Beneficial effects of rosuvastatin on insulin resistance, adiposity, inflammatory markers and non-alcoholic fatty liver disease in mice fed on a high-fat diet

Julio Cesar FRAULOB, Vanessa SOUZA-MELLO, Marcia Barbosa AGUILA and Carlos Alberto MANDARIM-de-LACERDA
Laboratory of Morphometry, Metabolism and Cardiovascular Disease, Biomedical Centre, State University of Rio de Janeiro, Brazil

ABSTRACT
The aim of the present study was to evaluate the effects of ST (rosuvastatin) and GZ (rosiglitazone) on IR (insulin resistance) and on liver as well as adipose tissue in mice fed on an HF (high-fat) diet. Our data show that treatment with ST resulted in a marked improvement in insulin sensitivity characterized by enhanced glucose clearance during the insulin tolerance test and a 70% decrease in the HOMA-IR (homoeostasis model assessment of insulin resistance) index level \((P = 0.0008)\). The ST-treated mice exhibited lower gains in BM (body mass; \(-8\%; P < 0.01\)) and visceral fat pad thickness (\(-60\%; P < 0.01\)) compared with the untreated HF group. In comparison with HF-diet-fed mice, HF + ST-treated mice showed a significant reduction in hepatomegaly and liver steatosis (\(-6\%; P < 0.05\); and \(-21\%; P < 0.01\) respectively). In HF + ST-treated mice, the hepatic TAG (triacylglycerol) levels were reduced by 58% compared with the HF group (\(P < 0.01\)). In addition, the expression of SREBP-1c (sterol-regulatory-element-binding protein-1c) was decreased by 50% in the livers of HF + ST-treated mice (\(P < 0.01\)) relative to the HF-diet-fed mice. The levels of resistin were lower in the HF + ST-treated group compared with the HF group (44% less, \(P < 0.01\)). In conclusion, we demonstrated that ST treatment improved insulin sensitivity and decreased liver steatosis in mice fed on an HF diet. Furthermore, ST reduced BM gains, improved the circulating levels of plasma cholesterol and TAG, and reduced hepatic TAG, which was concomitant with lower resistin levels.

INTRODUCTION
Most overweight and obese individuals develop hepatic steatosis, which is characterized by increased lipid [mainly TAG (triacylglycerol)] levels in the liver; hepatic steatosis leads to a condition known as NAFLD (non-alcoholic fatty liver disease) [1]. It is estimated that up to 70% of obese persons and subjects with T2DM

Key words: insulin resistance, non-alcoholic fatty liver disease (NAFLD), obesity, rosuvastatin, sterol-regulatory-element-binding protein 1 (SREBP-1).

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BM, body mass; CVD, cardiovascular disease; GLUT, glucose transporter; GZ, rosiglitazone; HF, high-fat; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HOMA-IR, homoeostasis model assessment of insulin resistance; IPITT, intraperitoneal insulin tolerance test; IR, insulin resistance; IRS, insulin receptor substrate; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; OGTT, oral glucose tolerance test; PPAR, peroxisome-proliferator-activated receptor; PUFA, polyunsaturated fatty acid; SC, standard chow; SREBP, sterol-regulatory-element-binding protein; ST, rosuvastatin; TAG, triacylglycerol; TC, total cholesterol; T2DM, Type 2 diabetes mellitus; TZD, thiazolidinedione.

Correspondence: Professor Carlos Alberto Mandarim-de-Lacerda (email mandarim@uerj.br).
(Type 2 diabetes mellitus) have hepatic steatosis, whereas the prevalence of hepatic steatosis is between 15% and 25% in the general population [2]. In addition, visceral fat accumulation plays a role in the pathogenesis and prognosis of NAFLD [3], as it is associated with increased cardiovascular risk [4,5].

NAFLD is the hepatic metabolic consequence of relative overnutrition and altered diet composition [6]. SREBP-1c (sterol-regulatory-element binding protein-1c) plays a unique role in the expression of genes involved in hepatic TAG synthesis and may also play a major role in the pathogenesis of NAFLD. Although the precise mechanisms leading to the activation of the SREBP-1c promoter have yet to be clarified, it is clear that SREBP-1c is induced by saturated fatty acids, possibly through their ability to promote inflammation [7]. Fatty acid synthesis is enhanced in NAFLD and participates in accumulation of fatty acids. The expression of SREBP-1c is nearly 5-fold greater in NAFLD patients than healthy controls [8].

HF (high-fat) diets account for the largest incidence of obesity in the world. Diet-induced weight loss and lifestyle modifications, including physical exercise and qualitative changes in the diet, have beneficial effects on NAFLD and help manage IR (insulin resistance) [9]. However, only a small percentage of patients with NAFLD can implement these measures efficiently [10]. Therefore the correction of IR is a relevant therapeutic strategy in this condition, and pharmacological treatment is one effective approach [11].

One of the most significant advances in drug therapy during the 20th century was the development of the statin class of drugs. ST (rosuvastatin) is a statin and a competitive inhibitor of HMG-CoA (3-hydroxy-3-methylglutaryl CoA) reductase. ST induces the up-regulation of cell surface LDL (low-density lipoprotein) receptors. HMG-CoA is the rate-limiting enzyme for de novo cholesterol biosynthesis.

It has been shown that ST is a highly effective hypo-lipidaemic agent in patients with the metabolic syndrome [13]. Indeed, it has demonstrated higher efficacy for reducing LDL-cholesterol than other statins at comparable doses [12] and therefore reduces cardiovascular risk [14].

