Singular effects of PPAR agonists on nonalcoholic fatty liver disease of diet-induced obese mice

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Aims: To assess the effects of peroxisome proliferator-activated receptor (PPAR) agonists on glucose tolerance and hepatic lipid metabolism in diet-induced obese mice.

Main methods: Male C57BL/6 mice received a standard chow diet (SC, 10% energy as lipids) or high-fat diet (HF, 50% energy as lipids) for 10 weeks, after which treatment was initiated, forming the groups: SC group, HF group, HF-BZ group (HF + bezafibrate, pan-PPAR agonist), HF-WY group (HF + WY-14643, PPARalpha agonist) and HF-GW group (HF + GW1929, PPARgamma agonist). Treatments lasted for four weeks. Insulin resistance and liver remodeling were evaluated by biochemical and molecular approaches.

Key findings: The HF and HF-GW mice were overweight. Conversely, the HF-BZ and HF-WY mice presented with body masses equal to those of the SC mice. All treatments restored insulin sensitivity and blood lipid and adiponectin levels. Hepatic steatosis was prevented in the HF-WY and HF-BZ mice as shown by the elevated mRNA levels of PPARalpha and Carnitine palmitoyl transferase-1a in both groups, which favored enhanced beta-oxidation. Marked decreases in liver triacylglycerol levels confirmed these findings. In contrast, the HF-GW mice exhibited increased PPARgamma and fatty acid translocase/CD136 mRNA levels, contributing to enhanced hepatic lipogenesis.

Significance: The WY14643 and bezafibrate treatments most effectively improved the adverse metabolic and hepatic effects caused by obesity and IR. The results reinforce the central role of PPARalpha, as well as its contrary relationship to PPARgamma in the regulation of metabolic homeostasis and lipolytic pathways in the liver.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) includes multiple conditions due to fat deposition within hepatocytes. The hepatic steatosis rate reveals the imbalance between input (lipolysis in white adipose tissue, de novo lipogenesis and lipogenesis), and output (lipoprotein synthesis and secretion) of free fatty acids in hepatic tissue [1]. Peroxisome proliferator-activated receptors (PPARs) are associated with nutrient metabolism and cellular proliferation [2]. PPARalpha activation is linked with nutrient metabolism and cellular proliferation [2]. PPARalpha activation is linked with enhanced lipolysis and glucose metabolism by inducing the transcription of several genes that participate in this process [3]. PPARbeta/delta activation in hepatic tissue participates in Kupffer cell regulation, acting as an anti-inflammatory molecule by decreasing the expression of genes encoding proteins involved in inflammatory pathways [4]. PPARalpha is somewhat expressed in hepatic tissue and is related to glucose and lipid metabolism along with PPARalpha and PPARbeta/delta [5]. The rise in obesity rates worldwide encourages a proposition of new approaches to reinforce the existing treatments [6]. Therefore, the work aims to investigate and compare the effects of treatment with WY14643, which is a selective PPARalpha agonist, GW1929, which is a selective PPARgamma agonist and bezafibrate, which is a pan-PPAR agonist, on glucose tolerance and hepatic lipid metabolism at the gene level in diet-induced obese mice.

Materials and methods

Animals and diet

The Animal Ethics Committee of the State University of Rio de Janeiro approved the present protocol (Number CEUA/012/2011), and the processes were run out according to the guidelines for animal experimentation (NIH Publications No. 85-23, revised in 1996).
Fifty 3 month old male C57BL/6 mice were put up at controlled temperatures (21 ± 1°C), humidity (60 ± 10%) and a 12 h light/dark cycle (1:00 AM to 1:00 PM light) with food and water ad libitum. Mice received standard chow (SC, 10% energy from lipids, 15 kJ/g of diet, n = 10) or HfTy diet (HF, 50% of energy as lipids, 21 kJ/g of diet, n = 40). Diets are shown in detail in Table 1. After ten weeks, the animals fed the high-fat (HF) diet were then randomly assigned to one of the following groups (treatment lasted four weeks and the drugs were combined into the diets as follows):

a) Standard chow (SC group; 10% energy from fat, n = 10);

b) High-fat diet (HF group; 50% energy from fat, n = 10);

c) High-fat diet + bezafibrate (HF-BZ group; pan-PPAR agonist, 100 mg/kg, n = 10);

d) High-fat diet + WY14643 (HF-WY group; PPARalpha agonist, 3 mg/kg, n = 10);

e) High-fat diet + GW1929 (HF-GW group; PPARgamma agonist, 5 mg/kg, n = 10).

