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Title: LIQUID FRUCTOSE DOWNREGULATES SIRT1 EXPRESSION AND ACTIVITY AND IMPAIRS THE OXIDATION OF FATTY ACIDS IN RAT AND HUMAN LIVER CELLS

Article Type: Regular Paper

Keywords: PPARalpha; steatosis; triglycerides; ChREBP

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Abstract: Fructose ingestion is associated with the production of hepatic steatosis and hypertriglyceridaemia. For fructose to attain these effects in rats, simultaneous induction of fatty acid synthesis and inhibition of fatty acid oxidation is required. We aimed to determine the mechanism involved in the inhibition of fatty acid oxidation by fructose and whether this effect occurs also in human liver cells. Female rats were supplemented or not with liquid fructose (10% w/v) for 7 or 14 days; rat (FaO) and human (HepG2) hepatoma cells, and human hepatocytes were incubated with fructose 25 mM for 24 hours. The expression and activity of the enzymes and transcription factors relating to fatty acid beta-oxidation were evaluated. Fructose inhibited the activity of fatty acid beta-oxidation only in livers of 14-day fructose-supplemented rats, as well as the expression and activity of peroxisome proliferator activated receptor alpha (PPARalpha). Similar results were observed in FaO and HepG2 cells and human hepatocytes. PPAR downregulation was not due to an osmotic effect or to an increase in protein-phosphatase 2A activity caused by fructose. Rather, it was related to increased content in liver of inactive, acetylated peroxisome proliferator activated receptor gamma coactivator 1alpha, due to a reduction in sirtuin 1 expression and activity. In conclusion, fructose inhibits liver fatty acid oxidation by reducing PPARalpha expression and activity, both in rat and human liver cells, by a mechanism involving sirtuin 1 down-regulation.

Response to Reviewers: Reviewer #1:
Major points:
1. In order for the reader to comprehend the nutritional setting in which the changes are being observed, far more detail needs to be included to support/strengthen table 1. Details on the nutritional content of the diet must be included (% calories from fat etc..). In the

same sense, it would be useful to see the data presented as total calories consumed from diet vs fructose in drinking water.
In this study the rats received a regular diet (Teklad Global 2018 Rodent Diet, fromHarlan Teklad), that provided 18% calories from fat, 24% from protein and 58% from carbohydrate. This information has been included in the new version of the manuscript (Materials and Methods section, pg 5, ln 22-23).
Regarding the calories consumed from diet or from fructose, we calculated the data from the area under the curve of food or drink consumption in g or ml/days/cage (containing two rats). Our results are the following:

	7days		14 days	
	Control	Fructose	Control	Fructose
Kcal from food	726.4	566.4	1609.6	1254.4
Kcal from drink	0	338	0	798
Total kcal	726.4	904.4	1609.6	2052.4

As we already stated in our first version of the manuscript, "rats increased their calorie intake in a similar way at 7 (x1.24-fold) and 14 days (x1.27-fold), mainly due to an increase in fructose calories (x1.37 and x1.39-fold at 7 and 14 days, respectively), which was not compensated by a reduction in the ingestion of solid food". Perhaps it was not clear enough, so we have rephrased the sentence (pg 11, ln 8-13): "rats increased their calorie intake in a similar way, from 726.4 to 904.4 kcal/7days/2 rats (increase of 1.24-fold) and from 1609.6 to 2052.4 kcal/14days/2 rats (x1.27-fold), at 7 and 14 days, respectively. The increase was mainly due to calories obtained from fructose, which represented a 37 and 39% of the total calories consumed at 7 and 14 days, respectively. This increase was not compensated by a sufficient reduction in the ingestion of solid food"
2. The authors state that body weight changes were not observed, but the values should be included for the readers' benefit, and it would also be beneficial to know roughly what was happening in other key metabolic tissues, such as adipose tissue. For instance leptin levels increase significantly at 14 weeks, suggestive of an expansion of adipose tissue lipid content. One might postulate that in the face of impaired lipid oxidation, the liver is exporting more triglyceride in VLDL particles which are being taken up by the white fat. Basic histological examination of WAT and tissue weights would be good and examination measurement of key lipid handling genes even better. Likewise, analysis of lipid synthetic genes or genes regulating triglyceride release (MTP) in liver would be illuminating in this sense. The increased serum TG have to be coming from somewhere - and the data indicate that it is not from the diet.
As the referee suggest, we have included body weight and white adipose tissue weight data in Table 1 of the new version of the manuscript. As can be seen in this table, there is no significant difference in total body weight (either expressed as area under the curve in g/7 or 14 days/rat or as body weight at the end of treatment) between control and fructose groups. Regarding adipose tissue weight, we neither observed statistically significant changes. On the other hand, plasma leptin levels are not significantly increased at 14 days
Further, we already measured the expression of the genes involved in lipid synthesis, specifically liver-pyruvate kinase (L-PK), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), glycerol phosphate

acyltransferase 1 (GPAT1) and acetyl-CoA carboxylase (ACC), and we found that fructose similarly induced their expression after 7- and 14-day supplementation (data in Table 2). Thus, serum and hepatic triglycerides come from the combined increase in lipid synthesis and reduction of fatty acid oxidation.

Regarding additional experiments suggested by the referee, we did not perform basic histological examination of WAT at the moment of sacrifice. Nevertheless, we could determine the mRNA levels of MTP in hepatic samples from treated animals. Our results were:

	7days		14 days	
	Control	Fructose	Control	Fructose
MTP	1 ± 0.14	1.21 ± 0.40	1 ±0.19	1.33 ± 0.33

The lack of increase of MTP expression is not surprising, as MTP expression is regulated by reduced lipid availability, but an increase in the amount of lipids would not necessarily induce MTP expression. It would have been better to determine MTP activity, but this has to be performed in freshly obtained hepatic samples.

3. Long chain fatty acid beta-oxidation is clearly impaired by fructose treatment, but is there any compensation via increased oxidation of short chain fatty acids? Are genes regulating fatty acid elongation down-regulated for instance?

In our study we fed rats with a regular chow diet that did not provide short chain fatty acids (SCFA) but long chain fatty acids (LCFA: palmitic, stearic, oleic, linoleic and linolenic acids). SCFA could also be formed from colonic fermentation of dietary fiber, but the regular chow that we used in our study has a low amount of crude fiber (3.5%), and moreover, its consumption was reduced in fructose-supplemented animals. Thus, the bulk of fatty acids reaching the hepatic cells of the rats in our study are LCFA. In this case, SCFA could only derive from the β-oxidation of these LCFA, but as this is reduced in fructose-fed rats, the amount of SCFA formed should also be reduced. Therefore, if a compensatory response of increased SCFA oxidation existed, it would not result in a meaningful metabolic effect.

Regarding fatty acid elongation, there are some reports showing that the expression of hepatic elongases (Elovl) might be controlled by several hormones and transcription factors, including PPARα, SREBP-1 and ChREBP (Wang et al., J Lipid Res. 2005, 46:706; Wang et al., J Lipid Res. 2006, 47: 2028). It has been shown that carbohydrates induce hepatic Elovl-6 along with L-PK and FAS through ChREBP (Wang et al., J Lipid Res. 2006, 47:2028). Thus, as the referee suggested, we decided to determine the expression of hepatic Elovl-6 in hepatic samples from our control and fructose-supplemented rats. According to the general induction of genes involved in lipid synthesis, we also found a significant increase in Elovl6 expression at both 7 and 14 days. These results have been included in the text (pg 12, ln 4 and in Table 2).

4. What effects are there on other key lipid-burning tissues? For instance what happens to beta-oxidative genes and UCP1 in the brown adipose tissue? This data would enable the specificity and relative contribution of the effects of fructose on hepatic lipid metabolism

Unfortunately, we did not obtain brown adipose tissue (BAT) from the rats of our study. UCP-1 is a hallmark of BAT and under basal conditions it is not expressed in white adipose tissue (WAT). A recent report (Li et al, Gastroenterology, Epub ahead of print 2013 Oct 31, doi:

10.1053/j.gastro.2013.10.059) showed that increased SIRT-1 activity increases the levels of the hepatocyte-derived hormone fibroblast growth factor 21 (FGF21), and this enhanced energy expenditure through white fat "browning" (with increased expression in WAT of typical BAT genes such as UCP-1). However, in our study we detected a decrease, not an increase in SIRT-1 expression and activity. Moreover, we did not observe changes in the mRNA expression of hepatic FGF21 after 14 days of fructose supplementation (data not shown in the paper). Thus, hepatic FGF21 mRNA levels, expressed in arbitrary units were 1.00 ± 0.44 (control rats) and 0.93 ± 0.27 (fructose-supplemented rats). These results suggest that UCP-1 would not be expressed in WAT from fructose-supplemented animals.

Minor points:

1. The concept that mitochondrial long-chain fatty acid oxidation is impaired by fructose is not novel. In fact a paper from 1976 already demonstrates this effect and should at least be cited. (Prager GN, Ontko JA. Direct effects of fructose metabolism on fatty acid oxidation in a recombined rat liver mitochondria-high speed supernatant system. Biochim Biophys Acta. 1976 Mar 26;424(3):386-95.)

According to the referee's suggestion we have cited this reference in the novel version of the manuscript (pg 4 ln 15-16)

2. Table one title should read "liver triglycerides".

The spelling error has been corrected in the new version of the manuscript.

3. Figure 6E does not appear to be discussed in the text?

This was also an error, as Figure 6E corresponds to the levels of acetylated ChREBP protein in liver samples from control and 14-day fructose-supplemented rats, but in the text it was cited as 4E. This error has been corrected in the new version of the manuscript (pg 19, ln 1).

Reviewer #2

General comments:

This is a short manuscript. The rationale is described clearly and the manuscript reports the important observation on the inhibition of fatty acid oxidation and underlying enzymes in female rat liver; however, a number of points need to be clarified.

In addition, the discussion is hard to follow. Therefore, a revision of manuscript as well as an addition of a diagram that summarizes the interaction between transcription factors leading to the activation or inhibition of PPARα and fatty acid oxidation would be very helpful.

According to the referee's suggestion, we have revised the discussion and added a figure (new Figure 7) to make it easier to follow.

Specific comments:

Introduction:

-The introduction is very short and in my opinion it is not complete. For instance, it doesn't give the logics behind measuring PPARα expression and activity. The role of PPARα in the fatty acid oxidation should be stated. This justifies why the authors measuring liver transcription factors and enzymes controlling PPARα. In addition, the authors should introduce the PP2A and its possible role in the in PPARα activity in Introduction. This justifies the use of okadaic acid and the Enzyme (PP2A) activity assays in Method section (2.5).

The role of PPARα on fatty acid oxidation, as well as the possible role of PP2A in the inhibition of the PPARα system has been clearly stated in

the introduction section in the new version of the manuscript (pg 4 ln 21-23 and pg 5 ln 1-3).
-Please rephrase the following statement:
Page 4: "Reported reasons are high energy intake, lack of adequate energy compensation and the special metabolism of fructose [1,5]."
The sentence has been rephrased as: “High energy intake, lack of adequate energy compensation and the special metabolism of fructose have been reported as reasons to explain this association [1,5].”
-Page 4: "As we wanted to understand the mechanism of fructose effects, we studied the effect of fructose.
You may rephrase it as "The aim of our study was to investigate the effect of fructose..
We have re-written the sentence as the referee suggests
Methods:
-It requires the justification for selection of use of 7 or 14 days and 10% fructose for 7-14 days or 25 mM for 24 h in vitro. How comparable or relevant these concentrations of fructose are to in vivo ingestion by human? Is it considered the medium range of ingestion or high?
Diets that incorporate 10 % weight/volume fructose concentrations in drinking water mimic the human pattern of fructose consumption, with daily fructose intake equivalent to that found in the upper quartile of fructose consumption in human populations. We have used this model of fructose administration during 14 days in several previous studies, e.g. Roglans N et al., Hepatology 2007, 45:778. In the present article we wanted also to study fructose effects at an earlier time point (7 days) and also, to avoid fluctuations in the amount of sugar ingested in the in vivo studies, to assess the effects of fructose on liver in vitro. Concentrations of fructose in the portal circulation can easily reach 5–10 mM (Du L, Heaney AP. Mol Endocrinol. 2012, 26:1773), but as for in vitro studies incubation times cannot be as long as in vivo exposure times, we decided to use higher fructose concentrations (25 mM), similar to previous studies of our group (Vilà et al., Hepatology 2008, 48:1506; Rodriguez-Calvo R et al., Hepatology 2009, 49:106).

-Page 6: In some experiments, 100 µM of Wy-14643 and 20 nM of Okadaic acid were added 3 hours and 30 minutes, respectively, before fructose. 100 nM of SRT1720 was added to the medium 12 hours after fructose supplementation.
Please state the action of these drugs when stated the first time in the manuscript. For instance, Wy-14643 (a PPARα ligand), Okadaic acid (an inhibitor of PP2A), SRT1720 (a selective activator of SIRT1), Okadaic acid (an inhibitor of PP2A)
According to the referee’s suggestion, we have defined the action of the drugs when first cited in the Methods section.
-Page 7, section 2.5: The concentration and the duration of treatment with TSA are missing.
TSA was added at a concentration of 1 µM for 12 h. This has been added in the new version of the manuscript (pg 7, section 2.5).
-Page 7, section 2.6: Why were different internal controls used for 7 and 14 days fructose supplemented rats? APRT was used for 7 days and 18s for 14 days group of rats.
The referee may be mistaken, as in fact we used APRT for 14 days and 18 s for 7 days. In our previous studies in rats treated with fructose for 14 days we always used APRT as an internal control. However, when we started

experiments using shorter treatment periods, we observed that the expression of APRT changed in samples from fructose-fed rats compared with controls. Therefore, we decided to use 18s as an internal control for these samples.

-Page 8, Section 2.7: It requires more details on the Western blot analysis.

In the new version of the manuscript we have provided more details, such as blocking conditions, dilutions of each primary antibody and conditions of incubation with secondary antibodies (pg 8 ln 22- pg 9 ln 11).

Results:

-According to the table 1, it seems there is a tendency for increase of leptin in both 7 and 14 days fructose supplementation to rats. Regardless the fatty acid oxidation is inhibited. This point should be discussed in Discussion. Have the authors also measured the level of adiponectin?

The differences in plasma leptin levels between control and fructose-treated rats are not statistically significant. Consequently, we consider that there is no need to speculate on this issue. On the other hand, we measured plasma adiponectin levels. These results have now been included in Table 1 and in page 11 in the new version of the manuscript. There is a significant increase in plasma adiponectin levels only in rats supplemented with fructose for 14 days. This increase was also detected in previous studies in male rats supplemented with either 10% glucose or 10% fructose (Roglans N et al., Hepatology 2007, 45:778), suggesting that this is not a specific effect of fructose. We do not believe that this data has enough relevance to be discussed in the article.

