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Liquid fructose in Western-diet-fed mice impairs liver insulin signaling and causes cholesterol and triglyceride loading without changing calorie intake and body weight☆,☆☆

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Abstract

Background/objectives: Liquid fructose associates with prevalence of type 2 diabetes mellitus and obesity. Intervention studies suggest that metabolically unfit individuals are more responsive than healthy individuals to liquid fructose. We determined whether mice consuming an obesogenic Western diet were more responsive than chow-fed mice to the alterations induced by liquid fructose supplementation (LFS).
Methods: C57BL/6N mice were fed chow or Western diet±*ad libitum* 15% fructose solution for 12 weeks. Food and liquid intake and body weight were monitored. Plasma analytes and liver lipids, histology and the expression of genes related to lipid handling, endoplasmic reticulum stress, inflammation and insulin signaling were analyzed.
Results: Western diet increased energy intake, visceral adipose tissue (vWAT), body weight, plasma and liver triglycerides and cholesterol, and inflammatory markers in vWAT *vs.* chow-fed mice. LFS did not change energy intake, vWAT or body weight. LFS significantly increased plasma and liver triglycerides and cholesterol levels only in Western-diet-fed mice. These changes associated with a potentiation of the increased liver expression of PPARγ and CD36 that was observed in Western-fed mice and related to the increased liver mTOR phosphorylation induced by LFS. Furthermore, LFS in Western-diet-fed mice induced the largest reduction in liver IRS2 protein and a significant decrease in whole-body insulin sensitivity.
Conclusions: LFS in mice, in a background of an unhealthy diet that already induces fatty liver visceral fat accretion and obesity, increases liver lipid burden, hinders hepatic insulin signaling and diminishes whole-body insulin sensitivity without changing energy intake.

Keywords: ChREBP; IRS2; PPARγ; CD36; mTOR; Visceral adipose tissue; Leptin

1. Introduction

Epidemiological studies indicate that the increased consumption of sugar-sweetened beverages is one of the key lifestyle modifications that occurred in the last decades that is associated to the increased prevalence of metabolic diseases, such as type 2 diabetes mellitus (T2DM) and obesity, in human populations all over the world [1,2]. Epidemiological studies in humans rely on data obtained from populations with a broad spectrum of genetic predisposition to metabolic diseases and highly varied dietary patterns. In addition, intervention studies in humans, although limited in duration and the number of participants, indicate that metabolically unfit individuals, for example, obese people, are more responsive than healthy people to the metabolic alterations induced by fructose ingestion [3–5]. Given the difficulties in performing intervention studies in humans in terms of design, cost and ethics, there is intense debate over the interpretation of these epidemiological studies on whether they are just merely detecting metabolic disturbances associated with excessive calorie intake or reflecting specific deleterious effects related to the way these calories are provided (liquid beverages), the very nature of the simple sugars used (fructose, glucose, sucrose, *etc.*) or the combination of both.

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Using healthy rodents maintained on solid diets supplemented with liquid solutions of simple sugars has shown that fructose consumption, when compared to equicaloric amounts of glucose consumption, is unique in inducing specific metabolic disturbances in the liver by affecting fatty acid metabolism and insulin signaling pathways [6,7]. Further, we have previously shown that liquid fructose supplementation, at a concentration below 30% (weight/volume) that has been shown to significantly alter intestinal permeability [8,9], significantly increased atherosclerosis and liver and plasma lipid content in LDLR$^{-/-}$ mice fed Western-type diet despite ingesting exactly the same amount of calories as LDLR$^{-/-}$ mice on Western-type diet only [10]. As these transgenic animals already presented a large fat deposition in the liver even when raised with standard rodent chow, we sought to investigate the metabolic response to the combined Western-diet feeding and liquid fructose supplementation in the background wild-type mice lineage used to generate the transgenic LDLR$^{-/-}$ mice we have previously used.

Here we show in a mouse model susceptible to diet-induced metabolic disturbances, the C57BL/6N mouse, that liquid fructose supplementation increases liver cholesterol and triglyceride burden, hinders hepatic insulin signaling and diminishes whole-body insulin sensitivity in mice with an unhealthy, Western-style solid diet that already induces fatty liver, visceral fat accretion and obesity. Fructose elicited these changes without affecting the total amount of energy consumed, as fructose-supplemented animals reduced their solid food intake to accommodate the ingested liquid calories.

2. Materials and methods

2.1. Animals and experimental design

Male mice (C57BL/6N) were purchased from Charles River (France) and maintained with water and food *ad libitum* at constant humidity and temperature with a light/dark cycle of 12 h. After 3 weeks of acclimatizing, animals were randomly separated into 4 groups of 12 mice each which received (a) control rodent diet without supplementary sugar: control group (C), (b) control rodent diet supplemented with 15% weight/volume fructose in drinking water: fructose-supplemented group (F), (c) Western-type diet without supplementary sugar: Western group (W) and (d) Western-type diet supplemented with 15% weight/volume fructose in drinking water: Western plus fructose group (W+F). The composition of control (2018 Teklad Global 18% protein, Harlan Laboratories) and Western-type (D12079B Open Source Diets, Research Diets, Inc.) diets was as detailed previously [10]. During a feeding period of 12 weeks, consumed food and beverage were measured every 2 days and body weight once a week. At the end of the study, animals were sacrificed under intraperitoneal ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia between 9 and 10 a.m. after being fasted for 2 h. All procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee, as stated in Law 5/1995 (21st July) from the Generalitat de Catalunya. These guidelines follow the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2. Sample preparation

Blood samples were obtained by intracardiac punction at the time of death and collected in microtubes containing anticoagulant as additive (Sarstedt AG & Co, Nümbrecht, Germany). Plasma was obtained by centrifugation and stored at −80°C until used. Liver and visceral adipose tissues were excised and fractionated. Ten to 100 mg was immediately frozen in liquid N_2 and stored at −80°C until used for protein and total RNA extraction. Another portion for hepatic histological analysis was obtained. An additional section of liver tissue (100 mg) was perfused and stored at −80°C for quantifying liver lipids content. Total and nuclear protein extracts from liver and visceral adipose tissue were isolated by the Helenius method [11], and protein concentrations were determined by the Bradford method [12].

2.3. Glucose, lipids, cholesterol, insulin and leptin analysis

Plasma glucose, triglycerides and cholesterol levels were measured using an Accutrend Plus System glucometer (Roche Farma, Barcelona, Spain). Plasma leptin and insulin levels were determined at the end of treatment using the EZRL-83K and EZRMI-13K kits from Millipore (Billerica, MA, USA), respectively. Insulin sensitivity index (ISI) was calculated as described by Qu et al. [13].

Liver lipids were extracted according to the Bligh and Dyer [14] method using the homogenate fraction. The lipid extract was evaporated under a stream of nitrogen gas and dissolved in absolute ethanol. Triglycerides and cholesterol contents in liver were determined by using colorimetric tests: Triglycerides-LQ no. 41030 and Cholesterol CHOD-POD no. 1001091 from Spinreact (Girona, Spain), respectively.