In addition, ST has several ‘pleiotropic’ effects that may result in clinical benefits. Recent studies have shown that statins may have additional effects that improve hepatic insulin sensitization in animal models [15] and humans [16]. Despite these reports, the use of statins to treat IR and NAFLD has been poorly investigated.

GZ (rosiglitazone) is in the class of TZDs (thiazolidinediones), which are insulin-sensitizing drugs used for the treatment of T2DM. At the molecular level, TZDs are highly specific ligands of the PPARγ (peroxisome-proliferator-activated receptor γ) [17,18]. GZ treatment of patients with poorly controlled T2DM and NAFLD improved liver function [10]. In that study, fatty liver improvement with GZ therapy suggested that TZDs could be useful for the treatment of NASH (non-alcoholic steatohepatitis) in overweight or obese patients. Despite the potential applicability of TZDs towards the treatment of NAFLD, it is important to note that liver injury has been reported in a few patients treated with GZ [19]. In murine models of T2DM and NAFLD, GZ has been shown to exacerbate pre-existing liver lesions [20–22]. Further, GZ treatment is associated with a higher increase in the incidence of serious heart failure [23–24]. Recently, the European Medicines Agency has recommended the suspension of GZ, and the U.S. Food and Drug Administration has recommended measures to determine the safety of GZ and to further restrict its use [25].

In the present study, we used an HF-responsive mouse model [26] to evaluate the physiological and molecular response of the liver and adipose tissues following ST treatment alone compared with ST treatment in combination with GZ. We investigated clinical IR, adiposity and hepatic steatosis.

**MATERIALS AND METHODS**

**Animals and diet**

Male C57BL/6 mice (at 8 weeks of age) were used in the study. The mice were housed under controlled conditions (21 ± 2 °C, 60% ± 10% humidity and a 12 h light/12 h dark cycle) and had free access to food and water. After 1 week of acclimatization, the mice were divided into five groups (n = 10 in each group) and were fed different diets during a 14-week period, which included a SC (standard chow; 10% lipids) or an HF (60% lipids) diet (details in Table 1). Fresh chow was provided daily, and any chow remaining from the previous day was discarded. The food intake was evaluated daily (at 13:00 h). The weekly (on Friday at 13:00 h) BM (body mass) was measured to calculate the daily medication intake; the drugs were mixed into the chow daily.

The drug treatments began on the seventh week of the experiment, and the drug-treated groups continued to have free access to the HF diet during the entire treatment period. The following treatments lasted 8 weeks: HF + ST [HF diet treated with ST (20 mg/kg of body weight; Crestor, AstraZeneca)]; HF + GZ [HF diet treated with GZ (5 mg/kg of body weight; Avandia, GlaxoSmithKline)]; and the combination HF + ST + GZ (HF diet treated with ST + GZ) at the same concentrations used in the monotherapies. Taking daily food consumption and BM into account, the drug doses were corrected to match the same concentrations as indicated.

The mineral and vitamin contents of the two diets were identical and were consistent with the American Institute of Nutrition’s recommendation (AIN 93M) [27]. The mouse chow was prepared by Pragsolucoes (Jau).
Table 1 Composition and energy content of the SC and HF diet

<table>
<thead>
<tr>
<th>Content</th>
<th>SC</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein ( (&gt;85% \text{ of protein}) ) (g/kg)</td>
<td>140.0</td>
<td>190.0</td>
</tr>
<tr>
<td>Cornstarch (g/kg)</td>
<td>620.7</td>
<td>250.7</td>
</tr>
<tr>
<td>Sucrose (g/kg)</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Soya-bean oil (g/kg)</td>
<td>40.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Lard (g/kg)</td>
<td>–</td>
<td>320.0</td>
</tr>
<tr>
<td>Fibre (g/kg)</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamin mix (g/kg)*</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Mineral mix (g/kg)*</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>L-Cysteine (g/kg)</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Choline (g/kg)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Antioxidant (g/kg)</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Total mass (g)</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Energy content (kcal/kg)</td>
<td>3573</td>
<td>5404</td>
</tr>
<tr>
<td>Carbohydrates (%)</td>
<td>76</td>
<td>26</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Lipids (%)</td>
<td>10</td>
<td>60</td>
</tr>
</tbody>
</table>

* Mineral and vitamin mixtures are in accordance with AIN 93M.

All procedures were performed in accordance with the conventional guidelines for experimentation with animals (National Institutes of Health Publication No. 85-23, revised in 1996), and all experimental protocols were approved by the Animal Ethics Committee of the State University of Rio de Janeiro.

**Glucose tolerance**

An OGTT (oral glucose tolerance test) was performed before treatments and after 8 weeks of treatment. The mice were fasted for 6 h, and the tests were performed at 07:00 h. Glucose (2.0 g/kg of body weight) was administered by orogastric gavage. Blood samples were drawn from the tail vein at 0, 15, 30, 60 and 120 min after glucose administration. Blood glucose levels were measured using an Accu-Chek Go glucometer (Roche Diagnostics).

**Insulin tolerance**

The IPITT (intraperitoneal insulin tolerance test) involved a 1 unit/kg of body weight intraperitoneal insulin injection (100 units/ml; Humalog Insulin Lispro, Lilly); the test was performed at 05:00 h, and the mice were fasted for 4 h. Blood glucose was measured in tail vein blood samples that were collected at 0, 15, 30, 60 and 120 min after insulin administration using an Accu-Chek Go glucometer (Roche Diagnostics).