The diets followed the American Institute of Nutrition recommendations (AIN-93M) [7] and were manufactured by PragSolucoes (Jau, SP, Brazil). Body mass (BM) was evaluated once a week, and food consumption and percent of body weight change were determined by multiplying the amount of food consumption by the energy content of the diet.

**Euthanasia**

After 14 weeks of testing, the mice were deeply anesthetized (i.p. sodium pentobarbital, 150 mg/kg) and killed by exsanguination. The serum was obtained via centrifugation (1200 g for 15 min) at room temperature and kept at −20°C until analysis. The liver was immediately removed, weighed, fixed and prepared as described [8]. Additional parts of the liver were quickly frozen for molecular evaluations. The left tibia was dissected and measured to correct for liver mass [9].

**Blood glucose test analysis**

Oral glucose tolerance tests (OGTTs) were taken at 13 weeks after induction of the dietary treatments. OGTTs were performed using a glucose overload of 1.0 g/kg, given after a six-hour fasting period through orogastric gavage. Blood glucose concentrations were measured before glucose administration (0 min) and at 15, 30, 60 and 120 min after administration. Blood samples taken from the tail vein were analyzed.

**Plasma analysis**

Total cholesterol (TC), triacylglycerols (TG), alanine aminotransferase (ALT) and gamma-glutamyl transferase (gGT) were measured using kinetic-colorimetric methods based on the manufacturer’s instructions (Bioclin System II, Quibasa, Belo Horizonte, MG, Brazil). The serum concentrations of insulin, leptin and proinflammatory adipokines (plasminogen activator inhibitor-1 (PAI-1), and resistin) were measured using Multiplex Biomarker Immunoassays with Luminex xMAP technology (Millipore, Billerica, MA, USA, Cat. No. MMHMAG-44K-08), whereas serum concentrations of adiponectin were evaluated with MADPK-71K-01. A Luminex 200 analyzer with the Xponent/Analyst software version 4.2 was used for result interpretation.

**Liver**

Liver TG was measured as described [10]. Briefly, frozen liver samples (around 50 mg) plus 1 ml of isopropanol were placed in an ultrasonic processor and homogenate was centrifuged at 2000 g and 5 ml of the supernatant was analyzed with an automatic analyzer (K55, Bioclin System II; Quibasa) using a kit for measuring TG.

Formalin-fixed liver specimens were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA), and 5-μm-thick sections were stained with hematoxylin and eosin and Sirius Red. The digital images were acquired blindly and randomly (Leica DMRE microscope, Wetzlar, Germany, and Lumenera Infinity 1-5c camera, Ottawa, Canada). The stereological estimation of liver steatosis was made by point-counting as mentioned previously [10,11].

**Immunofluorescence**

The liver sections (5 μm thick) were deparaffinized, and antigen retrieval was performed using citrate buffer, pH 6.0. Then, sections were blocked with glycine at 2% and incubated with anti-smooth muscle actin alpha antibody diluted in PBS/BSA 1% for 2 h. Afterward, samples were incubated with a secondary antibody conjugated with Alexa. DAPI was used to stain the nuclei. Coverslips were mounted with slow-fade (Invitrogen, Waltham, Massachusetts, USA) to keep fluorescence. Negative controls were obtained after the omission of the primary antibody. The confocal laser microscope C2 (Nikon Inc., Tokyo) was used to evaluate the results.