2.4. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from 60 mg of liver and adipose tissue using Trizol reagent (Invitrogen, Thermo-Fisher Scientific Inc., MA, USA) in accordance with the manufacturer's guidelines. Single-stranded cDNA was synthesized by mixing 1 μg of liver total RNA, 125 ng of random hexamers (Roche Farma, SA, Madrid, Spain) as primers in the presence of 5× First-Strand Buffer, 10 mM dithiothreitol, 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen), 20 U of RNase OUT from Invitrogen and 0.5 mM of each dNTP (Sigma-Aldrich, St. Louis, MO, USA) in a total volume of 20 μl. Samples were incubated at 37°C for 60 min in MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR was carried out in StepOnePlus Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Foster City, CA, USA). Twenty microliters of reaction mixture contained Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each specific primer (including forward and reverse primers) and 20 ng of cDNA for each gene. After an initial denaturation at 95°C for 10 min, 40 cycles of amplification were done. PCRs were performed in duplicate and normalized to a housekeeping gene, the TATA box binding protein (*tbp*) gene, using the $2^{-\Delta\Delta Ct}$ method. The GenBank number, primer sequences and PCR product length are listed in Table 1.

2.5. Western blot analysis

Thirty micrograms of different protein fractions from rat livers was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA, USA), blocked for 1 h at room temperature with 5% nonfat milk solution in 0.1% Tween-20-Tris-buffered saline (TBS) and incubated as described previously [6]. Detection was performed using the ECL chemiluminescence kit for HRP (Amersham GE Healthcare Europe GmbH, Barcelona, Spain). To confirm the uniformity of protein loading, blots were incubated with β-tubulin or β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) as a control. Primary antibodies for phospho- and total mTOR were supplied by Millipore (Billerica, MA, USA), those for phospho- and total IRE1 were obtained from Abcam (Cambridge, UK), and the antibody against phospho- and total JNK were purchased form Cell Signaling (Danvers, MA, USA). The rest of the antibodies used were from Santa Cruz Biotechnologies (Dallas, TX, USA).

2.6. Histological analysis

For hepatic histological analysis of Oil-Red-O-stained sections, the liver tissue was perfused and fixed in 10% paraformaldehyde solution before processing for paraffin embedding. Images were acquired with an Olympus BX71 microscope equipped with a DP72 camera and analyzed by a registered pathologist at BioBanc (Banc de tumors-IDIBAPS, Barcelona Spain) who was unaware of the treatment groups. The area of positive staining for Oil Red O was calculated as a percentage of stained cells/total section area in each sample. Eight-micrometer serial sections of liver segments obtained in a cryostat (Leyca CM-1900) at −24°C were prepared and stained with hematoxylin–eosin stain for histological evaluation of inflammatory structures. Lesion area was quantified following the standard procedure by using Image-J software.

2.7. Enzyme activity assays

Hepatic fatty acid β-oxidation activity was determined in mice livers as previously described [15] with 30 μg of postnuclear supernatant.

2.8. Statistical methods

Results are expressed as the mean of *n* values±standard deviation (SD). Plasma and lipid samples were assayed in duplicate. Significant differences between values from control, fructose and Western groups were established by the one-way analysis of variance test and Bonferroni posttest for selected comparisons; significant differences between values from Western and Western+fructose groups were established by the unpaired *t* test (GraphPad Software V5). The level of statistical significance was set at $P \leq .05$.

3. Results

3.1. Fructose supplementation did not change calorie intake, visceral adipose tissue amount and body weight

After 12 weeks of intervention, total calorie intake significantly increased in Western-diet-fed *vs.* normal chow-fed mice (×1.27-fold) but was not modified by supplementation with liquid fructose. Mice ingesting the 15% w/v fructose solution compensated for the ingested liquid calories by reducing the ingestion of the solid normal (×0.72-

Table 1
Primers used for reverse transcriptase PCR[a]

Gene	GenBank no.	Primer sequences	PCR product
Fos	NM_010234.2	Forward: 5′-TACTACCATTCCCCAGCCGA-3′ Reverse: 5′-GCTGTCACCGTGGGGATAAA-3′	113 bp
Ccr2	NM_009915.2	Forward: 5′-AGAGGTCTCGGTTGGGTTGT-3′ Reverse: 5′-CACTGTCTTTGAGGCTTGTTGC-3′	100 bp
Cd36	NM_001159558.1	Forward: 5′-CCAAGCTATTGCGACATGATTAAT-3′ Reverse: 5′-CAATGTCCGAGACTTTTCAACAAA-3′	75 bp
Chop	NM_007837.4	Forward: 5′-TATCTCATCCCCAGGAAACG-3′ Reverse: 5′-GGGCACTGACCACTCTGTTT-3′	219 bp
Dgat2	NM_026384.3	Forward: 5′-GCACAGACTGCTGGCTGATA-3′ Reverese: 5′-TTTCTTGGGCGTGTTCCAGT-3′	71 bp
Dnajb9	NM_013760.4	Forward: 5′-TCTGCCTCAGAGCGACAAAT-3′ Reverse: 5′-TCCGACTATTGGCATCCGAG-3′	145 bp
Edem1	NM_138677.2	Forward: 5′-CCAGCATGGGCTTCTACCAG-3′ Reverse: 5′-CCTTGGCCGATGAAGCCAG-3′	119 bp
F4/80	XM_011246272.1	Forward: 5′-GGAGGACTTCTCCAAGCCTATT-3′ Reverse: 5′-GGCCTCTCAGACTTCTGCTTT-3′	69 bp
Fasn	NM_007988.3	Forward: 5′-TCCTGGAACGAGAACACGATCT-3′ Reverse: 5′-GAGACGTGTCACTCCTGGACTTG-3′	138 bp
Gadd34	NM_008654.2	Forward: 5′-TGCAAGGGGCTGATAAGAGG-3′ Reverse: 5′-ATTCTCAGCTGGACCACCCT-3′	104 bp
Grp78	NM_001163434.1	Forward: 5′-ATTGGAGGTGGGCAAACCAA-3′ Reverse: 5′-TCGCTGGGCATCATTGAAGT-3′	150 bp
Grp94	NM_011631.1	Forward: 5′-GACCTTCGGGTTCGTCAGAG-3′ Reverse: 5′-AGCCTTCTCGGCTTTTACCC-3′	83 bp
Il-6	NM_001314054.1	Forward: 5′-ACACATGTTCTCTGGGAAATCGT-3′ Reverse: 5′-AAGTGCATCATCGTTGTTCATACA-3′	84 bp
Ccl2	NM_011333.3	Forward: 5′-GCTGGAGAGCTACAAGAGGATCA-3′ Reverse: 5′-CTCTCTCTTGAGCTTGGTGACAAA-3′	79 bp
Myd88	NM_010851.2	Forward: 5′-AGGCGATGAAGAAGGACTTTCC-3′ Reverse: 5′-TCAGTCTCATCTTCCCCTCTGC-3′	163 bp
Lep	NM_008493.3	Forward: 5′-AACCCTCATCAAGACCATTGTCA-3′ Reverse: 5′-CCTCTGCTTGGCGGATACC-3′	73 bp
Lepr	NM_146146.2	Forward: 5′-AACTGCAGTCTTCGGGGATG-3′ Reverse: 5′-ACTGAAAACTCACACCGGCA-3′	109 bp
Pparγ	XM_006505737.1	Forward: 5′-GCCCACCAACTTCGGAATC-3′ Reverse: 5′-TGCGAGTGGTCTTCCATCAC-3′	57 bp
Scd1	NM_009127.4	Forward: 5′-TTCCTTATCATTGCCAACACCAT-3′ Reverse: 5′-TGGGCGCGGTGATCTC-3′	71 bp
Tlr4	NM_021297.2	Forward: 5′-GGCTCCTGGCTAGGACTCTGA-3′ Reverse: 5′-TCTGATCCATGCATTGGTAGGT-3′	114 bp
Tnfα	NM_013693.3	Forward: 5′-GAAAAGCAAGCAGCCAACCA-3′ Reverse: 5′-CGGATCATGCTTTCTGTGCTC-3′	106 bp
Tbp	NM_013684.3	Forward: 5′-TGCCACACCAGCTTCTGAGA-3′ Reverse: 5′-TTTACAGCCAAGATTCACGGTAGA-3′	79 bp