**Killing and tissue extraction**

After 14 weeks on the diet, the animals were fasted overnight (food-deprived from 01:00 to 07:00 h) and then deeply anesthetized with sodium pentobarbital (150 mg/kg of body weight, intraperitoneal). Blood samples were rapidly obtained, and plasma was separated by centrifugation at 4 °C and stored at −20 °C until biochemical analyses were performed. Both the liver and fat deposits (inguinal, retroperitoneal and epididymal fat masses) were completely dissected, weighed and then rapidly fixed in freshly prepared fixative solution [4 % (w/v) formaldehyde and 0.1 M phosphate buffer, pH 7.2] for analysis by light microscopy.

**Metabolic parameters**

Fasting blood glucose was measured using a glucometer before and after treatment (Roche Diagnostics). Serum analysis of adiponectin was based on a commercially available mouse adiponectin ELISA kit (EZMADP-60K; Millipore). Leptin and resistin were analysed by a Milliplex Map (MDPK-71K; Millipore). The intra- and inter-assay coefficients of variation were 5.4 % and 1.4 % for adiponectin, and 4.5 % and 10.3 % for leptin and resistin. TC (total cholesterol), TAG and the enzymes ALT (alanine aminotransferase), AST (aspartate aminotransferase) and ALP (alkaline phosphatase) were measured using the kinetic-colorimetric method (Bioclin System II; Quibasa), according to the manufacturer’s instructions.

**RIA for insulin and HOMA-IR**

Serum insulin levels were determined using a commercially available insulin RIA kit (RI-13K; Linco Research). All the samples were analysed in a double assay format, for which the intra-assay coefficient of variation was 1.4 %. The HOMA-IR index was calculated as: (fasting serum glucose × fasting serum insulin/22.5) [28].

**Hepatic steatosis and TAG**

Random fragments of the liver were prepared for light microscopy. Small pieces of liver were embedded in Paraplast plus (Sigma–Aldrich), sectioned at 5 μm and then stained with haematoxylin and eosin. The liver sections were observed using light microscopy to investigate hepatocyte steatosis and the structure of the hepatic lobules. The images were acquired using Image-Pro Plus version 7.0 (Media Cybernetics) with a sample size of at least 75 fields per group (digital images, TIFF format, 36-bit colour, 1280 × 1024 pixels, LC Evolution camera and Olympus BX51 microscope). The volume density of steatosis was estimated by point counting as V_v[steatosis] = points hitting steatosis/total test-points, as described previously [29].
Several fragments of the liver of each animal were frozen at −80 °C for further biochemical analysis. The hepatic TAG levels were measured according to a protocol published previously [30]. Briefly, 50 mg of frozen liver tissue was placed in an ultrasonic processor with 1 ml of isopropanol. The homogenate was centrifuged at 2000 g and 5 μl of the supernatant was used with a kit for measuring TAG in a semi-automatic biochemical analyser (K55; Bioclin).

**Hepatic immunohistochemistry**

Antigen retrieval was performed using citrate buffer, pH 6.0, and endogenous peroxidase was quenched by 3% H₂O₂. Sections were then incubated with anti-SREBP-1c (68 kDa) (SC-367; Santa Cruz Biotechnology) antibodies for 2 h. Subsequently, the samples were treated with a biotinylated secondary antibody (K0679; Universal DakoCytomation LSAB + Kit, Peroxidase), which was detected by the reaction with an HRP (horseradish peroxidase)–streptavidin–biotin complex. Positive immunoreactions were identified following incubation with DAB (3,3′-diaminobenzidine tetrachloride) (K3466; DakoCytomation), and the sections were then counterstained with Mayer’s haematoxylin.

Digital images of the stained slices were obtained (LC Evolution camera, Olympus BX51 microscope, TIFF format, 36-bit colour, 1280 × 1024 pixels) and analysed with Image-Pro Plus version 7.0 software. The number of positive SREBP-1c-stained nuclei relative to the total number of hepatocyte nuclei was measured in ten fields per animal using a frame of known area with consideration for the edge effect, as described previously [11].

**Western blot analyses**

The total hepatic proteins were extracted in a homogenizing buffer with protease inhibitors. The liver protein content was detected according to the previously described method [31]. Then, the homogenates were centrifuged at 3200 g for 20 min at 4 °C, and the supernatants were collected. Equal quantities of total protein were resuspended in SDS-containing sample buffer, heated for 5 min at 100 °C and separated by SDS/PAGE. After electrophoresis, aliquots (15 μg) of the proteins were electroblotted on to PVDF transfer membranes (Hybond-P; GE Healthcare) and visualized with Ponceau solution staining. The membrane was then blocked by incubation in 6% (w/v) non-fat dried skimmed milk powder in TBS-T (Tris-buffered saline [20 mmol/l Tris/ HCl (pH 7.4) and 500 mmol/l NaCl]) and incubated with polyclonal antibodies against rabbit SREBP-1c (68 kDa) (SC-367; Santa Cruz Biotechnology), β-actin (57 kDa) (SC-130301; Santa Cruz Biotechnology), GLUT-2 (glucose transporter 2; 57 kDa) (AB1342; Chemicon), PPARγ (53–61 kDa) (SC-7273; Santa Cruz Biotechnology) and PPARα (53–61 kDa) (SC-9000; Santa Cruz Biotechnology). β-Actin served as a loading control for cytosolic proteins. Following the incubation with the primary antibody, the membranes were washed and incubated with an anti-(rabbit IgG) secondary antibody. Protein expression was detected using an ECL® advanced Western blotting detection kit and ECL® Hyperfilm (GE Healthcare). The signals were visualized by autoradiography and determined by quantitative analysis of digital images of gels using Image-Pro Plus version 7.0 (Media Cybernetics). The results are expressed as a percentage of the SC group.