**RT-qPCR**

RT-qPCR was executed as described elsewhere [12]. The qPCR primers were designed using the Primer3 online software, and endogenous control TATA-binding protein (TBP) was used to normalize the expression levels of the selected genes. The following primers were used: CPT-1 (FW AGGAATGCTCAAGTCCACATC; RV CAAGCTACATGGGACA TT), (fatty acid translocase)/CD36 (FW TACCTCCAGAATCCGACAAAC; RV GATATCCGACATGGTGTGG), PPARalpha (FW TGAGGAAAGGCGTACATACCT; RV TCTCCCAAAGCTCCCTTCAA), PPARgamma2 (FW ACGATCTGC TT), (fatty acid translocase)/CD36 (FW TACCTCCAGAATCCGACAAAC; RV GATATCCGACATGGTGTGG), SREBP-1c (FW GGTTTAGGCCTGCAGATGGAAT; RV TGGAGCTCGAACCATGAAA), TBP (FW TGAGGAAAGGCGTACATACCT; RV TCTCCCAAAGCTCCCTTCAA), PPARalpha (FW AGGAATGCTCAAGTCCACATC; RV CAAGCTACATGGGACA TT), (fatty acid translocase)/CD36 (FW TACCTCCAGAATCCGACAAAC; RV GATATCCGACATGGTGTGG), SREBP-1c (FW TGAGGAAAGGCGTACATACCT; RV TCTCCCAAAGCTCCCTTCAA), PPARgamma2 (FW ACGATCTGC TT), (fatty acid translocase)/CD36 (FW TACCTCCAGAATCCGACAAAC; RV GATATCCGACATGGTGTGG), TBP (FW TGAGGAAAGGCGTACATACCT; RV TCTCCCAAAGCTCCCTTCAA).

Negative controls were held after the blockade of cDNA in the racks. RQ (relative expression ratio) of mRNA was calculated by 2^−ΔΔCT equation, where ΔΔCT expresses the difference between the number of cycles (CT) of the target gene and the endogenous control. The expression of TBP was quantified for all samples via RT-qPCR and used for normalization.
When the HF-GW group did not show decreased BM after the treatment, and significantly different from that of the HF group (one-way ANOVA and Holm–Sidak post-hoc test).

### Data analysis

Data are expressed as the mean ± standard deviation. All data were examined for normality and homoscedasticity of variance, and then a 1-way analysis of variation (ANOVA) was established, followed by the Holm–Sidak post-hoc test. Statistical significance was considered when P-value ≤ 0.05 (GraphPad Prism v. 6.05 for Windows, GraphPad Software, La Jolla, CA, USA).

### Results

#### Body mass, energy intake and glucose tolerance (Table 2 and Fig. 1)

The supply of the HF diet for 10 weeks caused a progressive increase in BM for the HF group significantly higher than that in the SC group (+30%; P < 0.0001), which could be explained easily by the higher energy intake of the HF group (+50%; P < 0.05). Drug treatment for four weeks caused significant BM reductions in the HF-BZ and HF-WY groups in comparison with the HF group (−35% and −30%, respectively; P < 0.0001), although the energy intake levels of these two groups were similar to those of the untreated HF group. Conversely, the HF-GW group did not show decreased BM after the treatment, and when finalizing the experiment, the BM of this group was not significantly different from that of the HF group (P = 0.34).

The HF group was glucose intolerant as demonstrated by the increased AUC for the OGTT (+40%; P < 0.0001). In contrast, along with reductions in BMs, the HF-BZ, and HF-WY groups showed improved glucose tolerance because these groups were faced with a lower OGTT curve (−25%, P = 0.004) than that of the HF group. In addition, the HF-GW group showed improved glucose tolerance (−40%, P < 0.0001). The HF group showed hyperinsulinemia (+90%; P < 0.001), which was not seen in the SC group, while all treated groups showed reduced plasma insulin levels in contrast to the HF group (approximately −95% in the HF-BZ and HF-GW and −50% in the HF-WY groups; P < 0.001).

#### Adipokines and lipid profiles (Table 2)

Both groups HF-BA and HF-WY had small BM and showed improved levels of leptin, resistin and adiponectin, which were comparable to the SC group. Moreover, the HF-GW group exhibited improved adipokine levels compared to the HF group, but higher values than the SC group. None of the three treatments normalized PAI-1 levels, which remained comparable to those of the HF group.

Lipid profiles were assessed to confirm the beneficial effects of GW1929 independent of BM. The HF group showed increased TC (1.4-fold; P < 0.01) and TG (2-fold; P < 0.0001) than the SC group. In addition, the treated groups presented reduced TG (P = 0.02), but the treatments did not affect TC levels.