[a] All primers have been used at an efficiency between 85% and 110%.

fold) or Western diet (×0.82-fold) food. Only mice consuming the Western diet displayed increased final body weight (×1.29 *vs.* control) and visceral adipose tissue (vWAT) amount (×2.48 *vs.* control, as a percentage of body weight) (Table 2). Despite that, the solid Western diet and liquid fructose supplementation induced hyperleptinemia (×5.50 and ×2.29, respectively) *versus* normal chow-fed mice values (Fig. 1A) and increased expression of the *lep* gene in vWAT (Fig. 1B). The sustained hyperleptinemia probably resulted in a state of peripheral leptin resistance, as liver expression of the SOCS-3 protein, an endogenous inhibitor of leptin signaling [16], was also increased in solid Western-diet-fed mice and liquid-fructose-supplemented mice (Fig. 1C). Accordingly, liver *fos* expression, which is controlled by leptin activity, was decreased, while the expression of the long form of the leptin receptor was increased [17] (Fig. 1D and E).

3.2. Fructose supplementation did not result in hypertriglyceridemia or worsened histological signs of fatty liver

There is a wealth of information describing the induction of hypertriglyceridemia and fatty liver by fructose supplementation in rats and humans [3,18–20]. However, in our study, liquid fructose supplementation in C57BL/6N mice consuming either control or a Western diet did not induce hypertriglyceridemia or worsened fatty liver (Fig. 2A–B). Although fructose supplementation in Western diet mice significantly increased liver triglyceride accretion (Fig. 2C), histological analysis of Oil-Red-O-stained sections (Fig. 2B) showed exactly the same lipid percent area for Western-diet-fed mice, irrespective of fructose supplementation (11±12, 12±3, 100±0 and 100±0 lipid percent area, expressed as mean±SD, for control, fructose, Western and Western+fructose groups; $n=4$ for each group). Accordingly, the amount of the lipogenic transcription factors SREBP1c (mature form) and ChREBP in liver nuclear extracts was not increased by fructose supplementation (Fig. 2D and E). The same was observed with the hepatic expression of lipogenic enzymes (Fig. 2F) (*dgat2*, *scd1* and *fasn*). The lack of a clear lipogenic effect was not due to a deficit in fructose incorporation into liver metabolism, as fructokinase levels were increased in the livers of fructose-supplemented mice (Fig. 2G). Fructose is known for inducing its own metabolism by increasing the expression of the enzyme fructokinase [21].

3.3. Fructose supplementation potentiated the hepatic cholesterol burden in mice fed a solid Western diet

As the Western diet used in our experimental protocol contained 0.21% of cholesterol, mice consuming this diet showed hypercholesterolemia and increased cholesterol deposition in liver tissue (Fig. 3A and B). In addition, diets rich in saturated fat, like the Western diet used in the present work, are known to induce hypertriglyceridemia and fatty liver (Fig. 2) through the increased hepatic expression of the nuclear receptor *pparγ* and one of its target genes, *cd36* (Fig. 3C and D), a fatty acid translocase [22]. Fructose supplementation did not modify liver fatty acid β-oxidation activity (Fig. 3E). Surprisingly, despite not affecting fatty acid synthesis and catabolism, liquid fructose supplementation potentiated the increase of *pparγ and cd36* expression, as well as liver triglyceride and cholesterol accretion, in Western-diet-fed mice (Figs. 2 and 3). This was observed despite a significant reduction in the amount of the solid diet and thus dietary cholesterol consumed by these mice (see above and Table 2). In accordance with our previous results obtained in fructose-supplemented rats [23], only fructose-supplemented mice showed increased liver mTOR phosphorylation (Fig. 3F).

Table 2
Intake values (solid chow and supplemented beverage) and zoometric parameters for C, F, W and W+F expressed as mean±SD of the values obtained from at least eight different animals

Parameter	C	F	W	W+F
AUC[a] beverage (ml per mice per 12 weeks)	374±57	524±35 <.01 ×1.40	246±29 <.05 −34%	312±14 <.05 *vs.* W 1.27
AUC[a] solid (g per mice per 12 weeks)	274±31	197±13 <.05 −28%	230±17	197±10 <.05–14%
Ingested kcal from liquid	0 kcal	314±21	0 kcal	187±8 <.001 *vs.* F ×0.59
Ingested kcal from solid	852±94	611±41 <.05 ×0.72	1080±77 <.05 ×1.27	925±49 <.05 *vs.* W ×0.86
Total ingested calories	852±94	925±22	1080±37 <.05 ×1.27	1113±44
AUC[a] body weight (g per mice per 12 weeks)	318.8±21.5	315.8±21.9	369.2±26.5 <.001 ×1.17	358.3±33.9
Final body weight (BW) (g)	28.8±1.7	29.5±2.9	37.1±3.6 <.001 ×1.29	37.1±4.7
vWAT weight (g)	0.8±0.2	1.2±0.3	2.9±0.7 <.001 ×3.39	2.8±0.8
% vWAT/BW	3.2±0.06	4.2±0.9	7.8±1.3 <.001 ×2.48	7.7±1.3
Liver weight (g)	1.4±0.2	1.6±0.3	1.7±0.4	2.0±0.5

Statistical significance (*P* values *vs.* control group, if not indicated) and fold change are shown when appropriate.
[a] Area under the curve.