**Statistical analysis**

The data were tested for normality and homoscedasticity of the variances. The differences between the groups were tested by ANOVA, followed by a post-hoc Tukey test. Otherwise, the differences were tested with a Kruskal–Wallis test and a post-hoc Dunn test. Insulin and glucose tolerance data were analysed with a one-way ANOVA performed on the AUC (area under the curve) values. In all cases, P < 0.05 was considered statistically significant (GraphPad Prism version 5.03 for Windows).

**RESULTS**

**Energy intake, BM and fat pads**

The energy intake of the animals fed the HF diet surpassed the energy intake of the SC group before drug treatment (+ 100%, P < 0.0001). Following treatment, the animals in the HF + ST-treated group had a reduced energy intake compared with the untreated HF diet group (P < 0.001). In the fourth week of the HF diet, the animals fed on an HF diet had higher BM than the SC-fed animals (pre-treatment, P = 0.02). BM gain was significantly reduced in the HF + ST-treated group. It is worth mentioning that ST treatment prevented the mice from becoming overweight (Table 2, post-treatment, P < 0.01). The HF + ST-treated animals gained only 6.4 ± 3.4 % total BM during the final 7 weeks of the study, whereas the HF + ST + GZ-treated animals gained 11.2 ± 4.1 % BM during this period (n = 8). The changes in BM for all groups are shown in Figure 1.

Similarly, the fat pads of HF-diet-fed mice had significantly greater mass compared with the SC-diet-fed mice. ST treatment prevented the mice from becoming overweight (Table 2, post-treatment, P < 0.01). The HF + ST-treated animals gained only 6.4 ± 3.4 % total BM during the final 7 weeks of the study, whereas the HF + ST + GZ-treated animals gained 11.2 ± 4.1 % BM during this period (n = 8). The changes in BM for all groups are shown in Figure 1.

Similarly, the fat pads of HF-diet-fed mice had significantly greater mass compared with the SC-diet-fed mice. ST treatment prevented the mice from becoming overweight (Table 2, post-treatment, P < 0.01). The HF + ST-treated animals gained only 6.4 ± 3.4 % total BM during the final 7 weeks of the study, whereas the HF + ST + GZ-treated animals gained 11.2 ± 4.1 % BM during this period (n = 8). The changes in BM for all groups are shown in Figure 1.

Similarly, the fat pads of HF-diet-fed mice had significantly greater mass compared with the SC-diet-fed mice. ST treatment prevented the mice from becoming overweight (Table 2, post-treatment, P < 0.01). The HF + ST-treated animals gained only 6.4 ± 3.4 % total BM during the final 7 weeks of the study, whereas the HF + ST + GZ-treated animals gained 11.2 ± 4.1 % BM during this period (n = 8). The changes in BM for all groups are shown in Figure 1.
Body composition, adipokines and liver metabolism