-Page 10, Section 3.1: How was the degree of steatosis measured in the fructose ingested groups, and how compared between two groups of 7- and 14-days fructose supplemetation? The authors did not give any values but stated that liver steatosis was confirmed by histological analysis.

We quantified the amount of triglycerides in hepatic samples from fructose-supplemented and control rats. As shown in Table 1, the hepatic triglyceride content was not significantly increased after 7 days of fructose supplementation, but there was an increase of 1.9-fold (from 4.1 ± 2.3 to 7.7 ± 2.4 mg/g liver, $p<0.05$) after 14 days of treatment. Steatosis was confirmed qualitatively by histological analysis in liver sections stained with Oil Red O (Figure 1 C).

-Page 11, section 3.3: It is still unclear why the PPARα expression was increased in 7 days fructose supplementation to rats. This should be discussed in Discussion.

It is interesting to discuss why fatty acid oxidation is repressed at 7 days despite higher PPARα activity. It is probably due to higher ACC expression leading to malonyl-CoA production, which inhibits L-CPT1 and therefore reduces beta oxidation activity. We have included this in the Discussion section in the new version of the manuscript (page 16, ln 18-20). We are not certain about the mechanisms by which the expression and activity of PPARα at 7 days is increased. It is possible that in this situation there is an increase in endogenous PPARα ligands, or changes in the expression of co-activators and co-repressors leading to an increase of PPARα expression and its target genes.

-Page 11, Section 3.3: The authors mentioned about the possible mechanisms which are responsible for inhibition of fatty acid β-oxidation, despite opposite changes in PPARα. "This inhibition of fatty acid β-oxidation, despite opposite changes in PPARα, could be attributed to two mechanisms: 1. Livers from 7- and 14-day fructose supplemented

rats showed increased expression of total and phosphorylated ACC (Table 2), implying increased production of malonyl-CoA, a known allosteric inhibitor of L-CPT-I, whose activity controls the whole fatty acid β-oxidation system [24]; 2. ChREBP activity controls the expression of RGS16, a physiological inhibitor of the fatty β- oxidation system [25]."
First of all, the above statements should be moved to Discussion, and if I understand it correctly, in both 7- and 14-days fructose supplementation, the RGS16 was enhanced (Fig 2C). If the ChREBP activity controls the RGS16 (inhibitor of FA oxidation), please then clarify that as how the ChREBP was enhanced only in 14-days supplementation?
In order to follow the rationale behind the shown experiments, we consider that this piece of text is correctly placed in this section. The referee is right; the mRNA levels of RGS16 are equally increased at both time-points. To be sure of the physiological meaning of this induction, we measured the protein expression of RGS16 in hepatic samples from fructose-supplemented and control rats. The protein levels were not modified at any of the treatment times, ruling out the involvement of RGS16 in the inhibition of hepatic fatty acid oxidation:

7days:

Control	Fructose
1.00 ± 0.14	1.04 ± 0.36

14 days:

Control	Fructose
1.00 ± 0.17	1.09 ± 1.12

This information has been added in the new version of the manuscript (new Figure 2D) and in the discussion (pg 18, ln 9-12).
Here, the authors explaining the inhibition of fatty acid β-oxidation, despite opposite changes in PPARα through 1) enhanced production of malonyl-CoA through ACC phosphorylation and 2) Deacetylation and activation of PGC-1α (a cofactor of PPARα, necessary for the transcriptional control of genes related to fatty acid oxidation.
We know Malonyl CoA is synthesized in the liver by ACC, which in turn is phosphorylated and inhibited by AMPK. On the other hand, AMPK increases FA oxidation directly by PPARα activation. It would be interesting to measure the AMPK expression or activity in both 7 and 14-day fructose supplementation and to see whether its level is reduced in these groups. I am not suggesting additional experiments, but if the AMPK level was measured already, it would be nice to include it.
According to the referee's suggestion, we measured the expression of total and phosphorylated AMPK, and we did not observe significant differences between control and fructose-supplemented animals. These results have been included in the new version of the manuscript (Table 3).

	7days		14 days	
	Control	Fructose	Control	Fructose
p-AMPK	1.00 ± 0.32	1.06 ± 0.20	1.00 ± 0.24	0.91 ± 0.14
Total AMPK	1.00 ± 0.34	0.98 ± 0.17	1.00 ± 0.28	1.28 ± 0.20

The lack of increase in phospho-AMPK suggests that its kinase activity is not enhanced. Consequently, the ratio between phosphorylated and total ACC is not increased. Thus, the increase in ACC expression as a consequence of the activation of lipogenesis should lead to an increase in malonyl-CoA production.
A decrease in SIRT1 expression and activity (based on NAMPT) (Figs 6A-B) and consistently inactivity of PGC-1 (Fig 6D) could be responsible for the reduced PPARα and reduced FA oxidation in 14-day fructose supplementation group. However, it is still unclear how we get to an increased PPARα in 7-day fructose supplementation. Obviously we see no changes in SIRT1 expression and in NAMPT in this group.
We agree with the referee, the increase in the expression and activity of PPARα at 7 days is not related to changes in SIRT1 or NAMPT expression. Our hypothesis is that PPARα increase at 7 days could be a compensatory mechanism, an attempt to enhance fatty acid oxidation in the initial phases of fructose consumption, maybe related to an increase in endogenous PPARα ligands, or to changes in the expression of co-activators and co-repressors.
-The authors also mentioned that "The liver fatty acid β-oxidation system was probably repressed by products of the metabolism of fructose at the time of death, independently of the actual expression and transcriptional activity of PPARα."
This hypothesis need to be more clarified based on their data and observation.
As this sentence was confusing, we have deleted it in the new version of the manuscript.
-Figure 2C: How do you explain so much variability in mRNA bands of RGS16 of controls (n= 3)?
There was a mistake in the legend for Figure 2. Instead of “Each bar represents the mean±sd of three different samples” it should read “Each bar represents the mean±sd of values obtained from n=4 and n=5 animals (for control and fructose groups, respectively).” The autoradiography above the figure shows the bands corresponding to Rsg16 mRNA and that of the aprt gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of 3 animals from each treatment group. We have corrected the mistake in the figure legend. We cannot explain the variability, but we are confident in our results, because they come from 4-5 different animals per group of treatment. Moreover, when we performed statistical analysis, we found that the increase was statistically significant, despite the variability.
-Page 13, PGS16 needs to be replaced by RGS16. RGS16 stands for "Regulator of G protein signaling".This should be added to the Manuscript.
The complete name of the protein has been added in the new version of the manuscript.
-Page 14: The following statement should be moved to Discussion.
"Thus, the effect of fructose on the expression of these genes was independent of PP2A activity, corroborating previous research by Dentin et al. [28]."
The statement has been rephrased and moved
-Page 14, section 3.6: In Table 3, the authors showed that the expression of Foxa2, a transcription factor regulating fatty acid oxidation, was reduced only after 14-day fructose supplementation.

However β oxidation of fatty acid was reduced after both 7 and 14-day fructose supplementation. This requires further clarification.
The reduction of Foxa2 expression after 14 days of fructose treatment, although significant is just of 13%. This suggests that its biological significance is not relevant, and does not deserve further consideration.
-Page 14, Section 3.6: The authors stated "fructose was also able to efficiently block the increase in PPARα expression induced by incubating FaO cells with SRT1720, a potent and selective activator of SIRT1 [31] (Figure 6). "
Figure 6 must be replaced by Figure 6C.
The replacement has been done as indicated by the referee.
-There is no any text in this section referring to Figure 6E.
It is an error, as Figure 6E corresponds to the levels of acetylated ChREBP protein in liver samples from control and 14-day fructose-supplemented rats, but in the text it was cited as 4E. This error has been corrected in the new version of the manuscript (pg 19, ln 1).
Discussion
-In general, the Discussion is difficult to follow. It needs to be revised in a way that can be more interactive and clear. Perhaps an addition of a diagram would be helpful.
According to the referee's suggestion, we have revised the discussion and added a figure (new Figure 7) to make it easier to follow.
-In the first paragraph, the authors stated that "Here, we demonstrate that fructose inhibits liver fatty acid β-oxidation by reducing PPARα expression and activity, mainly by decreasing the expression and activity of SIRT1."
Please be more précised as this was shown only after 14-day fructose supplementation.
We have modified the sentence, and added some discussion on fructose effects at 7 days (pg 16, ln 18-20) of the new version of the manuscript).
-Page 16: Please rephrase following statement: "Thus, we were disappointed that fructose incubation of FaO cells, despite reducing PPARα expression, did not reduce SIRT1 and NAMPT.
We have rephrased the sentence as: "However, fructose incubation of FaO cells, despite reducing PPARα expression, did not reduce SIRT1 and NAMPT"
-Page 16: It is not clear to me as how a short incubation period of FaO cell with fructose is not sufficient to repress the SIRT1 expression, but reduces its deacetylase activity strongly enough to reduce the expression of PPARα. In addition, a question arises as how long incubation of FaO cells with fructose could be sufficient to repress the SIRT1 expression?
Our data shows that fructose at high concentrations directly or indirectly inhibits SIRT1 activity without modifying SIRT1 expression. We don't know how much time would be necessary to modify SIRT1 expression, but we could not extend incubation times for longer than 48 h in FaO cells.
-Page 17: The author stated that "we also found an increased amount of acetylated-ChREBP protein in livers of 14-day fructose-supplemented rats (Figure 4E)".
I assume the right figure for the above statement is 6E and not 4E. 4E should be replaced by 6E.
Yes, the referee is right; we have replaced 4E by 6E in the new version of the manuscript.

-Page 17: The authors concluded that “In conclusion, fructose depresses PPARα expression and activity, and thus fatty acid β-oxidation, in rat liver cells and human hepatocytes by a mechanism involving a reduction of SIRT1 expression and activity”
The decreased of PPARα expression and activity was observed after 14 days fructose supplementation and a reduction of SIRT1 was not observed in rat liver cells. Please revise the above statement to reflect the observations accordingly.
According to the referee’s suggestion, we have revised this final statement and reformulated it to reflect more precisely the main conclusions of our study: “In conclusion, fructose depresses PPARα expression and activity, in hepatic tissue from 14-days fructose-supplemented rats and in rat and human liver cells, by a mechanism that could involve a concerted increase in ChREBP and a reduction of SIRT1 expression and activity." This has been included in the new version of the manuscript (Discussion, pg 19, last paragraph).

Cover Letter

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Dr. Rudolf Zechner

Executive Editor
BBA – Molecular and Cell Biology of Lipids

Barcelona, December 13, 2013

Dear Dr. Zechner,

We are re-submitting to the *Biochimica and Biophysica Acta-Molecular and Cell Biology of Lipids* journal the manuscript entitled "LIQUID FRUCTOSE DOWNREGULATES SIRT1 EXPRESSION AND ACTIVITY AND IMPAIRS THE OXIDATION OF FATTY ACIDS IN RAT AND HUMAN LIVER CELLS", authored by Alba Rebollo, Núria Roglans, Miguel Baena, Rosa M Sánchez, Manuel Merlos, Marta Alegret and Juan C Laguna. As you will see in the new version of the manuscript, we have followed the majority of suggestions proposed by the referees, including new data and a new figure (Figure 7). We include a letter answering all the referees' queries, properly discussing our position when the referee's query is refuted.

We appreciate very much the opportunity of resubmission; we are convinced, and we deeply expect that you too, that the new version of the manuscript is much improved and will merit your approval.

Yours sincerely,

Dr. Juan C. Laguna

Reviewer #1:

Major points:

1. In order for the reader to comprehend the nutritional setting in which the changes are being observed, far more detail needs to be included to support/strengthen table 1. Details on the nutritional content of the diet must be included (% calories from fat etc..). In the same sense, it would be useful to see the data presented as total calories consumed from diet vs fructose in drinking water.

In this study the rats received a regular diet (Teklad Global 2018 Rodent Diet, fromHarlan Teklad), that provided 18% calories from fat, 24% from protein and 58% from carbohydrate. This information has been included in the new version of the manuscript (Materials and Methods section, pg 5, ln 22-23).

Regarding the calories consumed from diet or from fructose, we calculated the data from the area under the curve of food or drink consumption in g or ml/days/cage (containing two rats). Our results are the following:

	7days		14 days	
	Control	Fructose	Control	Fructose
Kcal from food	726.4	566.4	1609.6	1254.4
Kcal from drink	0	338	0	798
Total kcal	726.4	904.4	1609.6	2052.4

As we already stated in our first version of the manuscript, “rats increased their calorie intake in a similar way at 7 (x1.24-fold) and 14 days (x1.27-fold), mainly due to an increase in fructose calories (x1.37 and x1.39-fold at 7 and 14 days, respectively), which was not compensated by a reduction in the ingestion of solid food”. Perhaps it was not clear enough, so we have rephrased the sentence (pg 11, ln 8-13): “rats increased their calorie intake in a similar way, from 726.4 to 904.4 kcal/7days/2 rats (increase of 1.24-fold) and from 1609.6 to 2052.4 kcal/14days/2 rats (x1.27-fold), at 7 and 14 days, respectively. The increase was mainly due to calories obtained from fructose, which represented a 37 and 39% of the total calories consumed at 7 and 14 days, respectively. This increase was not compensated by a sufficient reduction in the ingestion of solid food”

2. *The authors state that body weight changes were not observed, but the values should be included for the readers' benefit, and it would also be beneficial to know roughly what was happening in other key metabolic tissues, such as adipose tissue. For instance leptin levels increase significantly at 14 weeks, suggestive of an expansion of adipose tissue lipid content. One might postulate that in the face of impaired lipid oxidation, the liver is exporting more triglyceride in VLDL particles which are being taken up by the white fat. Basic histological examination of WAT and tissue weights would be good and examination measurement of key lipid handling genes even better. Likewise, analysis of lipid synthetic genes or genes regulating triglyceride release (MTP) in liver would be illuminating in this sense. The increased serum TG have to be coming from somewhere - and the data indicate that it is not from the diet.*

As the referee suggest, we have included body weight and white adipose tissue weight data in Table 1 of the new version of the manuscript. As can be seen in this table, there is no significant difference in total body weight (either expressed as area under the curve in g/7 or 14 days/rat or as body weight at the end of treatment) between control and fructose groups. Regarding adipose tissue weight, we neither observed statistically significant changes. On the other hand, plasma leptin levels are not significantly increased at 14 days

Further, we already measured the expression of the genes involved in lipid synthesis, specifically liver-pyruvate kinase (L-PK), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), glycerol phosphate acyltransferase 1 (GPAT1) and acetyl-CoA carboxylase (ACC), and we found that fructose similarly induced their expression after 7- and 14-day supplementation (data in Table 2). Thus, serum and hepatic triglycerides come from the combined increase in lipid synthesis and reduction of fatty acid oxidation.