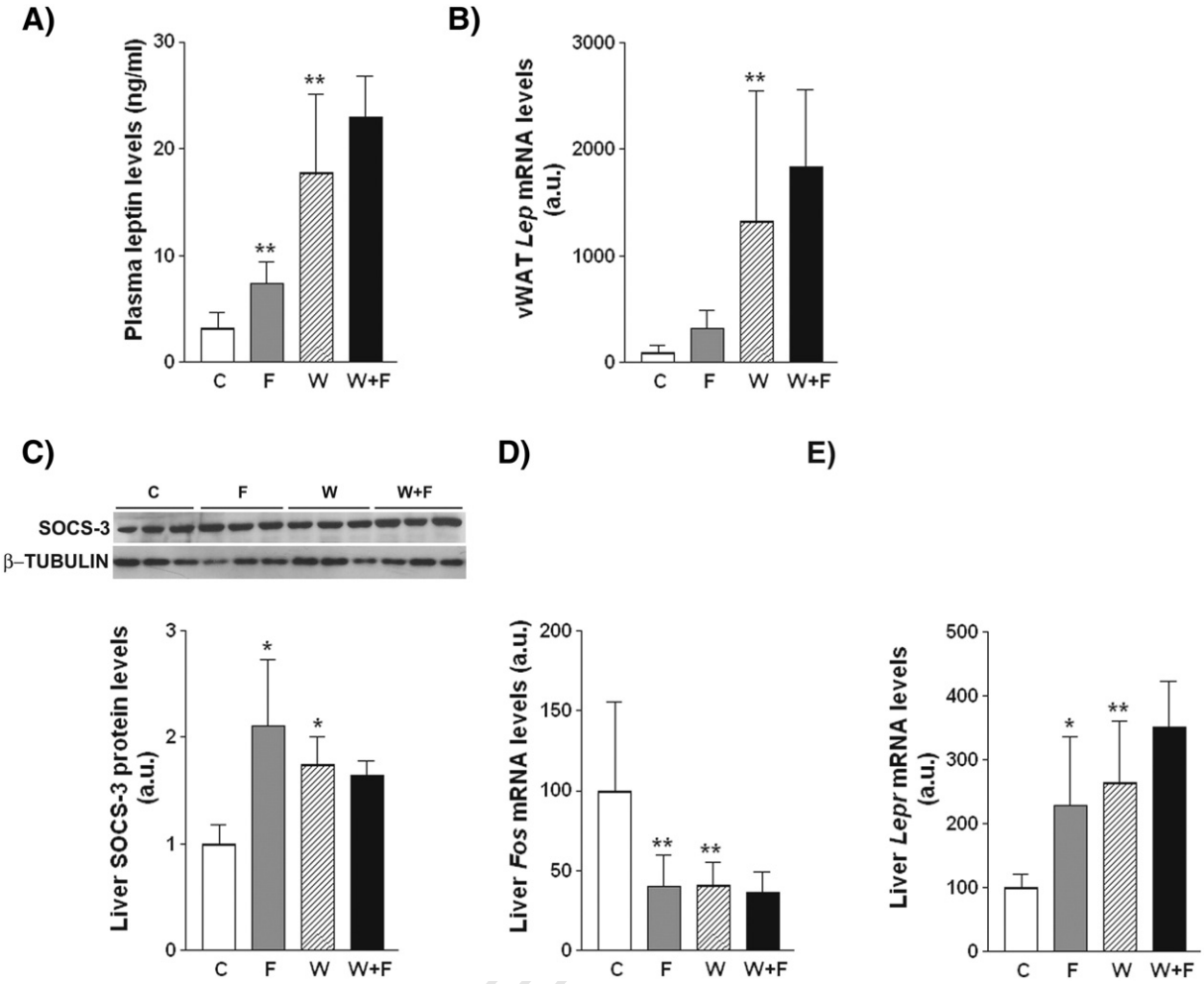


Fig. 1. Plasma leptin (A) and mRNA levels of *lep* gene in visceral adipose tissue (B) from C (mice fed standard solid chow), F (mice fed standard solid chow plus a 15% fructose solution *ad libitum*), W (mice fed Western solid chow), and W+F (mice fed Western solid chow plus a 15% fructose solution *ad libitum*) expressed as mean (a.u., arbitrary units)±SD of the values obtained from eight animals. (C) Western blot of SOCS-3 protein in liver samples obtained from the four experimental dietary groups of mice represented as the mean±SD of three to four different samples. Representative bands correspond to three different mice in each group. Bar plots showing the relative levels of *fos* (D) and *lepr* (E) mRNAs from C, F, W and W+F groups (mean±SD of four to six different liver samples). $^{*}P<.05$, $^{**}P<.01$ *vs.* C values.

3.4. Inflammation was present, mainly in visceral adipose tissue, in mice consuming a solid Western diet irrespective of liquid fructose supplementation

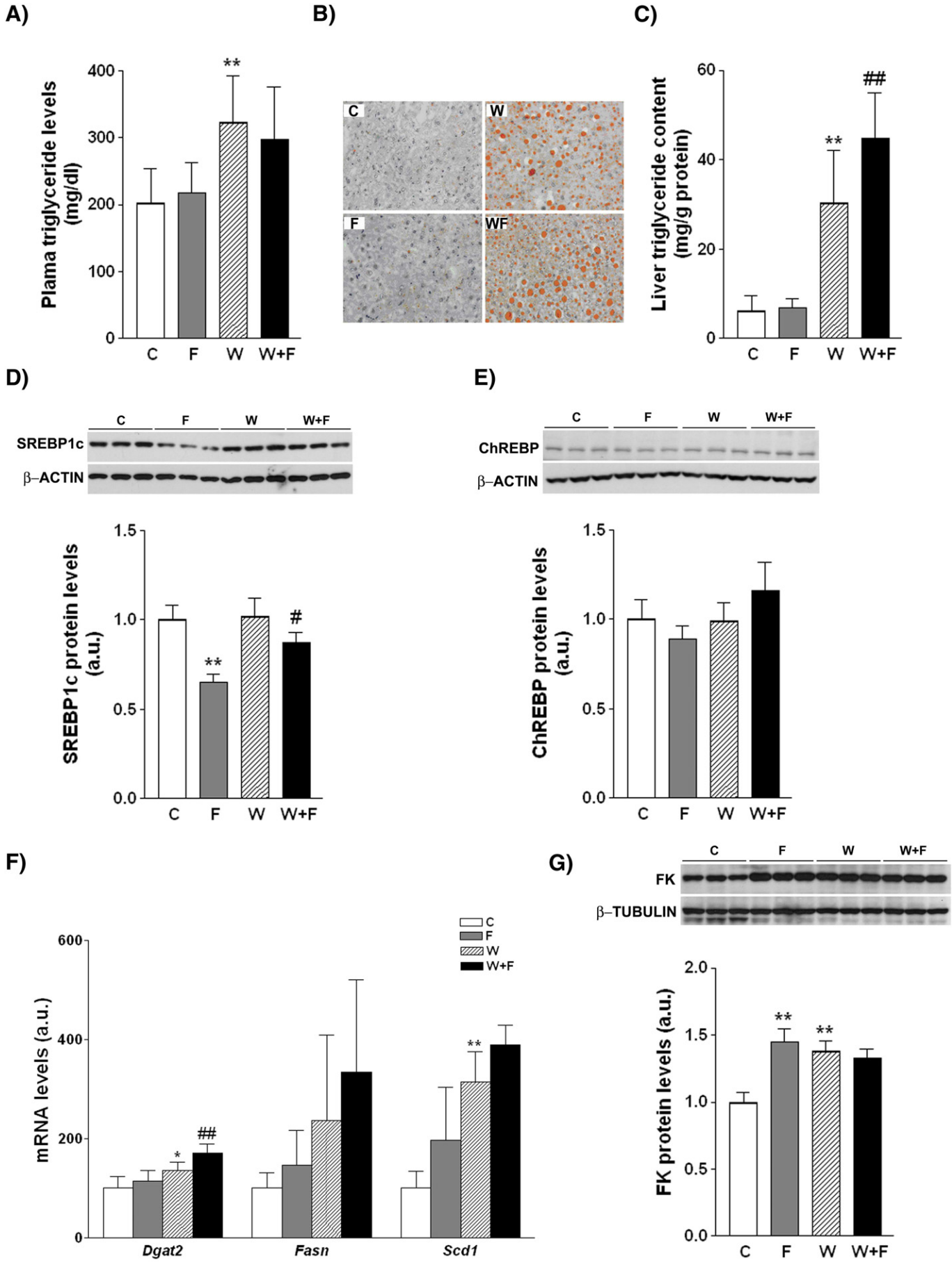
Western diet consumption over 12 weeks increased the expression of *f4/80*, a marker of macrophage infiltration and inflammatory markers, such as *ccl2* and *il-6*, in mouse vWAT (Fig. 4A). Although there was a tendency to increased expression of inflammatory markers in the livers of Western-diet-fed mice, this was not statistically significant (Fig. 4B). Liver nuclear expression of p65 (Fig. 4C) and histological analysis of liver samples (Fig. 4D) confirmed the lack of a clear inflammatory process in this tissue. The expression of inflammatory markers activated by bacterial endotoxins, such as *tlr4* or *myd88*, was not changed in the liver or adipose tissue of these mice. Moreover, fructose supplementation did not modify the expression of inflammatory markers, except in the cases of *tnfα* and the chemokine receptor *ccr2*, which were significantly increased in vWAT when compared to the levels in mice consuming a solid Western diet only.