Values are means ± S.E.M., n = 5–8 per group. In the indicated cases, P < 0.05 when compared with the SC group (*), HF group (†), HF + ST group (‡) and HF + GZ group (§) (one-way ANOVA and post-hoc Tukey test) ViscF/SubF, visceral fat/subcutaneous fat.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>SC</th>
<th>HF</th>
<th>HF + ST</th>
<th>HF + GZ</th>
<th>HF + ST + GZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body mass (g)</td>
<td>20.69 ± 0.79</td>
<td>22.74 ± 0.90*</td>
<td>30.06 ± 0.36</td>
<td>32.40 ± 0.97*</td>
<td>30.95 ± 0.65†</td>
<td>31.00 ± 0.39†</td>
</tr>
<tr>
<td>Food (g/day per mouse)</td>
<td>2.53 ± 0.08</td>
<td>3.30 ± 0.14</td>
<td>3.23 ± 0.36</td>
<td>3.55 ± 0.29</td>
<td>3.71 ± 0.39</td>
<td>3.69 ± 0.32†</td>
</tr>
<tr>
<td>Energy (kJ/day per mouse)</td>
<td>42.82 ± 1.58</td>
<td>85.79 ± 2.03*</td>
<td>73.08 ± 1.31†</td>
<td>81.35 ± 1.69†</td>
<td>83.90 ± 1.70‡</td>
<td>84.35 ± 1.69‡</td>
</tr>
<tr>
<td>Adipocyte size (μm)</td>
<td>53.60 ± 0.40</td>
<td>80.50 ± 1.07*</td>
<td>59.10 ± 0.60†</td>
<td>67.30 ± 0.90‡</td>
<td>56.30 ± 0.50¶</td>
<td>55.20 ± 0.60¶</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymal</td>
<td>0.40 ± 0.03</td>
<td>1.50 ± 0.23*</td>
<td>0.60 ± 0.07†</td>
<td>1.00 ± 0.26</td>
<td>0.60 ± 0.08†</td>
<td>0.60 ± 0.08‡</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>0.12 ± 0.01</td>
<td>0.54 ± 0.13‡</td>
<td>0.16 ± 0.02‡</td>
<td>0.34 ± 0.06</td>
<td>0.17 ± 0.03‡</td>
<td>0.17 ± 0.03‡</td>
</tr>
<tr>
<td>Inguinal</td>
<td>0.10 ± 0.01</td>
<td>0.32 ± 0.07*</td>
<td>0.13 ± 0.01‡</td>
<td>0.28 ± 0.05†</td>
<td>0.17 ± 0.03‡</td>
<td>0.17 ± 0.03‡</td>
</tr>
<tr>
<td>ViscF/SubF ratio</td>
<td>4.10 ± 0.15</td>
<td>5.30 ± 0.32*</td>
<td>4.60 ± 0.26</td>
<td>3.7 ± 0.19‡</td>
<td>3.6 ± 0.18‡</td>
<td>3.6 ± 0.18‡</td>
</tr>
<tr>
<td>Adipose tissue cytokines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistin (pg/ml)</td>
<td>3615.4 ± 60.6</td>
<td>4520.0 ± 442.5</td>
<td>2527.3 ± 470.3</td>
<td>4791.1 ± 164.6</td>
<td>2741.3 ± 459.0§</td>
<td>2741.3 ± 459.0§</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>14.10 ± 1.00</td>
<td>7.4 ± 0.40*</td>
<td>8.40 ± 0.60*</td>
<td>14.70 ± 1.60‡</td>
<td>13.50 ± 0.90†</td>
<td>13.50 ± 0.90†</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>1284.1 ± 159.3</td>
<td>142520.0 ± 33670.0</td>
<td>2054.2 ± 836.6</td>
<td>6531.1 ± 2484.0</td>
<td>3112.2 ± 1186.0†</td>
<td>3112.2 ± 1186.0†</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass (g)</td>
<td>1.10 ± 0.10</td>
<td>1.50 ± 0.04*</td>
<td>1.40 ± 0.04‡</td>
<td>1.40 ± 0.03*</td>
<td>1.30 ± 0.04†</td>
<td>1.30 ± 0.04†</td>
</tr>
<tr>
<td>Steatosis (%)</td>
<td>5.30 ± 1.20</td>
<td>16.10 ± 0.90</td>
<td>12.40 ± 0.50*</td>
<td>15.30 ± 0.60</td>
<td>10.00 ± 0.80‡</td>
<td>10.00 ± 0.80‡</td>
</tr>
<tr>
<td>TAGs (mg/ml per mg)</td>
<td>1800 ± 17.2</td>
<td>400.8 ± 62.2*</td>
<td>170.4 ± 23.6†</td>
<td>325.3 ± 31.7‡</td>
<td>142.5 ± 16.5§</td>
<td>142.5 ± 16.5§</td>
</tr>
<tr>
<td>SREBP-1c (nuclei/μm²)</td>
<td>8.20 ± 0.60</td>
<td>14.00 ± 0.50*</td>
<td>9.30 ± 0.40‡</td>
<td>12.70 ± 0.80†</td>
<td>9.40 ± 0.30§</td>
<td>9.40 ± 0.30§</td>
</tr>
</tbody>
</table>

Figure 1  BM evolution

Values are means ± S.E.M. Pre-treatment corresponds to the period until the 7th week (IR induction) when treatment began and extended until the 14th week. The differences were found by comparing the SC and HF groups (*P < 0.05), as determined using a one-way ANOVA and a post-hoc Tukey test.

Adipokines

The levels of resistin expression were 79% higher in the HF diet group as compared with the SC diet control group. In the HF + ST-treated group, the levels of resistin were lower compared with the HF diet group (44% less, P < 0.01). The levels of resistin were consistent with the results of the HOMA-IR index and IR (Table 2). Of all the experimental groups, the HF diet group showed the highest levels of leptin, but the levels were normalized after ST treatment. The leptin levels were 85% and 78% lower in the HF + ST + treated and the HF + ST + GZ-treated groups respectively compared with the HF diet group (P < 0.01). Interestingly, the levels of adiponectin were the lowest in the HF diet group and increased after GZ treatment. In the HF + ST-treated group, the levels of adiponectin were higher than those of the HF diet group (+13%), but not statistically significant. No statistically significant differences were found for the adiponectin levels of the HF + GZ, HF + ST + GZ and SC groups (Table 2).

Blood biochemistry

The plasma levels of TAG and TC were higher in the HF-diet-fed mice compared with the SC-diet-fed mice (P = 0.001). The plasma ALT (P < 0.001), AST
Post-treatment normalized the insulin levels and HOMA-IR (Table 3). HOMA-IR index ($\%$ of 80 %, $n$ insulin levels (from 16.96 to 2.80 units/l, a reduction in IR. ST treatment markedly reduced the fasting plasma glucose remained higher in the HF-diet-fed mice ($P < 0.0001$) and ALP ($P < 0.0001$) concentrations were also significantly higher in the HF-diet groups as compared with the SC-diet-fed group. In HF-diet-fed mice, treatment with ST significantly reduced the total plasma cholesterol levels by 16 % in the HF + ST-treated mice ($P < 0.05$) and by 41 % in the HF + ST + GZ-treated mice ($P < 0.0001$). However, TC increased in the HF + GZ mice as compared with the SC-diet-fed mice ($+ 27 \%, P < 0.001$). The TAG levels were normalized by ST treatment, and ST alone decreased the TAG levels by 22 % ($P < 0.05$) (Table 3).