Regarding additional experiments suggested by the referee, we did not perform basic histological examination of WAT at the moment of sacrifice. Nevertheless, we could determine the mRNA levels of MTP in hepatic samples from treated animals. Our results were:

	7days		14 days	
	Control	Fructose	Control	Fructose
MTP	1 ± 0.14	1.21 ± 0.40	1 ±0.19	1.33 ± 0.33

The lack of increase of MTP expression is not surprising, as MTP expression is regulated by reduced lipid availability, but an increase in the amount of lipids would not necessarily induce MTP expression. It would have been better to determine MTP activity, but this has to be performed in freshly obtained hepatic samples.

3. *Long chain fatty acid beta-oxidation is clearly impaired by fructose treatment, but is there any compensation via increased oxidation of short chain fatty acids? Are genes regulating fatty acid elongation down-regulated for instance?*

In our study we fed rats with a regular chow diet that did not provide short chain fatty acids (SCFA) but long chain fatty acids (LCFA: palmitic, stearic, oleic, linoleic and linolenic acids). SCFA could also be formed from colonic fermentation of dietary fiber, but the regular chow that we used in our study has a low amount of crude fiber (3.5%), and moreover, its consumption was reduced in fructose-supplemented animals. Thus, the bulk of fatty acids reaching the hepatic cells of the rats in our study are LCFA. In this case, SCFA could only derive from the β-oxidation of these LCFA, but as this is reduced in fructose-fed rats, the amount of SCFA formed should also be reduced. Therefore, if a compensatory response of increased SCFA oxidation existed, it would not result in a meaningful metabolic effect.

Regarding fatty acid elongation, there are some reports showing that the expression of hepatic elongases (Elovl) might be controlled by several hormones and transcription factors, including PPARα, SREBP-1 and ChREBP (Wang et al., J Lipid Res. 2005, 46:706; Wang et al., J Lipid Res. 2006, 47: 2028). It has been shown that carbohydrates induce hepatic Elovl-6 along with L-PK and FAS through ChREBP (Wang et al., J Lipid Res. 2006, 47:2028). Thus, as the referee suggested, we decided to determine the expression of hepatic Elovl-6 in hepatic samples from

our control and fructose-supplemented rats. According to the general induction of genes involved in lipid synthesis, we also found a significant increase in Elovl6 expression at both 7 and 14 days. These results have been included in the text (pg 12, ln 4 and in Table 2).

4. *What effects are there on other key lipid-burning tissues? For instance what happens to beta-oxidative genes and UCP1 in the brown adipose tissue? This data would enable the specificity and relative contribution of the effects of fructose on hepatic lipid metabolism*

Unfortunately, we did not obtain brown adipose tissue (BAT) from the rats of our study. UCP-1 is a hallmark of BAT and under basal conditions it is not expressed in white adipose tissue (WAT). A recent report (Li et al, Gastroenterology, Epub ahead of print 2013 Oct 31, doi: 10.1053/j.gastro.2013.10.059) showed that increased SIRT-1 activity increases the levels of the hepatocyte-derived hormone fibroblast growth factor 21 (FGF21), and this enhanced energy expenditure through white fat "browning" (with increased expression in WAT of typical BAT genes such as UCP-1). However, in our study we detected a decrease, not an increase in SIRT-1 expression and activity. Moreover, we did not observe changes in the mRNA expression of hepatic FGF21 after 14 days of fructose supplementation (data not shown in the paper). Thus, hepatic FGF21 mRNA levels, expressed in arbitrary units were 1.00 ± 0.44 (control rats) and 0.93 ± 0.27 (fructose-supplemented rats). These results suggest that UCP-1 would not be expressed in WAT from fructose-supplemented animals.

Minor points:

1. The concept that mitochondrial long-chain fatty acid oxidation is impaired by fructose is not novel. In fact a paper from 1976 already demonstrates this effect and should at least be cited. (Prager GN, Ontko JA. Direct effects of fructose metabolism on fatty acid oxidation in a recombined rat liver mitochondria-high speed supernatant system. Biochim Biophys Acta. 1976 Mar 26;424(3):386-95.)

According to the referee's suggestion we have cited this reference in the novel version of the manuscript (pg 4 ln 15-16)

2. Table one title should read "liver triglycerides".

The spelling error has been corrected in the new version of the manuscript.

3. Figure 6E does not appear to be discussed in the text?

This was also an error, as Figure 6E corresponds to the levels of acetylated ChREBP protein in liver samples from control and 14-day fructose-supplemented rats, but in the text it was cited as 4E. This error has been corrected in the new version of the manuscript (pg 19, ln 1).

Reviewer #2

General comments:

This is a short manuscript. The rationale is described clearly and the manuscript reports the important observation on the inhibition of fatty acid oxidation and underlying enzymes in female rat liver; however, a number of points need to be clarified.

In addition, the discussion is hard to follow. Therefore, a revision of manuscript as well as an addition of a diagram that summarizes the interaction between transcription factors leading to the activation or inhibition of PPAR<alpha> and fatty acid oxidation would be very helpful.

According to the referee's suggestion, we have revised the discussion and added a figure (new Figure 7) to make it easier to follow.

Specific comments:

Introduction:

-The introduction is very short and in my opinion it is not complete. For instance, it doesn't give the logics behind measuring PPAR<alpha> expression and activity. The role of PPAR<alpha> in the fatty acid oxidation should be stated. This justifies why the authors measuring liver transcription factors and enzymes controlling PPAR<alpha>. In addition, the authors should introduce the PP2A and its possible role in the in PPAR<alpha> activity in Introduction. This justifies the use of okadaic acid and the Enzyme (PP2A) activity assays in Method section (2.5).

The role of PPARα on fatty acid oxidation, as well as the possible role of PP2A in the inhibition of the PPARα system has been clearly stated in the introduction section in the new version of the manuscript (pg 4 ln 21-23 and pg 5 ln 1-3).

-Please rephrase the following statement:

Page 4: "Reported reasons are high energy intake, lack of adequate energy compensation and the special metabolism of fructose [1,5]."

The sentence has been rephrased as: "High energy intake, lack of adequate energy compensation and the special metabolism of fructose have been reported as reasons to explain this association [1,5]."

-Page 4: "As we wanted to understand the mechanism of fructose effects, we studied the effect of fructose.

You may rephrase it as "The aim of our study was to investigate the effect of fructose..

We have re-written the sentence as the referee suggests

Methods:

-It requires the justification for selection of use of 7 or 14 days and 10% fructose for 7-14 days or 25 mM for 24 h in vitro. How comparable or relevant these concentrations of fructose are to in vivo ingestion by human? Is it considered the medium range of ingestion or high?

Diets that incorporate 10 % weight/volume fructose concentrations in drinking water mimic the human pattern of fructose consumption, with daily fructose intake equivalent to that found in the upper quartile of fructose consumption in human populations. We have used this model of fructose administration during 14 days in several previous studies, e.g. Roglans N et al., Hepatology 2007, 45:778. In the present article we wanted also to study fructose effects at an earlier time point (7 days) and also, to avoid fluctuations in the amount of sugar ingested in

the in vivo studies, to assess the effects of fructose on liver in vitro. Concentrations of fructose in the portal circulation can easily reach 5–10 mM (Du L, Heaney AP. Mol Endocrinol. 2012, 26:1773), but as for in vitro studies incubation times cannot be as long as in vivo exposure times, we decided to use higher fructose concentrations (25 mM), similar to previous studies of our group (Vilà et al., Hepatology 2008, 48:1506; Rodriguez-Calvo R et al., Hepatology 2009, 49:106).

-Page 6: In some experiments, 100 <mu>M of Wy-14643 and 20 nM of Okadaic acid were added 3 hours and 30 minutes, respectively, before fructose. 100 nM of SRT1720 was added to the medium 12 hours after fructose supplementation.

Please state the action of these drugs when stated the first time in the manuscript. For instance, Wy-14643 (a PPAR<alpha> ligand), Okadaic acid (an inhibitor of PP2A), SRT1720 (a selective activator of SIRT1), Okadaic acid (an inhibitor of PP2A)

According to the referee's suggestion, we have defined the action of the drugs when first cited in the Methods section.

-Page 7, section 2.5: The concentration and the duration of treatment with TSA are missing.

TSA was added at a concentration of 1 µM for 12 h. This has been added in the new version of the manuscript (pg 7, section 2.5).

-Page 7, section 2.6: Why were different internal controls used for 7 and 14 days fructose supplemented rats? APRT was used for 7 days and 18s for 14 days group of rats.

The referee may be mistaken, as in fact we used APRT for 14 days and 18 s for 7 days. In our previous studies in rats treated with fructose for 14 days we always used APRT as an internal control. However, when we started experiments using shorter treatment periods, we observed that the expression of APRT changed in samples from fructose-fed rats compared with controls. Therefore, we decided to use 18s as an internal control for these samples.

-Page 8, Section 2.7: It requires more details on the Western blot analysis.

In the new version of the manuscript we have provided more details, such as blocking conditions, dilutions of each primary antibody and conditions of incubation with secondary antibodies (pg 8 ln 22- pg 9 ln 11).

Results:

-According to the table 1, it seems there is a tendency for increase of leptin in both 7 and 14 days fructose supplementation to rats. Regardless the fatty acid oxidation is inhibited. This point should be discussed in Discussion. Have the authors also measured the level of adiponectin?

The differences in plasma leptin levels between control and fructose-treated rats are not statistically significant. Consequently, we consider that there is no need to speculate on this issue. On the other hand, we measured plasma adiponectin levels. These results have now been included in Table 1 and in page 11 in the new version of the manuscript. There is a

significant increase in plasma adiponectin levels only in rats supplemented with fructose for 14 days. This increase was also detected in previous studies in male rats supplemented with either 10% glucose or 10% fructose (Roglans N et al., Hepatology 2007, 45:778), suggesting that this is not a specific effect of fructose. We do not believe that this data has enough relevance to be discussed in the article.

-Page 10, Section 3.1: How was the degree of steatosis measured in the fructose ingested groups, and how compared between two groups of 7- and 14-days fructose supplemetation? The authors did not give any values but stated that liver steatosis was confirmed by histological analysis.

We quantified the amount of triglycerides in hepatic samples from fructose-supplemented and control rats. As shown in Table 1, the hepatic triglyceride content was not significantly increased after 7 days of fructose supplementation, but there was an increase of 1.9-fold (from 4.1 ± 2.3 to 7.7 ± 2.4 mg/g liver, $p<0.05$) after 14 days of treatment. Steatosis was confirmed qualitatively by histological analysis in liver sections stained with Oil Red O (Figure 1 C).

-Page 11, section 3.3: It is still unclear why the PPAR<alpha> expression was increased in 7 days fructose supplementation to rats. This should be discussed in Discussion.

It is interesting to discuss why fatty acid oxidation is repressed at 7 days despite higher PPARα activity. It is probably due to higher ACC expression leading to malonyl-CoA production, which inhibits L-CPT1 and therefore reduces beta oxidation activity. We have included this in the Discussion section in the new version of the manuscript (page 16, ln 18-20). We are not certain about the mechanisms by which the expression and activity of PPARα at 7 days is increased. It is possible that in this situation there is an increase in endogenous PPARα ligands, or changes in the expression of co-activators and co-repressors leading to an increase of PPARα expression and its target genes.

-Page 11, Section 3.3: The authors mentioned about the possible mechanisms which are responsible for inhibition of fatty acid <beta>-oxidation, despite opposite changes in PPAR<alpha>. "This inhibition of fatty acid <beta>-oxidation, despite opposite changes in PPAR<alpha>, could be attributed to two mechanisms: 1. Livers from 7- and 14-day fructose supplemented rats showed increased expression of total and phosphorylated ACC (Table 2), implying increased production of malonyl-CoA, a known allosteric inhibitor of L-CPT-I, whose activity controls the whole fatty acid <beta>-oxidation system [24]; 2. ChREBP activity controls the expression of RGS16, a physiological inhibitor of the fatty <beta>- oxidation system [25]."

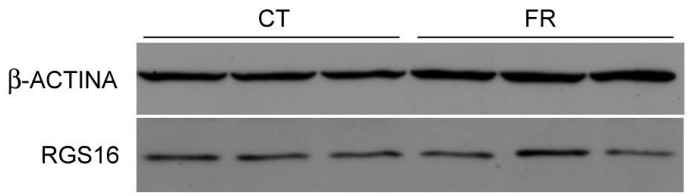
First of all, the above statements should be moved to Discussion, and if I understand it correctly, in both 7- and 14-days fructose supplementation, the RGS16 was enhanced (Fig 2C). If the ChREBP activity controls the RGS16 (inhibitor of FA oxidation), please then clarify that as how the ChREBP was enhanced only in 14-days supplementation?

In order to follow the rationale behind the shown experiments, we consider that this piece of text is correctly placed in this section. The referee is right; the mRNA levels of RGS16 are equally increased at both time-points. To be sure of the physiological meaning of this induction, we measured the protein expression of RGS16 in hepatic samples from fructose-

supplemented and control rats. The protein levels were not modified at any of the treatment times, ruling out the involvement of RGS16 in the inhibition of hepatic fatty acid oxidation:

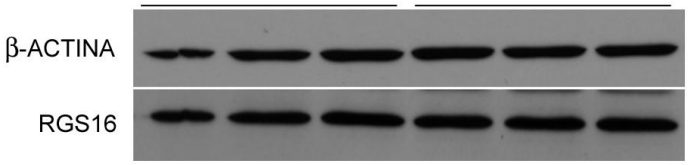
7days:

Control	Fructose
1.00 ± 0.14	1.04 ± 0.36



14 days:

Control	Fructose
1.00 ± 0.17	1.09 ± 1.12



This information has been added in the new version of the manuscript (new Figure 2D) and in the discussion (pg 18, ln 9-12).

Here, the authors explaining the inhibition of fatty acid <beta>-oxidation, despite opposite changes in PPAR<alpha> through 1) enhanced production of malonyl-CoA through ACC phosphorylation and 2) Deacetylation and activation of PGC-1<alpha> (a cofactor of PPAR<alpha>, necessary for the transcriptional control of genes related to fatty acid oxidation.