3.5. Endoplasmic reticulum stress was not activated by any dietary intervention

We have previously shown that fructose supplementation in rats does not activate the endoplasmic reticulum stress (ERS) response [23]. In the present study, neither liquid fructose supplementation nor Western diet consumption affected the activation state of the PERK and ATF6 branches of the ERS response (Fig. 5A and B). Consequently, the expression of the target genes for activated PERK and ATF6 transcription factors was not changed or even decreased (Fig. 5C).

Phosphorylation and activation of IRE-1 were increased by Western diet consumption and, similar to our previous observations in fructose-supplemented rats [23], liquid fructose supplementation

Fig. 2. Plasma triglyceride levels (A) from C, F, W and W+F mice (mean±SD of four to six different samples). (B) Representative liver sections stained with Oil Red O from each experimental group. (C) Liver triglyceride levels from the four experimental dietary groups of mice (mean±SD of four to six different samples). Western blot of SREBP1c (D) and ChREBP (E) proteins in nuclear liver samples from the four experimental groups of mice. Representative bands correspond to three different mice in each group; bar plots show the level of the protein expressed as the mean (a.u.)±SD of the values obtained from three to four animals. (F) Bar plots showing the relative levels of specific mRNAs from the four experimental dietary groups of mice (mean±SD of four to six different liver samples). (G) Western blot of fructokinase (FK) protein in liver samples obtained from C, F, W and W+F groups. Representative bands corresponding to three different mice in each group are shown; bar plots show the level of the protein expressed as the mean±SD of the values obtained from three to four animals. $^{*}P<.05$, $^{**}P<.01$ *vs.* C values; ## $P<.01$ *vs.* W values.


A)
Plama triglyceride levels (mg/dl)
400
300
200
100
0
**
C F W W+F
B)
C
W
F
WF
C)
Liver triglyceride content (mg/g protein)
60
40
20
0
**
##
C F W W+F
D)
C F W W+F
SREBP1c
β–ACTIN
SREBP1c protein levels (a.u.)
1.5
1.0
0.5
0.0
**
#
C F W W+F
E)
C F W W+F
ChREBP
β–ACTIN
ChREBP protein levels (a.u.)
1.5
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C F W W+F
F)
mRNA levels (a.u.)
600
400
200
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C
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Dgat2
Fasn
Scd1
G)
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FK protein levels (a.u.)
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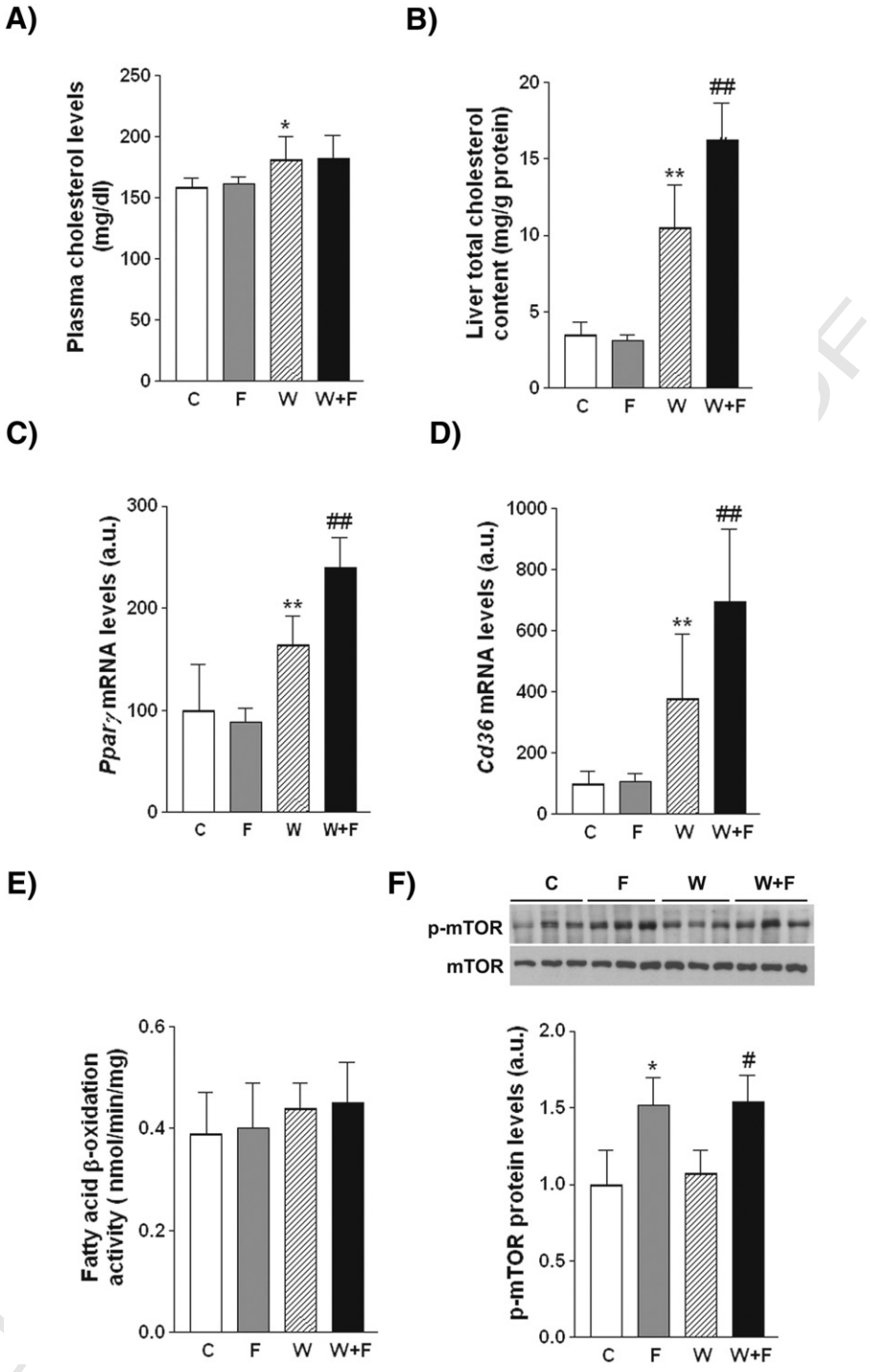