**Insulin and HOMA-IR**
The mice fed on the HF diet showed higher fasting plasma glucose ($P = 0.0001$) and insulin ($P = 0.0001$) levels compared with SC-diet-fed mice. Therefore the HF-diet-fed mice showed higher HOMA-IR ($P < 0.0001$), and all treatments reduced the plasma insulin levels and HOMA-IR. ST treatment markedly reduced the fasting plasma insulin levels (from 16.96 to 2.80 units/l, a reduction of 80 %, $n = 5$, $P = 0.002$), resulting in a 70 % lower HOMA-IR index ($P = 0.0008$). All of the drug treatments normalized the insulin levels and HOMA-IR (Table 3).

**OGTT and IPITT**
The plasma glucose levels increased to a maximum level in all groups at 15 min after oral administration of glucose, but the peak levels were higher in HF-diet-fed mice compared with the SC-diet-fed mice ($P < 0.0001$). The IPITT demonstrated a rapid decline in plasma glucose 15 min after insulin administration in both groups, but plasma glucose remained higher in the HF-diet-fed mice compared with the SC-diet-fed mice ($P < 0.0001$) at all time points up to 120 min; this classifies the HF-diet-fed mice as insulin resistant. Among the single agent therapies, the HF + ST-treated group demonstrated reduced glucose intolerance and IR (−12 % for AUC in OGTT, $P = 0.0004$ and −24 % for AUC in IPITT, $P = 0.0006$) (Figure 2).

**Liver**
The liver masses were larger (+38 %, $P < 0.05$) and steatosis was more common in the untreated HF diet group, which exhibited severe macro- and microvesicular steatosis within hepatocytes when compared with SC. The HF diet group also showed the greatest increase in hepatic TAG levels, which were 127 % higher than in the SC group ($P < 0.01$). In comparison with the HF-diet-fed mice, the HF + ST-treated mice showed significant less hepatomegaly and liver steatosis (−6 %, $P < 0.05$; and −21 %, $P < 0.01$ respectively). In the HF + ST-treated group, the hepatic TAG levels were 58 % lower than the HF group ($P < 0.01$) (Table 2). Figure 3 shows the photomicrographs of the hepatic tissue. In the HF + ST-treated group, liver steatosis returned to the levels observed in the SC diet group. Figure 4 shows photomicrographs of the liver. In the untreated HF diet group, we observed higher levels of nuclear SREBP-1c-positive hepatocytes compared with the SC diet group (+72 %, $P = 0.001$; Table 2). The HF + ST-treated group showed lower SREBP-1c immunostaining in comparison with the HF-diet-fed mice (−33 %, $P < 0.0001$). The HF + GZ-treated group did not have a significant reduction in liver steatosis or hepatic SREBP-1c immunostaining (Table 2 and Figure 4).

**Western blot analysis**
The expression levels of SREBP-1c in the livers of HF-diet-fed mice were 50 % higher than in the livers of SC-diet-fed mice ($P < 0.05$) and lower in the HF + ST-treated mice compared with the HF-diet-fed mice.

### Table 3  Biochemistry, lipid profile and carbohydrate metabolism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC</td>
</tr>
<tr>
<td>Pre-treatment</td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>136.7 ± 7.3</td>
</tr>
<tr>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>127.8 ± 10.0</td>
</tr>
<tr>
<td>Insulin (m-units/ml)</td>
<td>6.0 ± 1.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>113.0 ± 3.8</td>
</tr>
<tr>
<td>TAG (mg/dl)</td>
<td>28.0 ± 0.5</td>
</tr>
<tr>
<td>ALT (units/l)</td>
<td>30.2 ± 1.0</td>
</tr>
<tr>
<td>AST (units/l)</td>
<td>66.6 ± 2.8</td>
</tr>
<tr>
<td>ALP (units/l)</td>
<td>39.0 ± 2.6</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M., $n = 5$ per group. $P < 0.05$ when compared with the SC group (*), HF group (†) and HF + GZ group (§) (one-way ANOVA and post-hoc Tukey test).
Statin, insulin sensitivity, obesity and NAFLD

Figure 2  OGTT and IPITT curves before and after treatments
The AUC shows significant differences among the groups. In the indicated cases, \( P < 0.05 \) when compared with the SC group (†), HF group (‡), HF + ST group (§) and HF + GZ group (¶) (one-way ANOVA and post-hoc Tukey test). Values are means ± S.E.M., \( n = 5 \) per group.

Figure 3  Photomicrographs of the liver structure
Sections were stained with haematoxylin and eosin, and each photomicrograph is shown at the same magnification. (a) The usual liver appearance in the SC diet group; (b) macro- and micro-vesicular steatosis in the HF diet group; (c) steatosis was reduced in the HF + ST group; (d) alterations (macro- and micro-vesicular steatosis) in the HF + GZ group were similar to the untreated HF diet group (b); and (e) steatosis was almost completely cleared in the HF + ST + GZ group.

DISCUSSION

In the present study, we have demonstrated that ST treatment improved insulin sensitivity, adiposity and steatosis induced by an HF diet. Furthermore, ST reduced gains in BM, improved the circulating levels of plasma TC and TAG and reduced hepatic TAG, concomitant with lower resistin. This is the first report demonstrating that ST ameliorates hepatic steatosis and improves glucose intolerance in a murine model of IR.