We know Malonyl CoA is synthesized in the liver by ACC, which in turn is phosphorylated and inhibited by AMPK. On the other hand, AMPK increases FA oxidation directly by PPAR<alpha> activation. It would be interesting to measure the AMPK expression or activity in both 7 and 14-day fructose supplementation and to see whether its level is reduced in these groups. I am not suggesting additional experiments, but if the AMPK level was measured already, it would be nice to include it.

According to the referee's suggestion, we measured the expression of total and phosphorylated AMPK, and we did not observe significant differences between control and fructose-supplemented animals. These results have been included in the new version of the manuscript (Table 3).

	7days		14 days	
	Control	Fructose	Control	Fructose
p-AMPK	1.00 ± 0.32	1.06 ± 0.20	1.00 ± 0.24	0.91 ± 0.14
Total AMPK	1.00 ± 0.34	0.98 ± 0.17	1.00 ± 0.28	1.28 ± 0.20

The lack of increase in phospho-AMPK suggests that its kinase activity is not enhanced. Consequently, the ratio between phosphorylated and total ACC is not increased. Thus, the increase in ACC expression as a consequence of the activation of lipogenesis should lead to an increase in malonyl-CoA production.

A decrease in SIRT1 expression and activity (based on NAMPT) (Figs 6A-B) and consistently inactivity of PGC-1 (Fig 6D) could be responsible for the reduced PPAR<alpha> and reduced FA oxidation in 14-day fructose supplementation group. However, it is still unclear how we get to an increased PPAR<alpha> in 7-day fructose supplementation. Obviously we see no changes in SIRT1 expression and in NAMPT in this group.

We agree with the referee, the increase in the expression and activity of PPARα at 7 days is not related to changes in SIRT1 or NAMPT expression. Our hypothesis is that PPARα increase at 7 days could be a compensatory mechanism, an attempt to enhance fatty acid oxidation in the initial phases of fructose consumption, maybe related to an increase in endogenous PPARα ligands, or to changes in the expression of co-activators and co-repressors.

-The authors also mentioned that "The liver fatty acid <beta>-oxidation system was probably repressed by products of the metabolism of fructose at the time of death, independently of the actual expression and transcriptional activity of PPAR<alpha>."

This hypothesis need to be more clarified based on their data and observation.

As this sentence was confusing, we have deleted it in the new version of the manuscript.

-Figure 2C: How do you explain so much variability in mRNA bands of RGS16 of controls (n= 3)?

There was a mistake in the legend for Figure 2. Instead of “Each bar represents the mean±sd of three different samples” it should read “Each bar represents the mean±sd of values obtained from n=4 and n=5 animals (for control and fructose groups, respectively).” The autoradiography above the figure shows the bands corresponding to Rsg16 mRNA and that of the aprt gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of 3 animals from each treatment group. We have corrected the mistake in the figure legend. We cannot explain the variability, but we are confident in our results, because they come from 4-5 different animals per group of treatment. Moreover, when we performed statistical analysis, we found that the increase was statistically significant, despite the variability.

-Page 13, PGS16 needs to be replaced by RGS16. RGS16 stands for "Regulator of G protein signaling".This should be added to the Manuscript.

The complete name of the protein has been added in the new version of the manuscript.

-Page 14: The following statement should be moved to Discussion.

"Thus, the effect of fructose on the expression of these genes was independent of PP2A activity, corroborating previous research by Dentin et al. [28]."

The statement has been rephrased and moved

-Page 14, section 3.6: In Table 3, the authors showed that the expression of Foxa2, a transcription factor regulating fatty acid oxidation, was reduced only after 14-day fructose supplementation. However <beta> oxidation of fatty acid was reduced after both 7 and 14-day fructose supplementation. This requires further clarification.

The reduction of Foxa2 expression after 14 days of fructose treatment, although significant is just of 13%. This suggests that its biological significance is not relevant, and does not deserve further consideration.

-Page 14, Section 3.6: The authors stated "fructose was also able to efficiently block the increase in PPAR<alpha> expression induced by incubating FaO cells with SRT1720, a potent and selective activator of SIRT1 [31] (Figure 6). "

Figure 6 must be replaced by Figure 6C.

The replacement has been done as indicated by the referee.

-There is no any text in this section referring to Figure 6E.

It is an error, as Figure 6E corresponds to the levels of acetylated ChREBP protein in liver samples from control and 14-day fructose-supplemented rats, but in the text it was cited as 4E. This error has been corrected in the new version of the manuscript (pg 19, ln 1).

Discussion

-In general, the Discussion is difficult to follow. It needs to be revised in a way that can be more interactive and clear. Perhaps an addition of a diagram would be helpful.

According to the referee's suggestion, we have revised the discussion and added a figure (new Figure 7) to make it easier to follow.

-In the first paragraph, the authors stated that "Here, we demonstrate that fructose inhibits liver fatty acid <beta>-oxidation by reducing PPAR<alpha> expression and activity, mainly by decreasing the expression and activity of SIRT1."

Please be more précised as this was shown only after 14-day fructose supplementation.

We have modified the sentence, and added some discussion on fructose effects at 7 days (pg 16, ln 18-20) of the new version of the manuscript).

-Page 16: Please rephrase following statement: "Thus, we were disappointed that fructose incubation of FaO cells, despite reducing PPAR<alpha> expression, did not reduce SIRT1 and NAMPT.

We have rephrased the sentence as: "However, fructose incubation of FaO cells, despite reducing PPARα expression, did not reduce SIRT1 and NAMPT"

-Page 16: It is not clear to me as how a short incubation period of FaO cell with fructose is not sufficient to repress the SIRT1 expression, but reduces its deacetylase activity strongly enough

to reduce the expression of PPAR<alpha>. In addition, a question arises as how long incubation of FaO cells with fructose could be sufficient to repress the SIRT1 expression?

Our data shows that fructose at high concentrations directly or indirectly inhibits SIRT1 activity without modifying SIRT1 expression. We don't know how much time would be necessary to modify SIRT1 expression, but we could not extend incubation times for longer than 48 h in FaO cells.

-Page 17: The author stated that "we also found an increased amount of acetylated-ChREBP protein in livers of 14-day fructose-supplemented rats (Figure 4E)".

I assume the right figure for the above statement is 6E and not 4E. 4E should be replaced by 6E.

Yes, the referee is right; we have replaced 4E by 6E in the new version of the manuscript.

-Page 17: The authors concluded that "In conclusion, fructose depresses PPAR<alpha> expression and activity, and thus fatty acid <beta>-oxidation, in rat liver cells and human hepatocytes by a mechanism involving a reduction of SIRT1 expression and activity"

The decreased of PPAR<alpha> expression and activity was observed after 14 days fructose supplementation and a reduction of SIRT1 was not observed in rat liver cells. Please revise the above statement to reflect the observations accordingly.

According to the referee's suggestion, we have revised this final statement and reformulated it to reflect more precisely the main conclusions of our study: "In conclusion, fructose depresses PPAR<alpha> expression and activity, in hepatic tissue from 14-days fructose-supplemented rats and in rat and human liver cells, by a mechanism that could involve a concerted increase in ChREBP and a reduction of SIRT1 expression and activity." This has been included in the new version of the manuscript (Discussion, pg 19, last paragraph).

Highlights

. Fructose reduces PPARα expression and activity *in vivo* and in rat and human liver cells
. Fructose effect on PPARα is not related to an increased protein-phosphatase A2 activity
. Fructose reduces liver expression and activity of sirtuin 1 deacetylase
. Increased liver content of acetylated PGC 1α could be responsible for fructose-mediated effects on PPARα

*REVISED Manuscript (text UNmarked)
Click here to view linked References

LIQUID FRUCTOSE DOWNREGULATES SIRT1 EXPRESSION AND ACTIVITY AND IMPAIRS THE OXIDATION OF FATTY ACIDS IN RAT AND HUMAN LIVER CELLS

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Abstract:

Fructose ingestion is associated with the production of hepatic steatosis and hypertriglyceridaemia. For fructose to attain these effects in rats, simultaneous induction of fatty acid synthesis and inhibition of fatty acid oxidation is required. We aimed to determine the mechanism involved in the inhibition of fatty acid oxidation by fructose and whether this effect occurs also in human liver cells. Female rats were supplemented or not with liquid fructose (10% w/v) for 7 or 14 days; rat (FaO) and human (HepG2) hepatoma cells, and human hepatocytes were incubated with fructose 25 mM for 24 hours. The expression and activity of the enzymes and transcription factors relating to fatty acid β-oxidation were evaluated. Fructose inhibited the activity of fatty acid β-oxidation only in livers of 14-day fructose-supplemented rats, as well as the expression and activity of peroxisome proliferator activated receptor α (PPARα). Similar results were observed in FaO and HepG2 cells and human hepatocytes. PPARα downregulation was not due to an osmotic effect or to an increase in protein-phosphatase 2A activity caused by fructose. Rather, it was related to increased content in liver of inactive, acetylated peroxisome proliferator activated receptor gamma coactivator 1α, due to a reduction in sirtuin 1 expression and activity. In conclusion**,** fructose inhibits liver fatty acid oxidation by reducing PPARα expression and activity, both in rat and human liver cells, by a mechanism involving sirtuin 1 down-regulation.

Key words: PPARα, steatosis, triglycerides, ChREBP

Abbreviations: ACO, acyl-CoA oxydase; AMPK, AMP-activated protein kinase; APRT, adenosyl phosphoribosyl transferase); ACC, acetyl-CoA carboxylase); ChREBP, carbohydrate response element binding protein; CYP4A1, cytochrome P450 4A1; FAS, fatty acid synthase; Foxa2, forkhead box protein A2; GK, glucokinase; GPAT1; glycerol phosphate acyltransferase 1; HNF4, hepatic nuclear factor 4; L-CPT-I, liver carnitinepalmitoyl-CoA transferase I; L-PK, liver-pyruvate kinase; NAMPT, nicotinamide phosphoribosyltransferase; PGC-1α, peroxisome proliferator activated receptor gamma coactivator 1α; PP2A, protein phosphatase 2 A; PPARα, peroxisome proliferator activated receptor α; SCD1, stearoyl-CoA desaturase 1; SIRT1, sirtuin 1; SREBP1, sterol response element binding protein 1; T2DM, type 2 diabetes mellitus; Trib-3, mammalian *tribbles* homolog-3; TSA, trichostatin A.

1. Introduction

There is a world-wide pandemic of human diseases, such as obesity, related to an unbalanced energy intake. Further, the International Diabetic Federation projects 380 million adults suffering type-2 diabetes mellitus (T2DM) by the year 2025 [1]. There is also increased prevalence of risk factors for obesity and T2DM, such as hepatic steatosis [2] and hypertriglyceridaemia [3].

Epidemiological evidence suggests a causal association with the consumption of sugar-sweetened beverages by human populations [1,4]; fructose is used as a sweetener in liquid beverages [1,4,5]. High energy intake, lack of adequate energy compensation and the special metabolism of fructose have been reported as reasons to explain this association [1,5]. Fructose ingestion is also associated with the production of hypertriglyceridemia [6] and hepatic steatosis [7].

In vitro studies suggest that fructose directly inhibits the oxidation of fatty acids in rat liver mitochondria [8]. We showed, in a rat model of liquid-sugar feeding, that to induce hypertriglyceridemia and hepatic steatosis, a simultaneous induction of fatty acid synthesis and inhibition of fatty acid β-oxidation is required [9,10]. The reduction in the activity of the hepatic fatty acid β-oxidation system by fructose correlated with a decrease in the expression and activity of peroxisome proliferator–activated receptor α (PPARα), a nuclear receptor that plays a key role in the transcriptional control of genes encoding fatty acid β-oxidation enzymes [9, 10].Fructose, but not glucose, despite the latter’s being ingested in identical quantity, was the only sugar able to induce these changes [9]. The mechanisms by which fructose inhibits the PPARα

system are not fully elucidated. Recently, Ravnskjaer et al. proposed that glucose inhibits PPARα in pancreatic cells by activating protein phosphatase 2 A (PP2A) [11]. PP2A is also activated by other carbohydrates, such as fructose, through a common metabolite, xylulose-5-phosphate [10].

Sirtuin 1 (SIRT1) is a NAD^+-dependent deacetylase that modulates the functions of many proteins involved in the process of ageing and metabolic disorders. SIRT1 is activated by caloric restriction and selective drug activators [12,13]. Feige et al. [14] demonstrated that activation of SIRT1 in skeletal muscle and liver enhanced fatty acid oxidation, protecting from diet-induced fatty liver, obesity and insulin-resistance. The aim of our study was to investigate the effect of fructose supplementation on female Sprague-Dawley rats, rat (FaO) and human (HepG2) hepatoma cells and human hepatocytes. We examined liver transcription factors and enzymes controlling PPARα and fatty acid oxidation, including SIRT1 expression and activity.

2. Material and methods

2.1. Animals and experimental design

Female Sprague-Dawley rats (Charles River, Barcelona, Spain), were maintained at constant humidity and temperature, with a light/dark cycle of 12 hours, and had *ad libitum* access to water and to a regular diet (calories from fat 18%, from protein 24% and from carbohydrate 58%, cat. 2018, Harlan Teklad). Rats were randomly assigned to a control group and a fructose-supplemented group (8 and 12 rats per group, respectively). Fructose was supplied as a 10%

(weight/volume) solution in drinking water for 7 and 14 days. Then, food and fructose were removed at 8 a.m. and the animals were killed by decapitation under isoflurane anesthesia at 10 a.m. To reduce variability in plasma oestrogen concentrations, female rats were killed during the diestrus period.

Blood and liver tissue samples were collected and stored as described elsewhere [9]. All procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee (Autonomous Government of Catalonia's Act 5/1995, July 21).

2.2. Cell culture

Rat hepatoma FaO cells from the European Collection of Cell Cultures (ECACC) were cultured in low-glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS Gold, PAA) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Invitrogen). At 80% confluence, serum was reduced to 1%. Cells were incubated in the absence or presence of fructose, glucose or mannitol (Sigma-Aldrich, Madrid, Spain) at 25 mM for 24 hours [10]. Human hepatoma HepG2 cells from the EuCellBank (Barcelona, Spain) were cultured as FaO cells. Human hepatocytes (*Ready Heps™ Fresh Hepatocytes*) were from Lonza (Basel, Switzerland). They were cultured and treated in HMM™ medium (*Hepatocyte Medium Maintenance System* from Lonza). In some experiments, 100 µM of Wy-14643, a PPARα agonist (Sigma-Aldrich, Madrid, Spain), and 20 nM of Okadaic acid, a potent inhibitor of PP2A phosphatase, (Sigma-Aldrich, Madrid, Spain) were added 3 hours and 30 minutes, respectively, before fructose. 100 nM of the specific

SIRT-1 activator SRT1720 (Cayman Chemical, Ann Arbor, MI, USA) was added to the medium 12 hours after fructose supplementation.