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**Table 2**

Energy intake, liver mass, and blood and liver biochemistry data in mice of the groups studied. The groups are: standard chow, SC; high-fat diet, HF; bezafibrate, pan-PPAR agonist BZ; GW1929, PPARgamma agonist GW and WY14643, PPARalpha agonist WY. Values are mean ± SD. For superscripted values, P < 0.05 when: [a] compared to SC group, [b] compared to HF group, [c] compared to HF-BZ group and [d] compared to HF-WY group (one-way ANOVA and Holm–Sidak post-hoc test).

<table>
<thead>
<tr>
<th>Data</th>
<th>SC (n = 10)</th>
<th>HF (n = 10)</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake (kJ)</td>
<td></td>
<td></td>
<td>HF-BZ (n = 10)</td>
</tr>
<tr>
<td></td>
<td>43.05 ± 7.23</td>
<td>65.14 ± 9.02[a]</td>
<td>64.62 ± 10.85[a]</td>
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<tr>
<td>Liver mass (g)</td>
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<td>1.41 ± 0.21[a]</td>
<td>2.40 ± 0.11[b]</td>
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<td>Total cholesterol (mg/dL)</td>
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<td>158.70 ± 8.62[a]</td>
<td>165.50 ± 26.64[a]</td>
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<tr>
<td>Plasma triacylglycerol (mg/dL)</td>
<td>34.80 ± 12.13</td>
<td>69.50 ± 15.31[a]</td>
<td>55.40 ± 4.26[a]</td>
</tr>
<tr>
<td>Liver triacylglycerol (mg/dL)</td>
<td>1.63 ± 0.36</td>
<td>2.89 ± 1.59[a]</td>
<td>2.11 ± 0.24[b]</td>
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<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>8.40 ± 0.90</td>
<td>11.2 ± 0.84[a]</td>
<td>9.3 ± 0.84[b]</td>
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<tr>
<td>γ-Glutamyl transferase (IU/L)</td>
<td>8.6 ± 0.5</td>
<td>10.5 ± 1.1[a]</td>
<td>8.4 ± 0.9[b]</td>
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<tr>
<td>Leptin (pg/ml)</td>
<td>64.5 ± 12.28</td>
<td>7851.5 ± 445.00[a]</td>
<td>986.4 ± 193.00[a,b]</td>
</tr>
<tr>
<td>Adiponectin (pg/ml)</td>
<td>131.0 ± 17.84</td>
<td>78.0 ± 12.34[a]</td>
<td>141.0 ± 32.85[a,b]</td>
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<td>Resistin (pg/ml)</td>
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<td>1351.7 ± 250.40[a]</td>
<td>755.6 ± 109.70[b]</td>
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<td>PAI-1 (pg/ml)</td>
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<td>2767 ± 425.70[a]</td>
<td>2368 ± 440.30[b]</td>
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<tr>
<td>Insulin (UI/I)</td>
<td>21.46 ± 5.67</td>
<td>40.58 ± 6.11[a]</td>
<td>20.73 ± 6.41[a]</td>
</tr>
</tbody>
</table>

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Fig. 1. Body mass evolution of (A) all mice; oral glucose tolerance test curves (B). The groups are: standard chow, SD; HF diet, HF; bezafibrate, pan-PPAR agonist BZ; GW1929, PPARgamma agonist GW and WY14643, PPARalpha agonist WY. Data are presented as the mean ± SD, n = 5 for each group. *** and **** indicate P < 0.001 and P < 0.0001, respectively, compared to the SC group; † and ††† indicate P < 0.01 and P < 0.001, respectively, compared to HF group; $$$ and $$$$ indicate P < 0.01, P < 0.001 and P < 0.0001, respectively, compared to HF-BZ group; ‡‡‡‡ indicate P < 0.01, P < 0.001 and P < 0.0001, respectively, compared to HF-WY group.
Liver biochemistry and stereology (Table 2, Fig. 2)

The liver mass of the HF group was heavier than the SC group (+35%; $P < 0.0001$). Accordingly, the TG levels of the HF group were 80% higher ($P < 0.0001$) and the steatosis content was 150% higher ($P < 0.0001$) than the SC group. Interestingly, the HF-BZ group had increased liver mass (+25%; $P = 0.0009$), but TG levels and steatosis diminished compared to the HF group (−30% and −50%, respectively; $P < 0.0001$). To assess whether this increase in liver mass could cause liver damage, we measured plasma ALT and gGT concentrations and

