and GLC mice, even showing altered glucose homeostasis, didn’t reach a condition of hyperinsulinemia adequate to induce the de novo lipogenesis.

We then hypothesized a possible interference of CML on SCAP/SREBP system. Indeed, a direct correlation between AGE serum level and TG level was found in children and adolescents with Type 1 diabetes (13). Besides, the generation of CML has been observed during high-fat/high-sugar diets (20, 36), and this has been attributed to lipid peroxidation processes (12). A relationship between intracellular lipid accumulation and increase in CML levels has also been recently demonstrated in an in vitro model of steatosis (12). Moreover, CML accumulation in the liver of obese individuals has been involved in hepatosteatosis development (41). This is also supported by a study showing that administration of pyridoxamine, an inhibitor of CML formation, reduces plasma TG and cholesterol on Zucker obese rats (2).

Several studies have indicated that interaction of CML with RAGE causes oxidative stress and activation of NF-κB via multiple intracellular signal pathways (5, 16). Our results demonstrated that both fructose and glucose chronic exposure increased hepatic RAGE expression and consequently activates NF-κB and inflammatory/fibrogenic signaling, at the same level. Therefore, the CML involvement in the higher lipogenesis occurring in FRT mice is not mediated by RAGE binding.

A recent in vitro study in cultured mesangial cells highlights a direct causal role for CML in SREBP activation by interfering with SCAP and thus driving SREBP factors to elude its negative feedback control (52). The glycosylation of SCAP by Golgi enzymes plays an important role in the cycling of SCAP between the ER and the Golgi (31, 51). In physiological conditions, high intracellular concentrations of cholesterol prevent transport of the SCAP-SREBP complex from the ER to the Golgi and downregulate SREBP activation, avoiding intracellular cholesterol and lipid overloading (10). CML administration in mesangial cells disrupted the SCAP-mediated feedback regulation of SREBPs, increasing SCAP gene transcription and protein stability, thereby enhancing the cycling of SCAP between the ER and the Golgi and prolonging SREBP activation (52). Consistently, our immunofluorescence analysis suggested an interaction between CML and SCAP, which were extensively colocalized in the perinuclear zone of the hepatocytes in FRT mice. The result of coimmunoprecipitation technique further reinforced our hypothesis of a cross-link between CML and SCAP, indicating for the first time that a specific interference of CML in SCAP/SREBP system also occurs in vivo and, most notably, could be induced by fructose drinking. However, further experiments with specific CML inhibitors are needed for a conclusive demonstration of the causal role of fructose-derived AGEs in the activation of this specific signaling pathway. Recently, uric acid generated from fructose metabolism has been suggested as a further mechanism contributing at least in part to the lipogenic effect of fructose feeding (23, 24). Uric acid has been shown to induce mitochondrial oxidative stress and accumulation of citrate, being the substrate for the de novo lipogenesis (23). It is known that oxidative stress is an important element in the glycoxidation process that leads to AGE accumulation (15), and in some cases a direct positive correlation between uric acid and pentosidine levels has been reported (14, 34).

In summary, the present results improve our knowledge on fatty liver development and show an association between high levels of fructose-derived AGEs and activation of de novo lipogenesis, thus suggesting more caution in the even wider employment of fructose as added sweetener in foods and beverages.

GRANTS
This study was supported by a grant of CRT Foundation (2010.1954): “Consuming of sugar-added drinks as risk factor for metabolic diseases: emerging role of fructose” and by MIUR, Fondi ex-60: “High fructose intake: sweeter or health detrimental?”

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
R.M., M.C., and M.A. conception and design of research; R.M. and M.C. analyzed data; R.M. and M.C. interpreted results of experiments; R.M. prepared figures; R.M. drafted manuscript; M.C., G.B., and M.A. edited and revised manuscript; M.R., C.M., and D.N. performed experiments; M.A. approved final version of manuscript.

REFERENCES
12. Gaens KH, Niessen PM, Rensen SS, Buurman WA, Greve JW, Driessen A, Wolfs MG, Hofker MH, Bloemen JG, Dejong CH, Stehou-


Targher G. Non-alcoholic fatty liver disease, the metabolic syndrome and the risk of cardiovascular disease: the plot thickens. Diabetologia 51: 445–450, 2008.