2.3. Sample preparations

Total and nuclear extracts from rat liver and cells were isolated by the Helenius method [15]. Protein concentrations were determined by the Bradford method [16].

2.4. Lipids and Leptin analysis

Plasma triglycerides were measured by the Triglyceride L-Q test (Spinreact, Girona, Spain). Plasma leptin was determined with the RL-83K RIA kit (Millipore, Bedford, MA, USA). Liver triglycerides were extracted and measured as described by Roglans et al. [17].

2.5. Enzyme activity assays

Hepatic fatty acid ß-oxidation activity was determined in rat livers, as in [18], with 30 µg of postnuclear supernatant. PP2A activity in FaO cells was determined by the colorimetric kit *Sensolyte pNPP Protein Phosphatase Assay Kit* (AnaSpec, Fremont, CA, USA). A co-immunoprecipitation, using 100 µg of total protein and 4 µg of anti-PP2A antibody (Millipore, Bedford, MA, USA), was conducted to purify the PP2A protein. SIRT1 activity was analyzed in FaO cells

by the *HDAC Fluorimetric Cellular Activity Assay* (BML-AK503, Enzo Life Sciences, Ann Arbor, MI, USA). To evaluate SIRT-1 activity selectively, TSA (trichostatin A, 1µM, 12h) was used as a non-sirtuin deacetylase inhibitor.

2.6. RNA preparation and analysis

Total RNA was isolated by using the Trizol^{R} reagent (Invitrogen, Carlsbad, CA, USA). Specific mRNAs were assessed by reverse transcription polymerase chain reaction (RT-PCR) [17]. As internal control, adenosyl phosphoribosyl transferase (APRT) was used for samples from 14-day fructose-supplemented rats and FaO cells and 18s was used for HepG2, human hepatocytes and 7-day fructose-supplemented rats. The number of cycles, primer sequences, and resulting PCR products are listed in Supplementary Table 1. The mRNA levels were always expressed as the ratio to APRT or 18s mRNA levels.

2.7. Western blot analysis

Total and nuclear proteins (10 to 30 µg from rat livers and 10 to 20 µg from cells) were subjected to SDS-polyacrylamide gel electrophoresis [9,10]. Proteins were then transferred to Immobilon polyvinylidene diflouride transfer membranes (Millipore, Bedford, MA) and blocked for 1 hour at room temperature with 5% non-fat milk solution in TBS-0.1% Tween-20. Membranes were then incubated with the primary polyclonal antibody raised against ACC (dilution 1:1,000), ChREBP, Foxa2, HNF4 and NAMPT (dilution 1:500), PP2Ac,

PPARα (dilution 1:1,000), SIRT1 (dilution 1:500) and SREBP-1c (dilution 1:200). Incubations with primary antibodies were performed in TBS–0.1%Tween-20 with 5% non-fat milk (except for ACC, NAMPT and PP2Ac determination, which used 5% bovine serum albumin) at 4°C overnight. After several washes, they were incubated with horseradish peroxidase–conjugated anti-rabbit IgG or anti-goat IgG (1:3,000 dilution) for ChREBP determination. Detection was achieved using the ECL chemiluminescence kit for horseradish peroxidase (Amersham Biosciences). The size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Life Technologies). Antibodies were from Santa Cruz Biotechnologies (Dallas, Texas, USA), except those for phospho- and total ACC which were from Cell Signaling (Danvers, MA, USA) and PPARα from AbCam (Cambridge, UK).

2.8. Co-immunoprecipitation

100 µg of nuclear extracts from rat livers were incubated with 4 µg of anti-acetylated-lysine antibody in a final volume of 0.5 mL with buffer containing 10 mM PBS and 2% BSA for 4 h at 4 °C. Immunocomplexes were captured by incubating with protein A/G Plus-agarose suspension (Santa Cruz Biotechnology) overnight at 4°C on a rocker platform. Beads were collected by centrifugation at 1000xg for 5 min. and washed three times with PBS-containing protease, deacetylase and phosphatase inhibitors. After microcentrifugation, the pellet was resuspended with 40 µl of SDS-PAGE sample buffer and boiled for 5 min at 100°C. The supernatant was electrophoresed on 8% SDS-PAGE and

immunoblotted with an antibody against ChREBP (Santa Cruz Biotechnologies) and PGC-1α (Cayman).

2.9. Electrophoretic mobility shift assays (EMSA)

8 µg of nuclear extracts from FaO cells and rat livers were incubated with a PPRE-L-CPT-I probe obtained from the annealing of single-stranded complementary oligonucleotides spanning nucleotides –266 to -290 of the rat L-CPT-I gene (5'-AGTACGGGCATGGAGCAAAGAGCT-3'), exactly as described elsewhere [19].

2.10. Histological studies

Lipid accumulation, necrosis and fibrosis were analyzed in liver sections stained with Oil Red O, haematoxylin-eosin and trichromic acid, respectively. Images, acquired with an Olympus BX43 microscope, were interpreted at BioBanc (Banc de tumors-IDIBAPS, Barcelona Spain).

2.11. Statistics

The results are expressed as the mean of *n* values ± standard deviation. Plasma samples were assayed in duplicate. Significant differences were established by the unpaired *t*-test or the one-way ANOVA test, with analysis afterwards (GraphPad Software V2.03). When the number of samples was too small or variance was not homogeneous, a non-parametric test was performed. The level of statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Fourteen-day fructose supplementation to rats is necessary to induce fatty liver and hypertriglyceridaemia. Fructose-supplemented rats drank more liquid, reducing their ingestion of solid food (x0.78-fold). The induction of liver fructokinase, which controls the amount of fructose metabolized by cells [20], was the same at 7 and 14 days (x2.0-fold). Thus, rats increased their calorie intake in a similar way from 726.4 to 904.4 kcal/7days/2 rats (increase of 1.24-fold) and from 1609.6 to 2052.4 kcal/14days/2 rats (x1.27-fold), at 7 and 14 days, respectively. The increase was mainly due to calories obtained from fructose, which represented a 37 and 39% of the total calories consumed at 7 and 14 days, respectively. This increase was not compensated by a sufficient reduction in the ingestion of solid food. Visceral adipose tissue and body weight were not modified (Table 1). Despite this, only 14-day fructose-supplemented rats increased their percentage of liver weight (x1.2-fold), liver triglycerides (x1.9-fold), plasma triglycerides (x1.5-fold) and adiponectin levels (x1.7-fold) with unmodified plasma leptin levels (Table 1). While no clear signs of necrosis or fibrosis were detected, liver steatosis was confirmed by histological analysis (Figure 1). Thus, we proceeded to determine liver fatty acid synthesis and liver fatty acid β-oxidation in livers of female fructose-supplemented rats.

3.2. Seven days of fructose supplementation suffices to increase the expression of markers of liver fatty acid synthesis. Fructose similarly increased the liver expression of carbohydrate response element binding

protein (ChREBP) and its target genes related to fatty acid synthesis [21] liver-pyruvate kinase (L-PK), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), glycerol phosphate acyltransferase 1 (GPAT1), acetyl-CoA carboxylase (ACC), and elongation of very long chain fatty acids family member 6 (Elovl6), after 7- and 14-day supplementation (Table 2). As we showed elsewhere [10,20], the expression of the mature form of sterol response element binding protein 1 (SREBP1) and its target gene glucokinase (GK) [22], was not modified (Table 2).

3.3. Fructose induces a bimodal change in the expression and activity of PPARα and its target genes. Liver PPARα expression increased after 7-day supplementation of fructose to rats, while after 14 days, its expression was repressed x0.75-fold (Table 3). The expression of PPARα target genes, such as liver-carnitinepalmitoyl-CoA transferase-I (L-CPT-I), acyl-CoA oxidase (ACO), and the mammalian *tribbles* homolog-3 (Trib-3) [23,24], followed a similar pattern (Table 3), pointing to a similar bimodal change (increase-decrease) in the activity of PPARα. In accordance, the specific retention bands produced after incubation of liver nuclear extracts with an oligonucleotide reproducing the peroxisome proliferator response element (PPRE) of rat L-CPT-I showed a similar change in intensity after 7- and 14-day fructose supplementation (Figure 2A). On the other hand, the expression of total and phosphorylated AMP-activated protein kinase (AMPK), an enzyme which increases fatty acid oxidation in hepatic cells, was not modified (Table 3).

Surprisingly, the activity of the liver fatty acid β-oxidation system, under the transcriptional control of PPARα [25], was similarly reduced x0.55-fold after

7- and 14-day fructose supplementation (Figure 2B). To investigate the mechanisms by which fructose inhibits fatty acid β-oxidation, despite opposite changes in PPARα, we assessed the phosphorylation of ACC. Our results showed increased expression of total and phosphorylated ACC at both 7 and 14 days (Table 2), implying increased production of malonyl-CoA, a known allosteric inhibitor of L-CPT-I that controls the whole fatty acid β-oxidation system [26]. On the other hand, we assessed the expression of the regulator of G protein signaling 16 (RGS16), a physiological inhibitor of the fatty β-oxidation system [27]. Livers of 7- and 14-day fructose supplemented rats had increased expression of RGS16 (Figure 2C), but the protein expression was not modified at any time (Figure 2D).

To avoid variability in response due to fluctuations in the amount of sugar ingested in the *in vivo* studies, we assessed the PPARα system after the incubation of rat liver cells with fructose.

3.4. Fructose represses PPARα expression and activity in liver cells from rat and human origin. FaO rat hepatoma cells incubated for 24 hours with fructose 25 mM showed a reduction in the expression of PPARα and two of its target genes, ACO and cytochrome P450 4A1 (CYP4A1) (Figure 3A-1C). This was not produced by an osmotic shock, as a similar concentration of mannitol induced no change in the expression of these genes (Figure 3A-1C), and was specific to fructose, as glucose induced no significant changes (Figure 3A-1C), in accordance with our previous results *in vivo* [9].

As the production of PPARα ligands is probably minimal in cultured cells, we assessed the effect of fructose in the presence of Wy-14,643, a potent and

specific PPARα ligand [28]. 25 mM fructose incubation for 24 hours significantly reduced the induction of L-CPT-I, ACO and CYP4A1 elicited by Wy-14,643 (Figure 3D-1F). When we assayed the specific binding of FaO cell nuclear extracts to an oligonucleotide reproducing the PPRE of rat L-CPT-I, we observed a major band whose intensity was depressed in the presence of Wy-14,643 and further increased in the presence of fructose (Figure 3G). In naïve FaO cells, the PPRE in the L-CPT-I promoter is mainly occupied by an unproductive protein complex that drops in the presence of Wy-14,643, favoring the expression of L-CPT-I, and increases further on co-incubation with fructose, thus reducing the expression of L-CPT-I.

To determine whether fructose had similar effects in human cells, we incubated human HepG2 hepatoma cells and normal human hepatocytes for 24 hours with fructose 25 mM. Fructose reduced the expression of PPARα and L-CPT-I in the presence and in the absence of Wy-14,643 in these human liver cells, implying a similar effect of fructose on PPARα in rats and humans (Figure 4).

3.5. Protein Phosphatase 2A (PP2A) activity is not involved in the reduction of PPARα expression and activity by fructose. ChREBP dephosphorylated by PP2A is retained in the nucleus, increasing the transcription of its target genes, such as L-PK or RGS16 [21]. Sustained activation of PP2A by fructose could be responsible, through ChREBP activation, for the reduced expression and activity of PPARα. Only livers from 14-day fructose-supplemented rats showed an increase in the ChREBP protein (x2.01-fold) and in the catalytic subunit of PP2A (x1.28-fold) (Table 2). To test

this hypothesis, we determined the effect of fructose on these parameters in FaO cells.

Incubation of FaO cells with 25 mM fructose for 24 hours increased the expression and activity of PP2A and ChREBP (Figure 5A-B). Only fructose, but not glucose, was able to induce significantly the expression of L-PK (x2.06-fold), a prototypical ChREBP-driven gene, in FaO cells (Figure 5E). When FaO cells were incubated with okadaic acid, a known inhibitor of PP2A [29], although the fructose-induced increase in PP2A activity was blunted, the effect of fructose on ChREBP, L-PK and PPARα was preserved (Figure 5A-D).

3.6. Fructose supplementation reduces liver SIRT1 expression and activity. The liver expression of hepatic nuclear factor 4 (HNF4), a transcription factor also controlling the expression of L-CPT-I [30], paralleled that of PPARα. The expression of forkhead box protein A2 (Foxa2) [31], a transcription factor regulating fatty acid oxidation, was reduced only after 14-day fructose supplementation (x0.79 fold) (Table 3).

We found only in livers of 14-day fructose-supplemented rats a reduction in SIRT1 (x0.36-fold, Figure 6A) and nicotinamide phosphoribosyltransferase (NAMPT, x0.73-fold, Figure 6B), an enzyme essential for the synthesis of NAD^+, suggesting a deficit of SIRT1 activity, which could be responsible for the reduction in the expression and activity of the PPARα system.

Although in FaO cells incubation with 25 mM fructose for 24 hours did not modify the expression and activity of SIRT1 and NAMPT (data not shown), fructose was also able to efficiently block the increase in PPARα expression

induced by incubating FaO cells with SRT1720, a potent and selective activator of SIRT1 [32] (Figure 6C).

To confirm the reduction of SIRT1 activity *in vivo* by fructose, we determined the amount of the acetylated form of a well-known substrate of SIRT1 activity, peroxisome proliferator activated receptor coactivator 1α (PGC-1α) [13]. The amount of acetylated-PGC-1α was clearly increased in livers of 14-day fructose-supplemented rats (Figure 6D).

4. Discussion

We showed that hypertriglyceridaemia and fatty liver after carbohydrate ingestion in rats results from simultaneous induction of liver fatty acid synthesis and inhibition of fatty acid oxidation [9,10,20]. Simple sugars induce lipid synthesis, but only fructose, at least in rats, inhibits liver fatty acid oxidation. Here, we demonstrate that fructose inhibits hepatic fatty acid oxidation at both 7 and 14 days of supplementation (10% w/v) but through different mechanisms, involving the reduction of PPARα expression only at the latter time-point. In fact, at 7 days fructose supplementation increases PPARα expression and activity, and the reduction in fatty acid β-oxidation may be related to increased ACC expression leading to the production of malonyl-CoA and to CPT-1 inhibition. On the contrary, fructose supplementation for 14 days inhibits liver fatty acid β-oxidation also by reducing PPARα expression and activity, mainly by decreasing the expression and activity of SIRT1. This fructose-related inhibition of PPARα is reproduced in human HepG2 hepatoma cells and in human hepatocytes.