In the present study, the HF diet mouse model showed hypoadiponectinaemia and significantly higher
Figure 4  Photomicrographs of the liver structure immuno-stained for SREBP-1c
The marked nuclei in the test-area (frame) where the continuous line represents the ‘forbidden line’ were counted, i.e., all nuclei hitting this line were not counted: (A) SC group; (B) HF group; (C) HF + ST group; (D) HF + GZ group; and (E) HF + ST + GZ group. The same magnification is used in all of the photomicrographs.

Figure 5  Liver expression of SREBP-1c
Upper panels, representative Western blots with bands corresponding to the groups. Lower panel, quantification of the protein expression standardized to β-actin expression and expressed as a percentage of the SC group. Values are means ± S.E.M. P < 0.05 when compared with the SC group (*), HF group (†), HF + ST group (‡) and HF + GZ group (§) (one-way ANOVA and a post-hoc Tukey test).

Figure 6  Liver expression of PPARα
Upper panels, representative Western blots with bands corresponding to the groups. Lower panel, quantification of the protein expression standardized to β-actin expression and expressed as a percentage of the SC group. Values are means ± S.E.M. P < 0.05 when compared with the SC group (*), HF group (†), HF + ST group (‡) and HF + GZ group (§) (one-way ANOVA and a post-hoc Tukey test).

Figure 7  Ratio between SREBP-1c expression and PPARα expression in the liver
Results were standardized to β-actin expression and expressed as a percentage of the SC diet group. P < 0.05 when compared with the SC group (*), HF group (†), HF + ST group (‡) and HF + GZ group (§) (one-way ANOVA and a post-hoc Tukey test).

HOMA-IR, hypertriglyceridaemia and hyperinsulinemia, which delineates a phenotype of peripheral IR. The HF-diet-fed mice also showed adipocyte hypertrophy with a concomitant increase in serum resistin levels. It is widely accepted that overnutrition, mainly through consumption of a diet rich in saturated fatty acid,
may cause liver damage by TAG accumulation and elevated plasma levels of liver enzymes [32]. An increased incidence of NAFLD has been observed worldwide, and it has become one of the most common forms of liver disease. Furthermore, NAFLD is considered the hepatic manifestation of the metabolic syndrome [33], with IR as the primary characteristic of the association between obesity and NAFLD.

Our study showed that ST improves insulin sensitivity in HF-diet-fed mice and is in accordance with other studies in animals [15,34] and humans [35]. More specifically, statin treatment is associated with improved insulin action, increased insulin-stimulated IRS (insulin receptor substrate)/PI3K (phosphoinositide 3-kinase)/PKB (protein kinase B) signalling in the liver and muscles of HF-diet-fed rats and decreased inflammation related to IR [34]. In the present study, these findings were associated with HOMA-IR and the AUC in IPITT reduction. The effects of ST treatment on insulin sensitivity could be considered more relevant because this was followed by a reduction in weight gain. Furthermore, we also observed decreased abdominal fat deposits in the HF + ST-treated mice as compared with the HF diet or GZ single agent-treated mice. Moreover, ST decreased the circulating resistin levels, which was concordant with reduced adiposity. These observations are in accordance with studies that have shown increased serum resistin levels and resistin gene expression in abdominal deposits with increased adiposity [36].

The HF diet group in the present study also revealed high levels of plasma leptin. In contrast, ST single-agent treatment reduced leptin levels, which alleviated adipocyte hypertrophy and yielded a visceral fat/subcutaneous fat ratio similar to the SC diet group. Leptin is largely produced by adipose tissue. In mice, leptin expression is higher in visceral fat, whereas, in humans, leptin is more highly expressed in subcutaneous adipose tissue [37]. The increased hepatic TAG accumulation and high TAG concentrations in plasma of the HF diet group observed in the present study could be partly attributable to diminished leptin entry into cells in addition to insulin and leptin resistance [38]. Leptin is a sensor of fat mass and a regulator of energy homoeostasis [39]. Leptin acts on the brain and on peripheral tissues such as the pancreas and liver and may prevent lipid accumulation in non-adipose tissues [40] through the modulation of hepatic β-oxidation [41]. Leptin also regulates the expression of SREBP-1 [42]. This beneficial effect of leptin depends on enhanced insulin sensitivity and the actions of leptin itself. Conversely, in obesity, resistance to leptin may promote ectopic lipid storage, which may further impair insulin sensitivity [43].

Another important finding of the present study is that ST effectively treated hepatic steatosis. ST treatment decreased SREBP-1c expression and attenuated hepatic steatosis compared with the HF diet and GZ treatment. Moreover, in the livers of the HF diet group, the levels of the pro-lipogenic transcription factor SREBP-1c were 50% higher compared with the SC-diet-fed mice,
indicating the induction of lipogenesis and ectopic lipid storage. Several mechanisms may account for ectopic lipid storage in the liver. In addition to leptin, expression of SREBP-1c is dependent on nutritional status.

It is widely described that SREBP-1c controls endogenous fatty acid synthesis. The accumulation of lipids has been linked to functional disturbances in various tissues and organs, often referred to as lipotoxicity. SREBP-1c controls endogenous fatty acid synthesis and therefore the expression of SREBP-1c is highly up-regulated by dietary intake of carbohydrates and sugars [44,45]. It is conceivable that a positive energy imbalance, which happened in our model, chronically activates SREBP-1c, causing lipotoxicity in various tissues and organs. It has been reported that SREBP-1c is involved in NAFLD, as shown in the HF group, and pancreatic β-cell dysfunction [46,47].