Fig. 3. Plasma (A) and liver (B) cholesterol levels from from C, F, W and W+F mice (mean±SD of four to six different samples). Bar plots showing the relative levels of *pparγ* (C) and *cd36* (D) mRNAs from the four experimental dietary groups of mice (mean±SD of four to six different liver samples). Fatty acid β-oxidation activity in liver samples obtained from C, F, W and W+F groups represented as the mean±SD of the values obtained from four to six animals. (F) Western blot of phospho- and total mTOR protein in liver samples obtained from the four experimental dietary groups of mice. Representative bands corresponding to three different mice in each group are shown; bar plots show the level of the protein expressed as the mean±SD of the values obtained from three to four animals. *P<.05, **P<.01 *vs.* C values; # P<.05, ## P<.01 *vs.* W values.

(Fig. 5D). However, this activation did not translate into an increased expression of the spliced XBP1 (XBP1s) transcription factor in all the dietary intervention groups (Fig. 5E), as demonstrated by the unaltered or even decreased expression of XBP1s target genes (Fig. 5C). Moreover, there was no clear increase in the activated, phosphorylated form of JNK (Fig. 5F).

3.6. Only the combination of a Western diet and liquid fructose supplementation significantly reduced the ISI in mice

No dietary intervention significantly modified plasma glucose (Fig. 6A) and insulin (Fig. 6B) concentrations, although mice fed a Western diet showed a tendency towards increased glucose and insulin levels, an

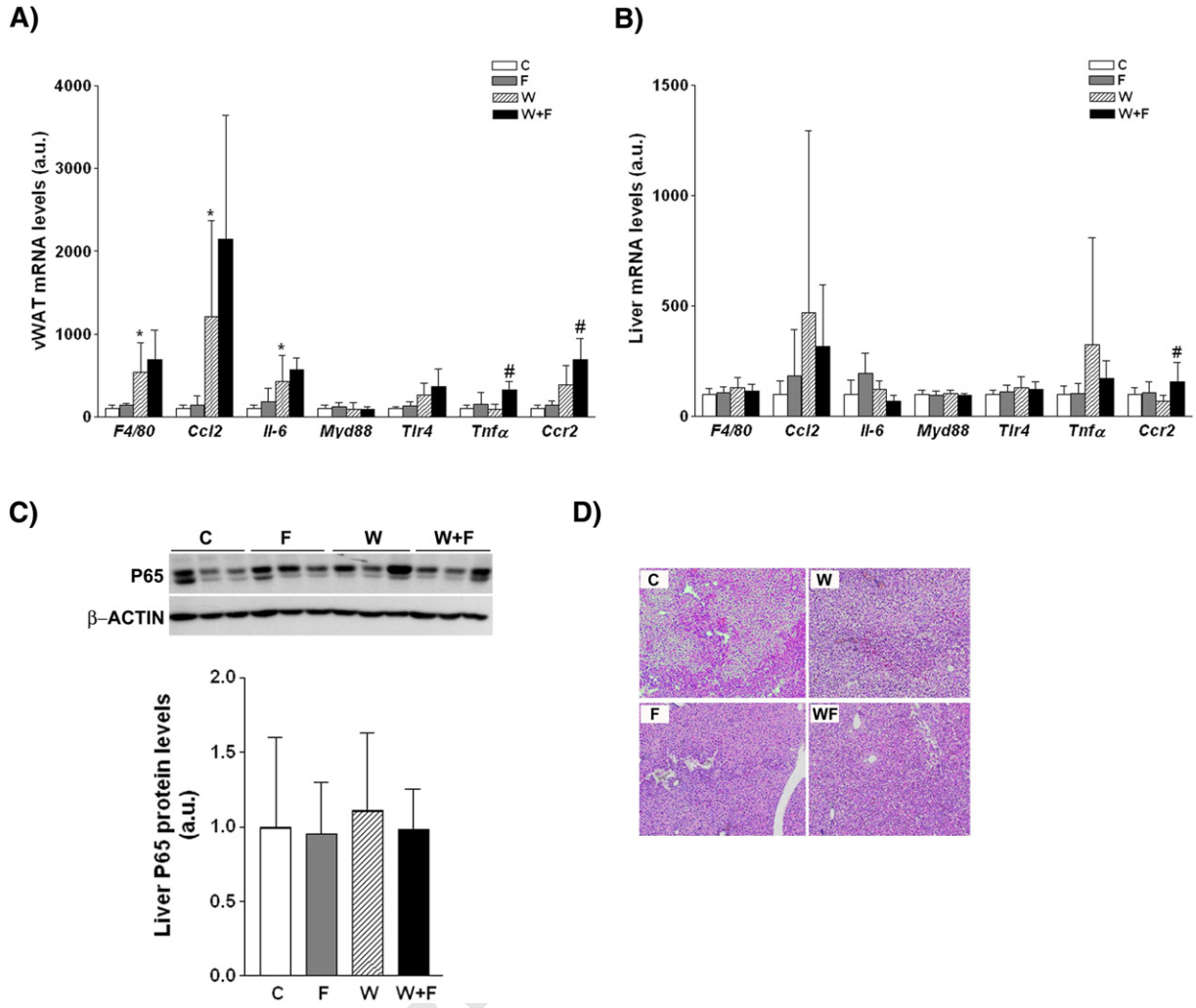

Fig. 4. Bar plots showing the relative levels of specific mRNAs from from C, F, W and W+F mice represented as mean (a.u.)±SD of four to six different visceral adipose tissue (A) or liver (B) samples. Western blot of p65 protein in liver samples obtained from C, F, W and W+F groups (C). Representative bands corresponding to three different mice in each group are shown; bar plot shows the level of the protein expressed as the mean±SD of the values obtained from three to four animals. (D) Representative hematoxylin–eosin liver sections from C, F, W and W+F groups. #$P<.05$ *vs.* W values.

effect that was further magnified with liquid fructose supplementation. As a consequence, the ISI was only significantly reduced in the Western-diet-fed mice supplemented with fructose (W+F mice) (Fig. 6C). We have previously shown that liquid fructose supplementation reduces rat liver IRS2 protein content [24,25]. As IRS2 is a key molecule in the insulin signaling pathway [26], we measured IRS2 protein levels in our liver samples. We found a progressive reduction in IRS2 protein levels across the different intervention dietary groups that was significant in Western-fed mice, further increased to less than half the value present in controls in W+F animals (Fig. 6D).