Activation of SIRT1 protects against diet-induced metabolic disorders by enhancing fatty acid oxidation [14]. This effect could be mediated by two complementary mechanisms: 1. Activation of AMPK [33], which we can rule out from our present results, and 2. deacetylation and activation of PGC-1α [34], a cofactor of PPARα necessary for the transcriptional control of genes related to fatty acid oxidation [35]. Conversely, hepatocyte-specific deletion of SIRT1 impairs PPARα signaling and decreases fatty acid β-oxidation [36]. We showed that there was a reduction in SIRT1 expression, as well as in nicotinamide phosphorybosyl-transferase (NAMPT), the rate-limiting enzyme in NAD^+ biosynthesis [37], pointing to a decrease in SIRT1 activity in the livers of 14-day fructose-supplemented rats. Consistently, a well-known substrate of SIRT-1, PGC-1α, remained hyperacetylated in the same samples. The inactivity of acetylated PGC-1α could be responsible for the reduced expression and activity of PPARα and the fatty acid β-oxidation system. However, fructose incubation of FaO cells, despite reducing PPARα expression, did not reduce SIRT1 and NAMPT. Nevertheless, FaO cells were incubated with fructose for 24 hours, in comparison with the 14-day fructose supplementation in rats. Moreover, the fluorimetric assay used to detect SIRT1 is designed for evaluation of total deacetylase activity and not specifically the SIRT1 activity present in a complex cellular mixture. Thus, it is relevant that the incubation of FaO cells with a SIRT1 agonist, SRT1720 [14], increased PPARα expression, an effect that was blunted by fructose. In FaO cells, a short incubation period with fructose is not sufficient to repress the expression of SIRT1, but probably reduces its deacetylase activity strongly enough to reduce the expression of PPARα.

The mechanism involved in the fructose-mediated reduction of SIRT1 expression remains to be determined. Our results suggest ChREBP as a potential candidate. It has been previously suggested that ChREBP is activated by fructose-induced PP2A activity. Previous work by Dentin et al [38] and our present data suggest that this is not the case, given that in our in vitro studies OA inhibited PP2A activity without changing the effect of fructose on target genes. It has also been suggested that ChREBP indirectly represses the liver expression of PPARα-target genes through the increased expression of RGS16 [27]. However, although we observed increased mRNA levels of RGS16 in fructose-fed rats, protein levels were not modified by the treatment, suggesting that the effects of fructose on genes controlled by PPARα is independent from RGS16. Moreover, Boergesen et al. [39] showed that glucose reduces PPARα expression in pancreatic β-cells, but not in rat hepatoma FaO cells, by activating ChREBP. We found that glucose does not increase the expression of the *l-pk* gene in FaO cells (Figure 5E), suggesting a lack of ChREBP activation. On the contrary, fructose efficiently increased the expression of the *l-pk* gene in FaO cells and human hepatocytes (Figure 4C), as well as reducing PPARα expression in these cells. Indeed, recent research by Noriega et al. [40] has shown that ChREBP does repress SIRT1 expression. Conversely, liver-specific knock-out of SIRT1 increases ChREBP, promoting steatosis [41]. Thus, fructose could impair PPARα expression and activity by activating ChREBP, which could be responsible for the direct increase in the expression of lipogenic genes, as well as, indirectly by repressing SIRT1, for the reduction of fatty acid oxidation in liver. Although there is no data suggesting ChREBP as a SIRT1 substrate, we also found an increased amount of acetylated-ChREBP protein in

livers of 14-day fructose-supplemented rats (Figure 6E). Sustained consumption of fructose could generate a feed-forward cycle, as the transcriptional activity of acetylated ChREBP is higher, at least in lipogenic target-genes, than the corresponding activity of the deaceylated form [42]. A diagram of the proposed involvement of SIRT1 as a key molecule in the production of fructose-related effects on lipid synthesis and fatty acid oxidation is shown in Figure 7.

The fructose-mediated reduction in liver PPARα expression and activity is reproduced in human hepatocytes, probably by a SIRT1-related mechanism, suggesting that a similar reduction in hepatic fatty acid oxidation could be present in humans consuming fructose-sweetened beverages. In fact, a fructose-rich diet reduces liver fatty acid oxidation in healthy male subjects [43]. Patients suffering from metabolic alterations (metabolic syndrome, T2DM) are frequently treated with fibrate-type hypolipidemic drugs, whose pharmacological properties are due to PPARα activation [28]. Our results suggest that consumption of fructose-sweetened beverages by this population could reduce the efficacy of drug-therapy, worsening their metabolic control.

In conclusion, fructose depresses PPARα expression and activity in hepatic tissue from 14-days fructose-supplemented rats, and in rat and human liver cells, by a mechanism that could involve a concerted increase in ChREBP and a reduction of SIRT1 expression and activity. The possible role of ChREBP activation, as the key molecular switch for the production of the two main effects of fructose in liver, increased lipid synthesis and decreased fatty acid oxidation, deserves further studies.

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Figure Legends

Figure 1. Histological study of liver sections from control and 14-day fructose-supplemented rats: Livers were cryosectioned and processed for histological examination of inflammation, collagen deposition and fat infiltration. Images are representative of liver sections from control (n = 4) and fructose-supplemented rats (n = 4) stained with H&E (**A**), trichrome Masson (**B**) and oil red O (**C**).

Figure 2. ***In vivo*** **effect of fructose on rat liver binding to a PPRE oligonucleotide, fatty acid β-oxidation activity and RGS16 expression:** (**A**) Representative EMSA autoradiography showing the binding of pooled nuclear extracts (NE) from control (n=4) and fructose-supplemented rats (n=6) to a PPRE oligonucleotide. (**B**) Fatty acid β-oxidation activity, expressed as nmols of oxidized palmitoyl-CoA/min/mg of protein in liver postnuclear supernatant, in control (n=4) and fructose-supplemented (n=6) rats. (**C**) mRNA of RGS16 in control and fructose-supplemented rats. Each bar represents the mean±sd of

values obtained from n=4 and n=5 animals (for control and fructose groups, respectively). The autoradiography above the figure shows the bands corresponding to Rsg16 mRNA and that of the aprt gene, used as an internal control in the PCR reaction to normalize the results, from liver samples of 3 animals from each treatment group. (**D**) Bar plot showing the levels of RSG16 protein in hepatic samples from control and fructose-supplemented rats. Each bar represents the mean±sd of values obtained from n=4 and n=5 animals (for control and fructose groups, respectively). Above the figure, a representative Western blot shows the RSG16 bands corresponding to 3 different control and fructose-fed rats.* $p<0.05$; ** $p<0.01$

Figure 3. Fructose represses PPARα expression and activity in FaO rat hepatoma cells: The mRNA levels of PPAR α (**A**), ACO (**B**, **D**), CYP4A1(**C**,**F**) and L-CPT-I (**E**) in control (CT) or in FaO cells cultured with 25 mM fructose (FRC), 25 mM glucose (GLC), 25 mM mannitol, 100 µM Wy-14.643 or fructose plus Wy-14.643 for 24 hours. Each bar represents the mean±sd of three different assays performed in duplicate. (**G**) Representative EMSA autoradiography showing the binding of nuclear extracts (NE) from control, Wy-14.643 and Wy-14.643 plus fructose-treated FaO cells to a PPRE oligonucleotide.* $p<0.05$; ** $p<0.01$

Figure 4. Fructose represses PPARα expression and activity in human liver cells: mRNA of PPARα (**A**), L-CPT-I (**B**) and L-PK (**C**) in human hepatocytes, and L-CPT-I (**D**) in HepG2 cells in control cells (CT) or in cells treated with fructose (FRC), Wy-14.643, or fructose plus Wy-14.643. Each bar

represents the mean±sd of three different assays performed in duplicate. * p<0.05; ** p<0.01

Figure 5. Protein Phosphatase 2A (PP2A) activity is not involved in the reduction of PPARα expression and activity by fructose: (**A**) PP2A activity, ChREBP protein (**B**) and L-PK (**C**) and PPARα (**D**) mRNA in FaO cells incubated in the absence (CT) or in the presence of 25 mM fructose (FRC), 20 nM okadaic acid (OkA) or 25 mM fructose plus 20 nM okadaic acid (F+OkA) for 24 h. (**E**) mRNA of L-PK in control cells (CT) or in cells treated with 25 mM fructose (FRC) or 25 mM glucose (GLC) for 24 hours. Each bar represents the mean±sd of three different assays performed in duplicate. **p<0.01, ***p<0.001 *vs* CT; #p<0.01 *vs* FRC.

Figure 6. Fructose reduces liver SIRT1 expression and increases the amount of acetylated proteins: SIRT1 (**A**) and NAMPT (**B**) proteins in the livers of control and fructose-supplemented female rats. Each bar represents the mean±sd of values obtained from n=4 and n=5 animals (for control and fructose groups, respectively). Above the figure, a representative Western blot shows the SIRT1 and NAMPT bands corresponding to 3 different control and fructose-fed rats (**C**) mRNA of PPARα in control FaO cells (CT) or in cells treated with fructose (FRC), SRT1720, or fructose plus SRT1720. Each bar represents the mean±sd of three different assays performed in duplicate. Acetylated PGC1α (**D**) and ChREBP (**E**) proteins in liver samples from control and 14-day fructose-supplemented rats.

Figure 7. Fructose increases liver lipid synthesis by directly activating ChREBP and indirectly by inhibiting SIRT1, thus promoting the accumulation of active, acetylated-ChREBP. Further, inhibition of SIRT1 also promotes the accumulation of inactive, acetylated-PGC1α, reducing the expression and activity of PPARα and its target genes controlling liver fatty acid oxidation.

Table 1

Table 1. Fructose effects on líquid and food ingestion, triglyceride and leptin plasma analytes, liver triglycerides and body and tissue weight values and fructokinase protein levels at 7 and 14 days.

	7 Days		14 Days	
	Control (n = 4)	Fructose (n = 6)	Control (n = 4)	Fructose (n = 6)
AUC ingested líquid (ml/days/2 rats)	360 ± 8	845 ± 198*	706 ± 91	1995 ± 430*
AUC consumed diet (g/days/2 rats)	227 ± 2	177 ± 13**	503 ± 12	392 ± 44*
AUC body weight (g/days/rat)	1478 ± 53	1495 ± 94	3575 ± 380	3687± 257
Final body weight (g)	249.5 ± 7.0	257.0 ± 19.4	263.7 ± 25.9	276.7 ± 20.7
Adipose tissue weight (g)	1.49 ± 0.69	1.53 ± 0.69	1.92 ± 1.24	2.57 ± 1.05
% liver weight	3.5 ± 0.2	3.5 ± 0.3	3.2 ± 0.2	3.8 ± 0.2**
FK protein (a.u.)	1 ± 0.32	1.78 ± 0.28**	1 ± 0.17	2.18 ± 0.20**
Hepatic Triglycerides (mg/g liver)	6.8 ± 0.6	8.2 ± 3.0	4.1 ± 2.3	7.7 ± 2.4*
Plasma Triglycerides (mg/dl)	58 ± 27	57 ± 13	63 ± 12	94 ± 17**
Plasma Leptin (ng/ml)	2 ± 1	3.2 ± 2.3	2.8 ± 2.7	4.7 ± 2.2
Plasma Adiponectin (µg/ml)	2.7 ± 0.7	3.9 ± 1.5	2.3 ± 0.7	4.0 ± 0.7*

*P<0.05, **P<0.01

Table 2

Table 2. Expression of genes / proteins involved in lipogenic pathways after 7- and 14-day fructose supplementation.

	7 Days		14 Days	
Gene	Control (n = 4)	Fructose (n = 5)	Control (n = 4)	Fructose (n = 5)
Fas	1 ± 0.20	6.28 ± 5.34**	1 ± 0.39	4.66 ± 1.52**
Gk	1 ± 0.34	1.09 ± 0.62	1 ± 0.43	1 ± 0.46
Gpat1	1 ± 0.26	1.57 ± 0.18**	1 ± 0.04	1.74 ± 0.81**
L-pk	1 ± 0.29	1.59 ± 0.14**	1 ± 0.05	2.12 ± 0.64**
Scd1	1 ± 0.52	11.98 ± 5.06**	1 ± 0.39	4.39 ± 2.53*
Elovl6	1 ± 0.40	2.88 ± 1.47*	1 ± 0.35	5.29 ± 1.73*
Protein				
p-ACC	1 ± 0.25	1.78 ± 0.11**	1 ± 0.10	1.28 ± 0.19*
ACC	1 ± 0.27	2.01 ± 0.15**	1 ± 0.16	1.49 ± 0.36*
ChREBP	1 ± 0.14	1.15 ± 0.23	1 ± 0.36	2.01 ± 0.87*
PP2Ac	1 ± 0.07	0.9 ± 0.08	1 ± 0.13	1.28 ± 0.15**
SREBP-1c 68KD	1 ± 0.12	0.80 ± 0.14	1 ± 0.24	1.12 ± 0.16

*P<0.05, **P<0.01

Table 3

Table 3. Expression of genes / proteins involved in fatty acid oxidation pathways after 7- and 14-day fructose supplementation.