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observed that the HF group had augmented levels of both hepatic enzymes (+30% and +20%; \( P < 0.001 \)) than the SC group. The HF-BZ group preserved values similar to the SC group. The HF-WY group showed lower TG levels (−65%; \( P < 0.0001 \)) and lower steatosis (−135%; \( P < 0.0001 \)) than the HF group, but no significant difference in liver mass between the HF and HF-WY groups. In contrast, GW1929 failed to override the effects of the HF diet on the liver, and no substantial differences were found between the HF and HF-GW groups. Similar to HF-BZ, both the HF-WY and HF-GW groups presented improved plasma ALT levels compared to the HF group (−15%; \( P = 0.02 \) for the HF-WY group, and −30%; \( P = 0.0001 \) for the HF-GW group). The HF-WY and HF-GW groups showed reduced levels of plasma gGT compared to the HF group (−30% and −20%, respectively, \( P < 0.01 \)).

**Inflammation and fibrosis in the liver (Figs. 3 and 4)**

Immunofluorescence demonstrated positive reactions for smooth muscle alpha actin in groups fed HF diet, implying the role that chronic dietary intake of excessive saturated fatty acids has in hepatic inflammation. Of note, the HF-BZ group presented a marked reaction, suggesting a greater activation of hepatic stellate cells (HSCs) in these animals. This observation complies with the higher gene expression of PPARbeta, induced by the treatment with bezafibrate. Hepatic fibrosis demonstrated by Sirius Red staining suggests HSC activation once all HF fed groups presented clusters of fibrosis within hepatic tissue, confirming that the diet is a significant factor in the progression of NAFLD. However, the treated groups showed less expressive fibrosis than the untreated HF group.

**PPARalpha, PPARbeta, PPARgamma and target gene equilibrium in liver (Figs. 5, 6 and 7)**

In order to determine the influence of disrupted equilibrium of key transcription factors associated with the NAFLD/NASH spectrum, we performed RT-qPCR to evaluate PPAR isoforms and some target gene levels. As expected, the HF group presented increased mRNA levels of PPARgamma (\( P = 0.03 \)) and SREBP-1c (\( P < 0.01 \)), which are lipogenic transcription factors in the liver, and diminished mRNA levels of PPARalpha (\( P < 0.05 \)). The HF-WY group presented high PPARalpha (\( P < 0.0001 \)) and low PPARgamma (\( P < 0.01 \)) and SREBP-1c (\( P < 0.01 \)) mRNA levels as expected. The HF-GW group was not different from the HF group concerning PPARgamma and PPARalpha expressions, but showed an increased expression of SREBP-1c (\( P < 0.03 \)). The HF-BZ group had high PPARalpha (\( P < 0.001 \)) and low PPARgamma (\( P < 0.01 \)) mRNA levels, but SREBP-1c was similar to the HF group. When the PPARalpha:PPARgamma ratio was performed, a higher hepatic beta oxidation capacity was found on the HF-WY group (\( P = 0.002 \)) and on the HF-BZ group (\( P = 0.004 \)) in comparison with the untreated HF group. This finding correlates with the marked increase in PPARalpha gene expression in these groups compared to the untreated HF group.

**Fig. 4.** Liver immunofluorescence for smooth muscle alpha actin, same magnification. The groups are: standard chow, SD; HF diet, HF; bezafibrate, pan-PPAR agonist BZ; GW1929, PPARgamma agonist GW and WY14643, PPARalpha agonist WY. Negative immunoreaction was observed in the SC group (A), positive immunoreaction was found in the HF group (B), HF-BZ group (C), HF-WY group (D) and HF-GW group (E). Activated hepatic stellate cells are marked (arrows).
The RT-qPCR of the expression levels of CPT-1a, fatty acid translocase (FAT)/CD36 and fatty acid synthase (FAS) was performed to investigate whether the decreased TG and steatosis levels were due to enhanced beta-oxidation. The HF and HF-GW groups had decreased mRNA levels of CPT-1a \( (P < 0.01) \), and increased mRNA levels of FAT/CD36 \( (P < 0.01) \). In contrast, the HF-WY group had increased CPT-1a mRNA levels \( (P < 0.0001) \) and decreased FAT/CD36 mRNA levels \( (P < 0.01) \). Moreover, the HF-BZ group showed increases in both CPT-1a \( (P < 0.0001) \) and FAT/CD36 \( (P < 0.01) \) gene expression. A higher beta oxidation capacity was observed with the CPT-1a:SREBP-1c ratio. This ratio was over, the HF-BZ group showed increases in both CPT-1a \( (P < 0.0001) \) and FAS gene levels \( (P < 0.01) \) than the untreated HF group.