4. Discussion

Epidemiological studies in humans point to the high consumption of fructose-enriched beverages as a key factor in the development of obesity, T2DM and associated cardiovascular comorbidities. Here we show in an experimental dietary intervention model, the C57BL/6N mouse, whose metabolism is responsive to dietary manipulation that liquid fructose supplementation promotes hepatic cholesterol and triglyceride accretion, a deficit in liver insulin signaling and a reduction in whole-body insulin sensitivity in a background of an unhealthy, Western-style diet. These effects were observed despite total calorie intake remaining exactly the same between nonsupplemented and supplemented animals.

Increased energy intake without a corresponding increase in calorie expenditure by physical activity, thermoregulation, *etc.*, leads to an energy imbalance that results in increased body weight, mainly through an expansion of visceral adipose tissue [27]. Thus, in our present work, mice consuming a high-energy-density diet, such as the Western-type diet we used, increased their energy intake by ×1.27 when compared to control animals, consequently increasing their body weight mainly through a marked accretion of visceral adipose tissue (Table 2). Although Western-diet-fed mice were hyperleptinemic, they did not compensate for the excess of calories ingested by reducing the total amount of solid food consumed, thereby indicating a state of leptin resistance. Indeed, we provide evidence for leptin resistance in the peripheral tissues of Western-diet-fed animals (Fig. 1). Hypertriglyceridemia has been proposed to be key in reducing leptin penetration in the central nervous system and thus inducing central leptin resistance [28]. Given that Western-diet-fed mice showed marked hypertriglyceridemia (Fig. 2), this could be responsible for the lack of calorie compensation. This is in accordance with the fact that, although mice supplemented with liquid fructose showed hyperleptinemia and manifestations of peripheral leptin resistance (Fig. 1), they

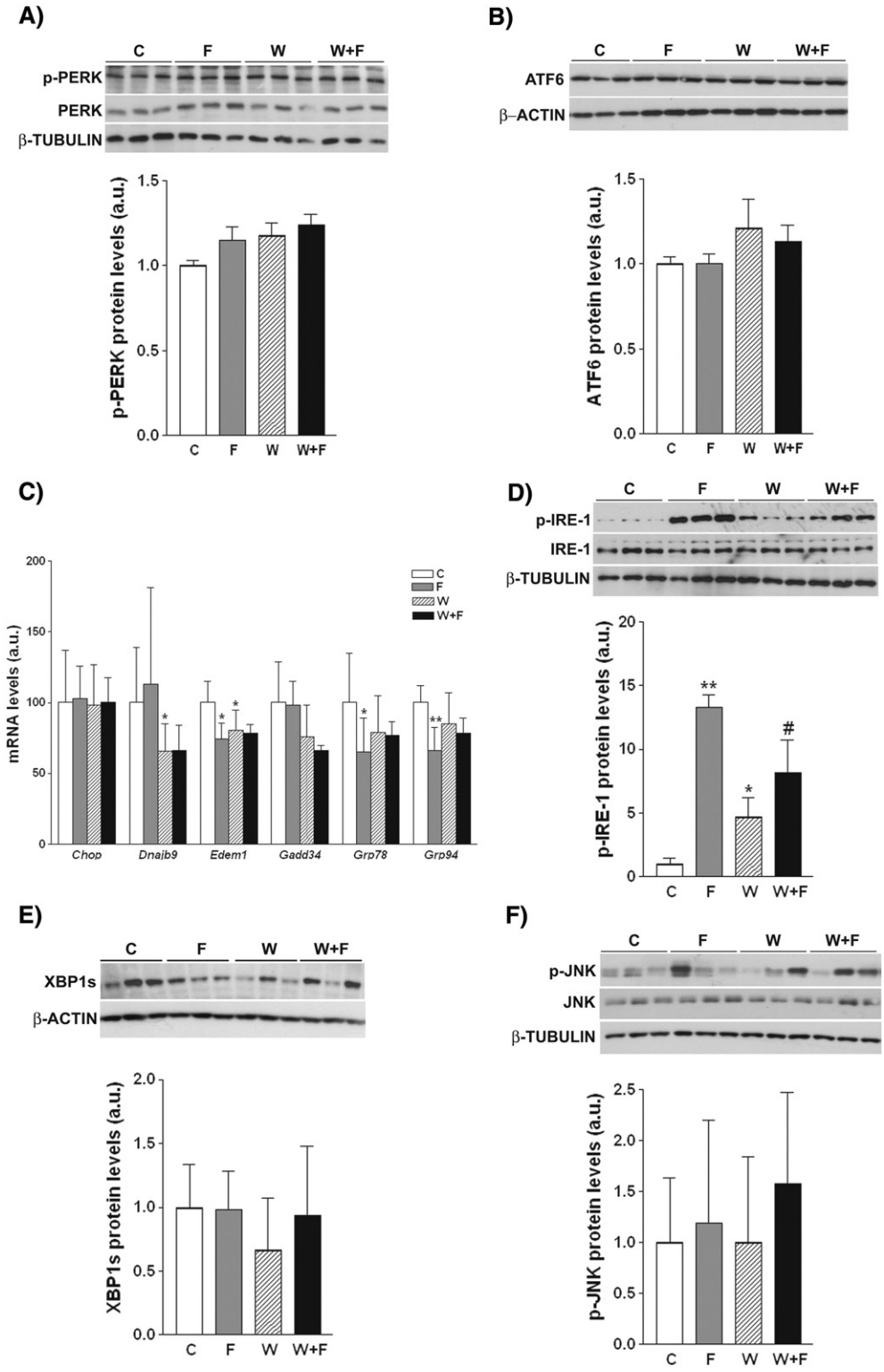

Fig. 5. Western blot of phospho- and total PERK (A) and ATF6 (B) proteins in liver samples obtained from C, F, W and W+F mice. Representative bands corresponding to three different mice in each group are shown; bar plots show the level of the protein expressed as the mean (a.u.)±SD of the values obtained from three to four animals. (C) Bar plots showing the relative levels of specific mRNAs from the four experimental groups of mice (mean±SD of four to six different liver samples). Western blot of phospho- and total IRE-1 (D), XBP1s (E) and phospho- and total JNK (F) in liver samples obtained from C, F, W and W+F groups. Representative bands corresponding to three different mice in each group are shown; bar plots show the level of the protein expressed as the mean±SD of the values obtained from three to four animals. *P<.05, **P<.01 *vs.* C values; #P<.05 *vs.* W values.

did not present hypertriglyceridemia, and consequently, they did compensate for the increased amount of fructose-derived calories by reducing the quantity of solid food consumed. Thus, body weight and the amount of visceral adipose tissue did not change in liquid-fructose-supplemented mice with respect to their corresponding controls (Table 2).