	7 Days		14 Days	
Gene	Control (n = 4)	Fructose (n = 5)	Control (n = 4)	Fructose (n = 5)
L-cpt-1	1 ± 0.48	1.68 ± 0.69	1 ± 0.37	0.56 ± 0.21*
Aco	1 ± 0.09	1.26 ± 0.12**	1 ± 0.02	0.80 ± 0.11*
Trib-3	1 ± 0.61	3.34 ± 0.89**	1 ± 0.25	0.65 ± 0.21*
Pparα	1 ± 0.72	2.08 ± 0.40*	1 ± 0.27	0.36 ± 0.22**
Protein				
PPARα	1 ± 0.50	1.10 ± 0.44	1 ± 0.12	0.75 ± 0.16*
p-AMPK	1 ± 0.32	1.06 ± 0.20	1 ± 0.24	0.91 ± 0.14
AMPK	1 ± 0.34	0.98 ± 0.17	1 ± 0.28	1.28 ± 0.20
HNF4	1 ± 0.51	1.76 ± 0.49*	1 ± 0.23	0.52 ± 0.19**
Foxa2	*1 ± 0.16*	*1.13 ± 0.20*	*1.1 ± 0.11*	0.87 ± 0.12*

*P<0.05, **P<0.01

Figure 1

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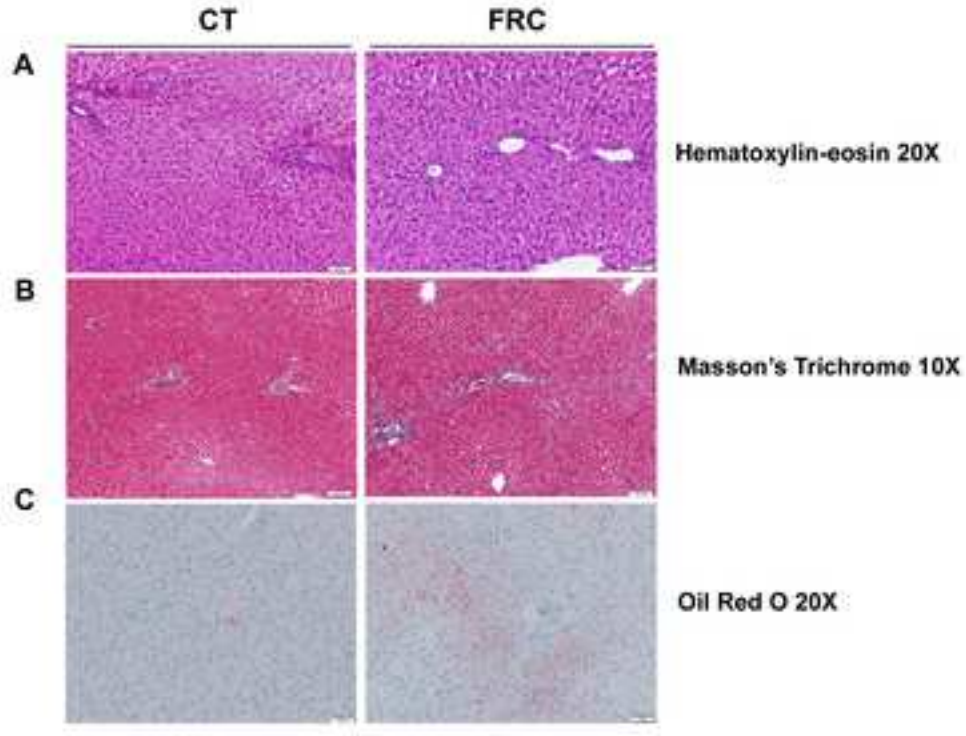

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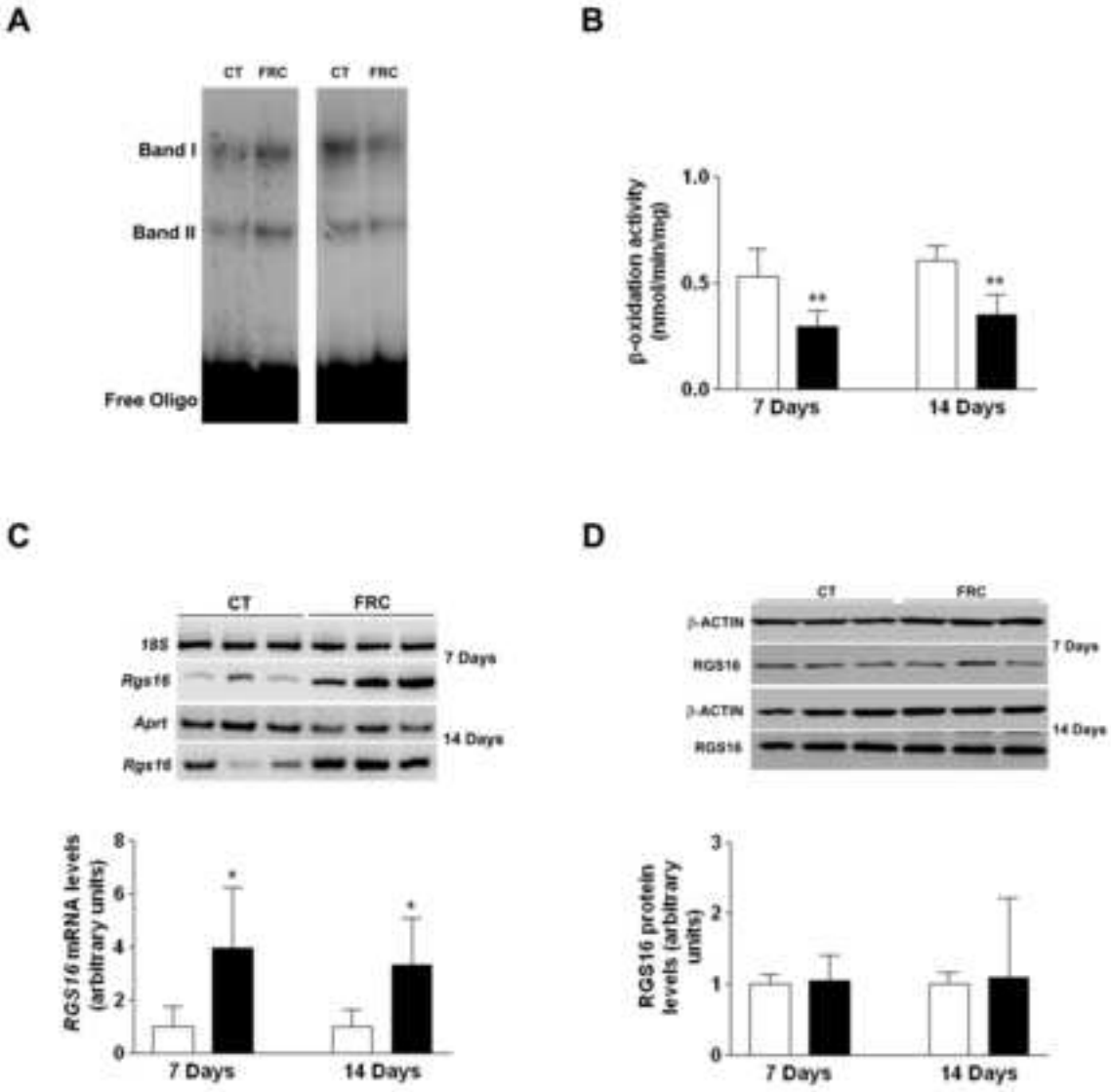

Figure 3

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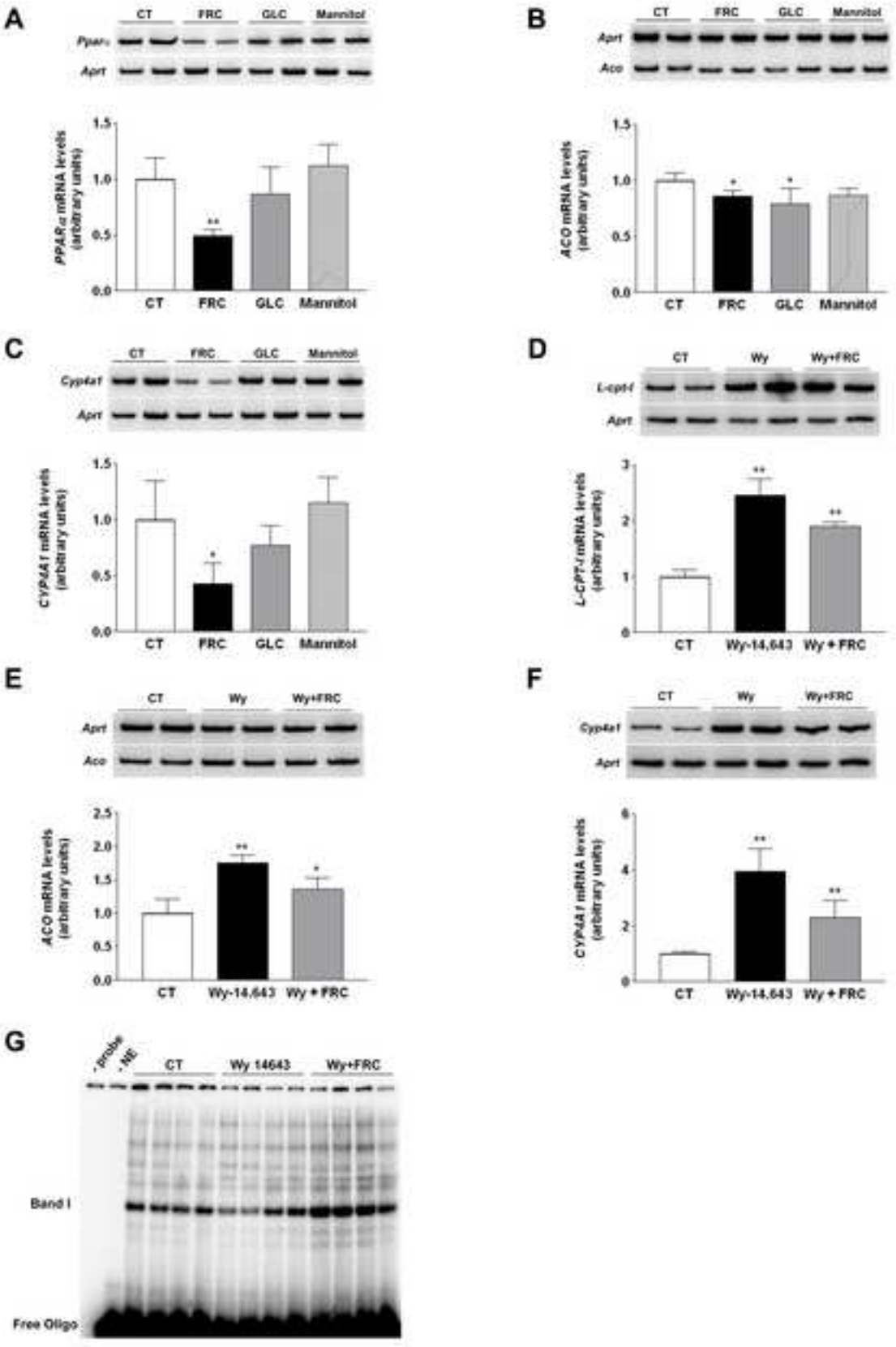

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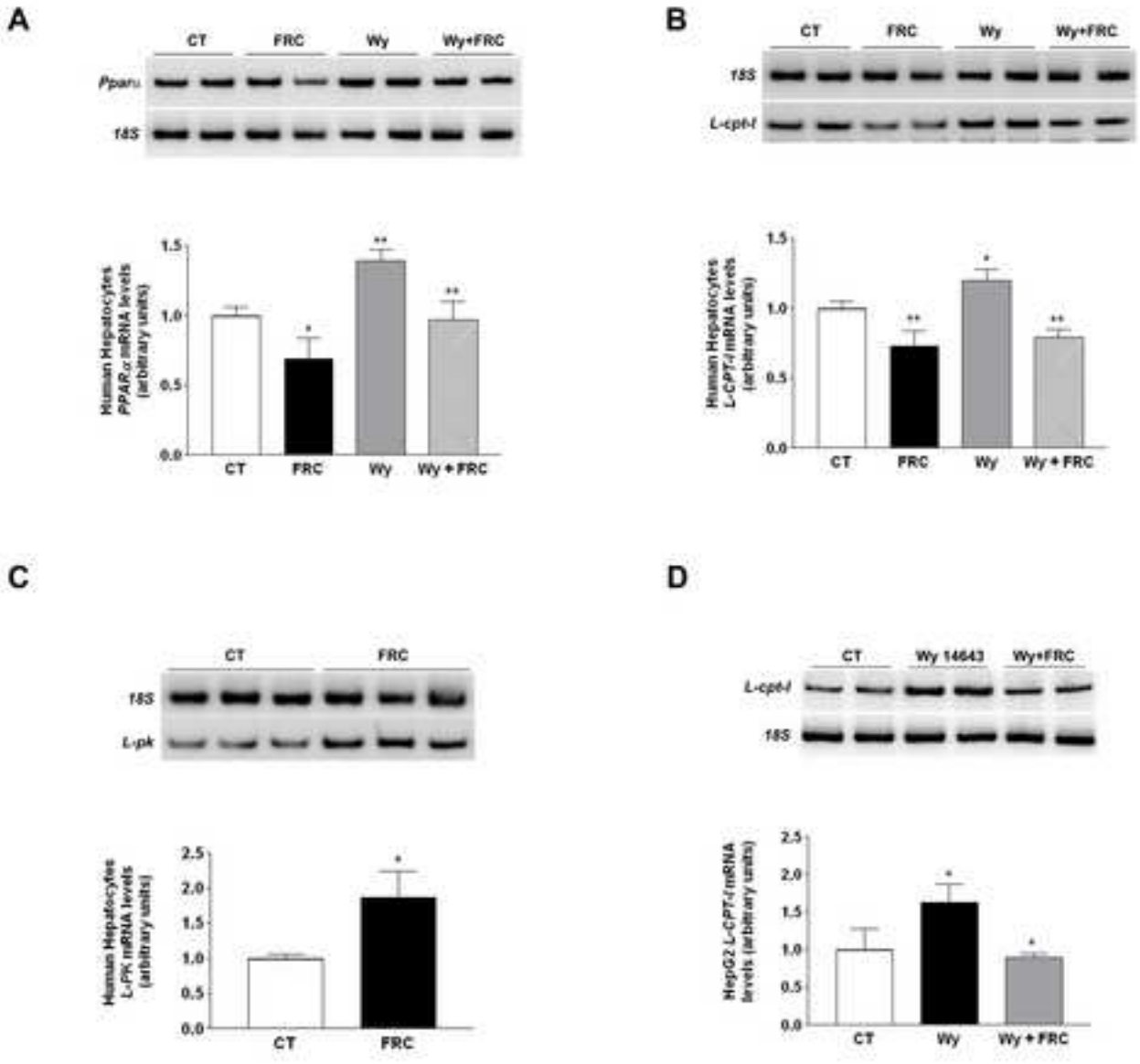