A FAS gene expression followed PPARgamma gene expression, being enhanced in the HF and HF-GW groups when compared to the SC group \( (P < 0.02) \). Curiously, pan-PPAR activation by bezafibrate elicited enhanced FAS gene levels \( (P < 0.01) \). However, the higher PPAR-alpha and CPT-1a gene expression guaranteed higher metabolization of hepatic lipids, avoiding accumulation. In the agreement to this, the HF-WY group presented the weakest FAS gene expression.

Finally, PPARbeta gene expression was diminished in the HF group in comparison to SC group \( (P = 0.003) \). Only treatment with bezafibrate was able to induce PPARbeta gene expression significantly compared to HF group \( (P < 0.0001) \). When the PPAR balance was analyzed, considering the three PPAR isoforms, bezafibrate induced all PPAR isoform gene expression, but predominantly the alpha and beta isoforms. As expected, HF-WY presented a greater activation of PPARalpha as well as HF-GW induced PPARgamma.

Discussion

The current study compared the effects of treatments with different PPAR agonists (PPARalpha, PPARgamma, and pan-PPAR agonist) on hepatic liver metabolism using a dietary murine model of obesity. The three treatments led to improved glucose tolerance, plasma insulin, and adipokine levels but disagreed with respect to their effects on hepatic structure, inflammation, lipid metabolism and biochemistry.

Animal model and different dietary approaches are useful to mimic the NAFLD observed in humans. However, the degree of hepatic steatosis, inflammation and fibrosis depend on the type of animal, the nutritional manipulation and the duration of diet administration [13]. The HF diet is suitable to mimicize in the animal model the hepatic alterations observed in humans. The HF diet is more appropriate than methionine choline-deficient diet, because it elicits insulin resistance and proinflammatory adipokine profile prior to lipid accumulation within hepatocytes [13,14].

The HF diet induced pronounced weight gain during the observational period. It has been extensively demonstrated that being overweight and being obese are directly linked to IR, glucose intolerance and increases in inflammatory adipokines [15], as observed in the HF group. Treatment with WY14643 and bezafibrate significantly reduced BM. This reduction has been shown to be an important contributor to improved glucose tolerance and IR [16] and reduced inflammatory adipokines [17], all of which were found in the HF-WY and HF-BZ groups. Interestingly, GW1929 attenuated glucose intolerance, IR and inflammation in a BM-independent manner as shown by the similar BMs of the HF-GW and the untreated HF group during the experimental period. We have previously demonstrated that rosiglitazone, which is a total PPARgamma agonist, can improve these parameters without altering BM [5]. Furthermore, PPARgamma agonists, such as pioglitazone and rosiglitazone, have improved metabolic parameters in individuals with NASH [18], but the clinical use of these agents has been limited by off-target adverse effects, such as weight gain [19].

The high energy diets contribute significantly to the development of NAFLD/NASH in human and animal models. Concomitant with the