Some therapies, such as the use of fish oil, rich in PUFAs (polynsaturated fatty acids), against CVD (cardiovascular disease) has been well established. However, the molecular mechanisms by which PUFA inhibit SREBP-1c are complex and still under investigation. In pancreatic β-cells, palmitate impairs insulin secretion, and studies conducted on SREBP-1c-deficient islets have found that these effects are mediated through the regulation of SREBP-1c. Concerning the liver, findings have suggested that hepatic SREBP-1c is also induced by dietary saturated fatty acids [48,49]. Furthermore, SREBP-1c directly represses IRS-2 expression and leads to hepatic IR as a part of the underlying pathogenesis of the metabolic syndrome [50]. The saturated fatty acids found in HF diets increase hepatic expression of SREBP-1, possibly because of the capacity of the saturated fatty acids to promote inflammation. Additionally, HF diets induce lipogenesis and increase SREBP-1 activity, concomitant with an increase in the expression of downstream targets of SREBP [51]. The excess fat promotes a rapid stimulation of de novo lipogenesis and ectopic lipid accumulation in the liver [42,52].

In our study, we found that treatment of hepatic steatosis prevented a decline in glucose metabolism. Hepatic GLUT2 expression was significantly lower in the HF + ST-treated group, suggesting normalization of post-receptor insulin signalling and restoration of not only hepatic but also whole-body insulin sensitivity. In NAFLD, it is expected that the increased expression of GLUT2 in liver occur, as clearly demonstrated in liver steatosis induced in human HepG2 cells incubated in vitro at high concentration of oleic acid [53]. In hyperglycaemia, the expression of GLUT2 increases in the liver due to the activation of the transcriptional factor SREBP [54]. The untreated HF group had higher GLUT2 expression, which is consistent with NAFLD.

PPARγ is a member of the PPAR subfamily of nuclear hormone receptors, whose activation promotes adipogenesis, improves insulin sensitivity, induces the development of fatty liver as a downstream effect of SREBP-1c activation and up-regulates lipogenic enzymes. The up-regulation of lipogenesis observed in the HF + GZ-treated mice may be due to the action of GZ as a PPARγ agonist [18]. ST lowered SREBP-1c expression, increased PPARα expression and altered the pattern of ectopic lipid and adipose tissue storage. Although GZ increased PPARα expression and adiponectin, only ST treatment resulted in a visceral fat/subcutaneous fat ratio and SREBP-1c/PPARα protein ratio similar to that of the SC diet group. These findings are consistent with studies in obese patients, which have demonstrated high SREBP-1c/PPARα ratios and IR, a condition that may favour lipogenesis over fatty acid oxidation, thereby leading to steatosis [55]. PPARα is a transcription factor that up-regulates the oxidative enzymes CPT (carnitine palmitoyltransferase)-1 and acyl CoA oxidase [56]. Down-regulation of liver PPARα expression without significantly altering SREBP-1 expression has been observed in NAFLD [57,58]. In addition, PPARα expression in the liver positively correlates with plasma adiponectin levels in obese insulin-resistant NAFLD patients, whereas PPARα expression negatively associates with HOMA. Therefore hypoadiponectinaemia in obese individuals may impair liver PPARα function [55].

ST is well recognized for its benefits in the treatment of dyslipidaemia. Our results are in agreement with other studies. Our findings also showed a clear reduction in plasma TC and TAG levels in the HF + ST-treated group as compared with the HF diet group. These results were associated with decreased TAG synthesis and the production of VLDL (very-low-density lipoprotein) particles by the liver. Simultaneously, biliary bile acid, cholesterol and phospholipid excretion from the liver were increased in ST-treated mice, suggesting that ST induces a redirection of lipid transport in the liver [59].

In conclusion, the overfed mouse model displays the characteristics of human NAFLD within the appropriate metabolic setting of CVD risk factors, i.e. obesity, IR and hypertriglyceridaemia, through overcaloric intake of dietary fat. The present study demonstrated a significant decrease in adiponectin and SREBP-1c expression in the livers of mice with NAFLD, suggesting the importance of the functional pathway of this adipokine. ST was effective in improving IR and reversing hepatic steatosis with decreased adiposity. Furthermore, ST reduced gains in BM, improved the circulating levels of plasma TC and TAG and reduced hepatic TAG, in addition to lower resistin levels.

**AUTHOR CONTRIBUTION**

Julio Cesar Fraulob carried out the immunohistochemistry and RIAs, performed the statistical analysis and
drafted the paper. Vanessa Souza-Mello made substantial contributions to the study and was involved in revising the paper. Marcia Barbosa Aguil made substantial contributions to the research conception and design and was involved in revising the paper. Carlos Alberto Mandarim-de-Lacerda was critically involved in writing, revising, drafting the paper and has given final approval of the version of the paper to be published. All authors read and approved the final manuscript.

ACKNOWLEDGEMENT

We are grateful to Mrs Thatiany Marinho for her technical assistance.

FUNDING

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 300396/2008-2 (to C.A.M.-d.-L.)] and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) [grant number E-26/102.828/2008 (to C.A.M.-d.-L.)].

REFERENCES

3 Eguchi, Y., Mizuta, T., Sumida, Y., Ishibashi, E., Kitajima, Y., Isoda, H., Horie, H., Tashiro, T., Iwamoto, E., Takahashi, H. et al. (2011) The pathological role of visceral fat accumulation in steatosis, inflammation, and progression of nonalcoholic fatty liver disease. J. Gastroenterol. 46 (Suppl. 1), 70–78


45 hypothetical