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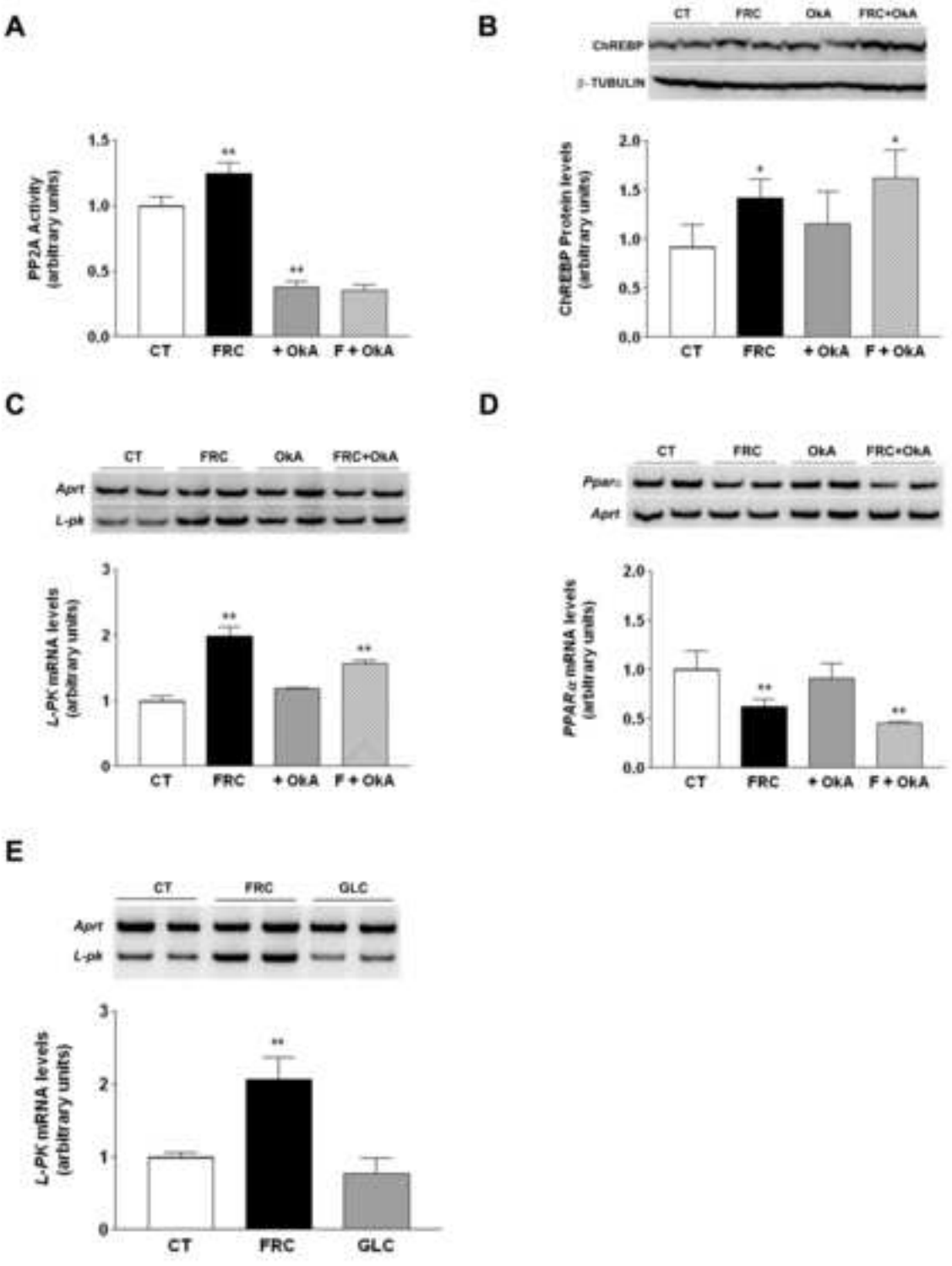

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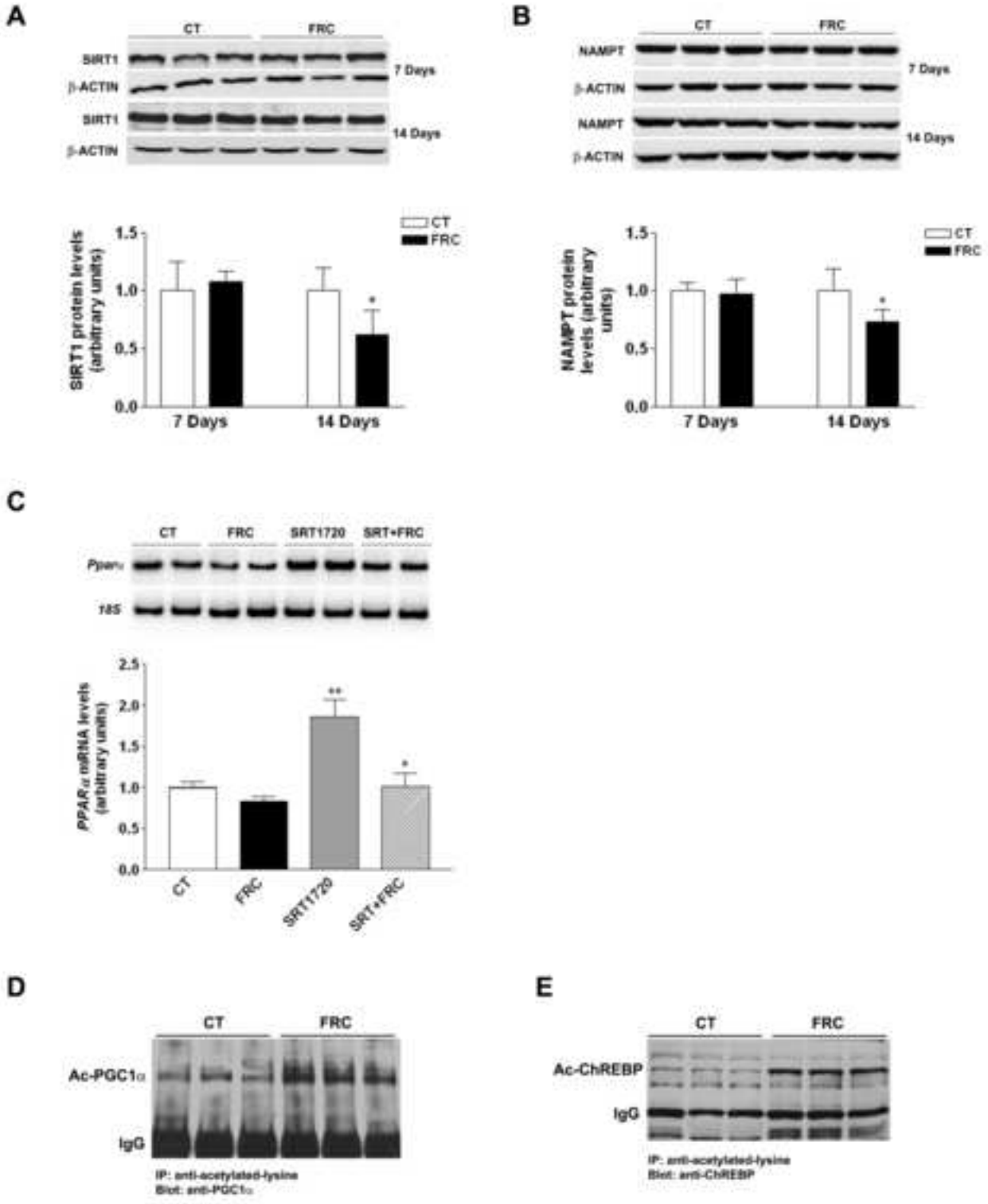

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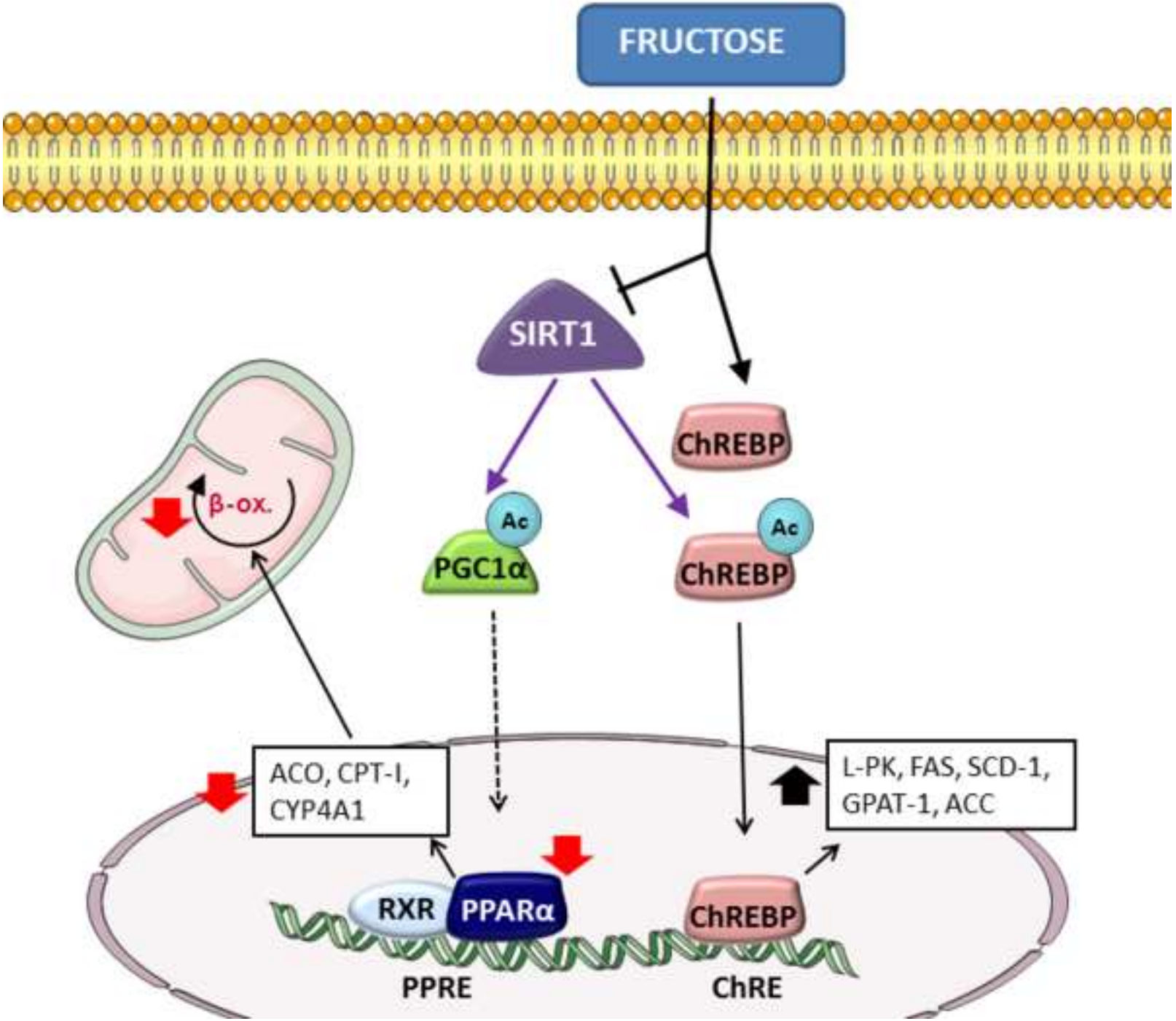

Supplementary Table

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Supplemental table 1. Primers used for the PCR reaction: A. Rat samples and FaO cells. B. Human hepatocytes (H.H.) and HepG2 cells.

A	*GenBank™ nº*	*Primer sequences*	*PCR product (bp)*	*Amplif. Cycles Rat / FaO*
18S	M-100098.1	Forward: 5'-CCAAAGTCTTTGGGTTCCGGG-3' Reverse: 5'-GCTCAATCTCGGGTGGCTGAA-3'	337 bp	18 / -
APRT	L04970	Forward: 5'-AGCTTCCCGGACTTCCCCATC-3' Reverse: 5'-GACCACTTTCTGCCCCGGTTC-3'	329 bp	23 / 21
ACO	NM_017340	Forward: 5'-ACTATATTTGGCCAATTTTGTG-3' Reverse: 5'-TGTGGCAGTGGTTTCCAAGCC-3'	195 bp	23 / 24
CYP4A1	NM_175837	Forward: 5'-CTGGCTTCCTCCAAGTGGCCT-3' Reverse: 5'-TTGCTTCCCCAGAACCATCGA-3'	509 bp	- / 25
ELOVL6	NM_ 134383	Forward: 5'-AGCCATCCAATGGTGCAGGA-3' Reverse: 5'-TGCTTTGCTGAGCACAAACGC-3'	301 bp	22 / -
FAS	M76767	Forward: 5'-GTCTGCAGCTACCCACCCGTG-3' Reverse: 5'-CTTCTCCAGGGTGGGGACCAG-3'	214 bp	20 / -
GPAT1	AF021348	Forward: 5'-ATCCGCAACGCTGAAATGGAA-3' Reverse: 5'-GGCAAACATGCCCTTGTGGAC-3'	244 bp	22 / -
GK	J04218	Forward: 5'-AGAAGGAGATGGACCGTGGCC-3' Reverse: 5'-TCCCTTCTGCTCCAGCGGCCT-3'	421 bp	23 / -
L-CPT-I	L07736	Forward: 5'-TATGTGAGGATGCTGCTT-3' Reverse: 5'-CTCGGAGAGCTAAGCTTG-3'	629 bp	23 / 23
L-PK	M11709	Forward: 5'-TATGGCGGACACCTTCCTGGA -3' Reverse: 5'-GCTGAGTGGGGAGGTTGCAAA-3'	250 bp	23 / 21
PPARα	M88592	Forward: 5'-GGCTCGGAGGGCTCTGTCATC-3' Reverse: 5'-ACATGCACTGGCAGCAGTGGA-3'	654 bp	23 / 25
RGS16	AY651775	Forward: 5'-CACCTGCCTGGAAAGAGCCAA-3' Reverse: 5'-GGCCAGCCAGAACTCCAGGTT-3'	259 bp	26 / -
SCD1	J02585	Forward: 5'-GCTCATCGCTTGTGGAGCCCAC-3' Reverse: 5'-GGACCCCAGGGAAACCAGGAT-3'	521 bp	18 / -
SREBP1C	L16995	Forward: 5'-TCACAGATCCAGCAGGTCCCC-3' Reverse: 5'-GGTCCCTCCACTCACCAGGGT-3'	180 bp	23 / -
Trib3	NM_144755	Forward: 5'-TGCTCTTTGGCAAGATCCGTA-3' Reverse: 5'-CAACCTGGTCCATCTCCCTTC-3'	204 bp	26 / -

B	*GenBank™ nº*	*Primer sequences*	*PCR product (bp)*	Amplif. Cycles HepG2 / H.H.
18S	M-100098.1	Forward: 5'-CCAAAGTCTTTGGGTTCCGGG-3' Reverse: 5'-GCTCAATCTCGGGTGGCTGAA-3'	337 bp	18 / 18
L-CPT-I	U66828	Forward: 5'-TGATCCGCATGAAGAATGGCA-3' Reverse: 5'-GCGGAAGAAGAAGATGCCCGT-3'	274 bp	34 / 28
L-PK	M15465	Forward: 5'- AGGAGCTGGGCACTGCCTTCT-3' Reverse: 5'-GTGGGAGCCGTGGGAGAAGTT-3'	227 bp	- / 26
PPARα	EU650667	Forward: 5'-TCTGAAGAGTTCCTGCAAGAAATGG-3' Reverse: 5'-AGCATCCCGTCTTTGTTCATC-3'	953 bp	- / 24