Fig. 5. Gene expression levels of peroxisome proliferator-activated receptor (PPAR)alpha (A), PPARbeta (B), PPARgamma (C) and PPAR balance (D) in the liver. The groups are: standard chow, SD; HF diet, HF; bezafibrate, pan-PPAR agonist BZ; GW1929, PPARgamma agonist GW and WY14643, PPARalpha agonist WY. TBP was used as an internal control to normalize the expression levels of the selected genes. Data are presented as the mean ± SD, n = 5 for each group. *, **, *** and **** indicate \( P < 0.05, P < 0.01, P < 0.001 \) and \( P < 0.0001 \), respectively, compared to SC group; †, †† and ††† indicate \( P < 0.01, P < 0.001 \) and \( P < 0.0001 \), respectively, compared to HF group; $$$ and $$$$ indicate \( P < 0.001 \) and \( P < 0.0001 \), respectively, compared to HF-BZ group and ‡‡‡ indicates \( P < 0.001 \) compared to the HF-WY group.
development of IR, liver damage includes lipogenesis activation through overexpression of PPARgamma and SREBP-1c [20,21]. The current study confirmed that the HF diet accentuated the liver mass, steatosis percentage and TG levels in the liver and demonstrated that the treatments led to different results. First, WY14643 markedly diminished steatosis, and consequently, TG levels in the liver without causing significant differences in liver mass compared with the HF group. Bezaflibrate also diminished steatosis and TG levels, but enhanced liver mass. Finally, GW1929 did not alter these data compared to the HF group.

PPARalpha activation by fibrates in the liver can cause hepatic peroxisome proliferation and hepatomegaly, but these effects are lost in humans, even when the fibrates preserve PPARalpha activation.

Fig. 6. Gene expression levels of carnitine palmitoyl transferase (CPT1a) (A), sterol regulatory element-binding protein (SREBP)-c (B), fatty acid translocase (FAT)/CD36 (C) and fatty acid synthase (D) in the liver. The groups are: standard chow, SD; HF diet, HF; bezafibrate, pan-PPAR agonist BZ; GW1929, PPARgamma agonist GW and WY14643, PPARalpha agonist WY. TATA binding protein (TBP) was used as an internal control to normalize the expression levels of the selected genes. Data are presented as the mean ± SD, n = 5 for each group. *, **, *** and **** indicate $P < 0.05, P < 0.01, P < 0.001$ and $P < 0.0001$, respectively, compared to the SC group; †† and †††† indicate $P < 0.01$ and $P < 0.0001$, respectively, compared to the HF group; $\$ and $\$$\$$ indicate $P < 0.01$ and $P < 0.0001$, respectively, compared to the HF-BZ group and $\$\$ and $\$$\$$\$$ indicate $P < 0.001$ and $P < 0.0001$, respectively, compared to the HF-WY group.

Fig. 7. The groups are: standard chow, SD; HF diet, HF; bezafibrate, pan-PPAR agonist BZ; GW1929, PPARgamma agonist GW and WY14643, PPARalpha agonist WY. PPARalpha/PPARgamma mRNA ratio in the liver (A) and CPT1a/SREBP1c mRNA ratio in the liver (B). Data are presented as the mean ± SD, n = 5 for each group. *, **, *** and **** indicate $P < 0.05, P < 0.01, P < 0.001$ and $P < 0.0001$, respectively, compared to the SC group; †† and †††† indicate $P < 0.01$ and $P < 0.0001$, respectively, compared to the HF group; $\$ and $\$$\$$ indicate $P < 0.01$ and $P < 0.0001$, respectively, compared to the HF-BZ group and $\$\$ and $\$$\$$\$$ indicate $P < 0.001$ and $P < 0.0001$, respectively, compared to the HF-WY group.
Particularly, the role of PPARalpha in lipid metabolism is well conserved in both species [12]. Both the HF-BZ and HF-WY groups presented enhanced PPARalpha mRNA levels, but only the HF-BZ group showed increased liver mass, confirming that not only PPARalpha activation, but also fibrates, can cause this effect in mice. Although animals treated with bezafibrate showed increased liver mass, this was not associated with high levels of ALT or gGT, which is indicative of the absence of liver damage in this group [22].

PPAR activation in the liver is associated with increased beta-oxidation or the increased activity of lipogenic pathways [12]. PPARalpha is the primary activator of beta-oxidation through its target genes, such as CPT-1a [23]. In contrast, PPARgamma participates in lipogenic activities in the liver through its target genes, FAT/CD36 and SREBP-1c [5,24]. CPT-1a is the mitochondrial gateway for fatty acid entry into the mitochondrial matrix. As such, it is considered the master regulator of hepatic mitochondrial beta-oxidation [25]. Both the HF-BZ and HF-WY groups presented elevated mRNA levels of CPT-1a, which favors beta-oxidation. On the other hand, only the HF-BZ group showed elevated mRNA levels of FAT/CD36, which is responsible for promoting a long-chain fatty acid (LCFA) transport into mitochondria, augmenting oxidative capacity as long as CPT-1 is also upregulated [24,26].

Although FAT/CD36 is not crucial for fatty acid uptake in the liver, it enables the initial metabolism of LCFAbs, which involves a chain shortening to allow fatty acid entry into the mitochondria by CPT-1 activation (for molecules with a maximum of 20 carbons) [24]. The HF-WY group had the weakest FAT–CD36 gene expression. However, this fact did not compromise the beta-oxidation capacity of the group, because the HF diet was based on lard (predominantly composed of saturated fatty acids with 16 and 18 carbons in length). These fatty acids do not rely on FAT/CD36 to enter the mitochondria [24,27]. In contrast, the GW1929 and untreated HF groups favor the lipogenic pathway, as proven by the enhanced mRNA levels of PPARgamma and FAT/CD36 and the diminished mRNA levels of PPARalpha and CPT-1a. In addition, FAT/CD36 is a common target for various lipogenic genes, such as LXR, PXR and PPARgamma and its upregulation leads to hepatic steatosis [28].

PPARgamma and SREBP-1c are essential for triggering hepatic lipogenesis [29,30]. Using a metabolic programming model, we have previously demonstrated that PPARgamma, but not SREBP-1c, plays a role in lipogenic activity in liver [12]. We confirmed these data in the current study by showing that bezafibrate treatment optimizes beta-oxidation without altering SREBP-1c mRNA levels. The PPARalpha activation (WY14643 treatment) was able to decrease the expression levels of PPARgamma and SREBP-1c in the liver, and consequently the lipogenic activity.

Despite being a benign condition, NAFLD can progress to harmful diseases such as NASH and liver fibrosis [31]. This progression relies on chronic inflammation, which was identified in all HF fed groups after Sirius Red staining. However, the three treatments attenuated this parameter, being efficient to avoid NAFLD progression. In the presence of chronic liver injury, hepatic stellate cells (HSCs) receive signals that cause their transdifferentiation into a fibroblast-like cell that produce a temporary scar to protect the liver from further injuries [32].

Activated HSCs cause hepatic fibrosis if the triggering stimulus of their activation is prolonged [33]. PPAR activation may interfere with the quality and amount of extracellular matrix synthesis due to HSC activation. Thus, PPARgamma activation may maintain HSCs quiescent phenotype [34]. In fact, the HF–GW group showed the highest body mass and steatosis degree and although activated HSCs were identified after positive immunoreactions for smooth muscle cell alpha actin, this group showed the lowest fibrosis. This observation is supported by the suppressive effect that the PPARgamma agonist exerts upon the fibrogenic potential of HSCs [35,36]. Activated HSCs were also observed in animals treated with fenofibrate, which can be accounted for by the recently described greater phosphorylation of p38 and c-Jun N-terminal kinases by the activation of PPARbeta [37].

This study does have some limitations. We aimed to perform a translational research, and as such, we did not use a PPARbeta/delta agonist because it is not currently available in clinical practice due to its procarcinogenic effects [38]. As a result, we attempted to mimic the clinical uses of fenofibrate (PPARalpha agonist), pioglitazone (PPARgamma agonist) and bezafibrate (pan-PPAR agonist) in the treated mice.

Conclusion

The present findings highlight the importance of PPARalpha activation to counteract liver steatosis, insulin resistance, liver inflammation, and overweight/obesity. The WY14643 and bezafibrate treatments emerged as the most powerful approaches to overcoming the adverse metabolic and hepatic effects caused by obesity and IR. These observations confirmed that PPARalpha is the master regulator of the beta-oxidation target genes and elucidated the influence of Pan-PPAR activation on PPARgamma gene expression. Although bezafibrate activated the three PPAR isoforms, PPARalpha, and PPARbeta/delta levels seemed to surpass PPARgamma. This observation emphasizes the pivotal role of PPARalpha and its balance with PPARgamma gene expression in the regulation of metabolic homeostasis and lipolytic pathways in the liver.

Conflict of interest statement

The authors declare no conflicts of interest.

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