The lack of hypertriglyceridemia and liver steatosis in fructose-supplemented mice was rather surprising given the wealth of

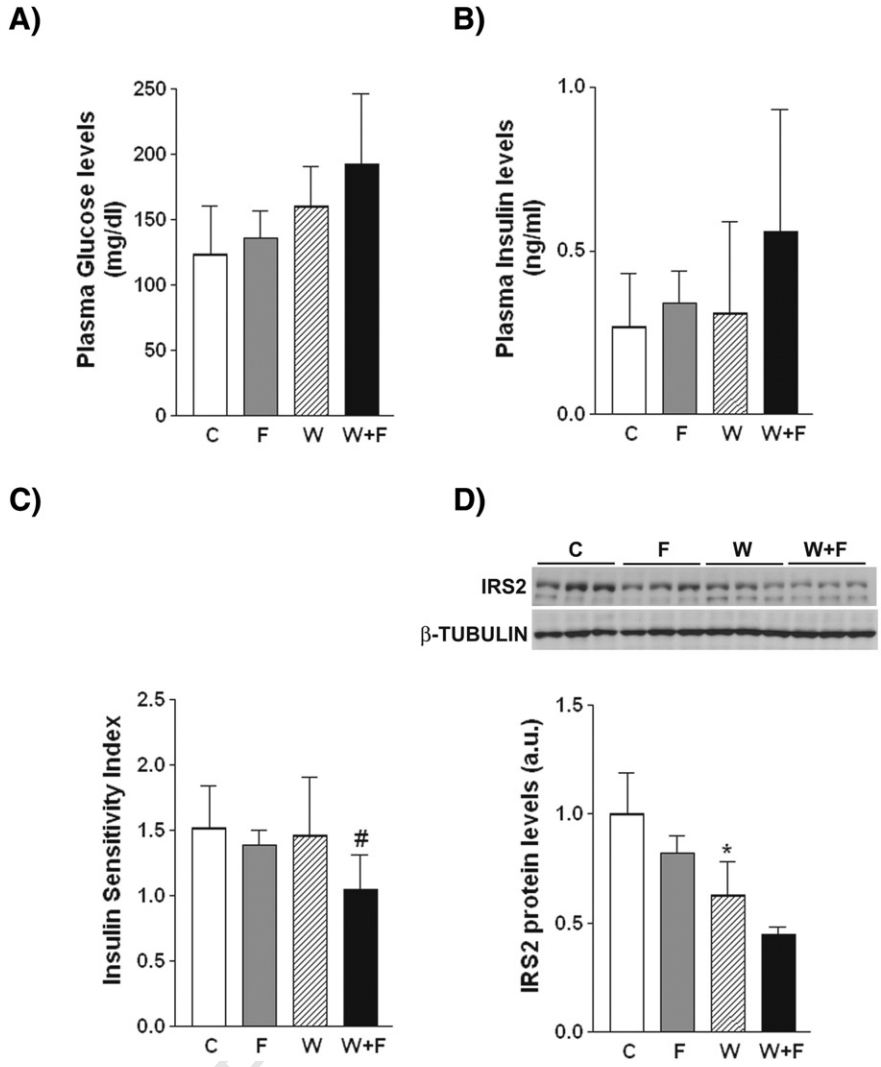

Fig. 6. Glucose (A) and insulin (B) plasmatic levels and ISI (C) from C, F, W and W+F mice (expressed as mean±SD of 6–12 different samples). (D) Western blot of IRS2 protein in liver samples obtained from C, F, W and W+F groups. Representative bands corresponding to three different mice in each group are shown; bar plot shows the level of the protein expressed as the mean (a.u.)±SD of the values obtained from three to four animals. *P<.05; #P<.05 *vs.* W values.

information on fructose and increased liver lipogenesis [20] and our previous study with 10% w/v liquid-fructose-supplemented rats [19]. We have previously shown in rats that fructose is more lipogenic than glucose because the former not only directly increases liver lipogenesis but also reduces liver fatty acid β-oxidation activity [6,7]. As mice, unlike rats and humans, are able to transform a considerable amount of fructose into glucose in the intestinal tract [29], one possible explanation for our present results is that the total amount of fructose reaching the liver was not enough to stimulate lipogenesis and also reduce fatty acid oxidation. In fact, when a higher fructose solution is used (30% w/v, see work from the Bergheim group [9]), a clear lipogenic effect is observed in mice but at the expense of changing intestinal permeability that allows the presence of bacterial endotoxins in portal blood and the development of liver TLR4-mediated inflammation [8]. In our mice, fructose supplementation did not increase the expression of inflammatory markers in liver, including endotoxin-related markers such as *tlr4* and *myd88*. An increase in inflammatory markers not related to bacterial endotoxins was only detected in visceral adipose tissue from Western-diet-fed mice. Similarly to our previous observations in rat [23], fructose supplementation did not induce an ERS response. Only the IRE-1 pathway seemed to be activated; however, there were no discernible changes in the downstream targets of IRE-1, either as a kinase, leading to the activation of JNK, or as RNA splicing enzyme, leading to XPB1s' increased expression and transcriptional activity. The consumption of a solid Western diet had a similar, but small, effect on IRE-1 activation as fructose supplementation in the mice. Again, like our previous results in rats [23], we observed increased mTORC1 phosphorylation in the livers of fructose-supplemented mice. mTORC1 activation has been linked to specific stimulation of the IRE-1 pathway [30], possibly explaining the specific and intense increase in IRE-1 phosphorylation observed in fructose-supplemented mice. It is interesting to note that IRE-1 activation has been directly linked to the prevention of hepatic steatosis [31]. In this sense, the strong increase in IRE-1 phosphorylation in fructose-supplemented mouse livers could explain the absence of a lipogenic response in these animals.

Despite the lack of a lipogenic effect, fructose supplementation clearly increased liver triglyceride and cholesterol deposition in mice consuming a solid Western diet, although these animals did reduce their consumption of the solid diet and maintained an isocaloric intake

compared to nonsupplemented Western-diet-fed animals. This effect of fructose supplementation was probably due to the potentiation in the increase of *pparγ* and *cd36* expression in the livers of W+F animals. CD36 contributes to the development of fatty liver in mice fed high-fat diets, and ablation of its expression in liver attenuates fat deposition [32]. mTORC1 activation seems to be directly associated with this potentiating effect of fructose on *cd36* expression and liver cholesterol accretion in the livers of Western-diet-fed mice. Indeed, mTORC1 has been linked to an increased expression of cholesterol biosynthetic [33] and *cd36* [34,35] genes. The combined effects of the Western diet and fructose supplementation, especially on *cd36* expression, resulted in the largest liver lipid deposition in the studied animals. We had previously observed a similar effect of fructose supplementation in LDL-R$^{-/-}$ mice, although these transgenic animals already presented a large fat deposition in the liver even when raised with standard rodent chow [10].

The strong induction of *cd36* in W+F mice could also be linked to the impaired liver insulin signaling and reduced whole-body insulin sensitivity in these animals. Fructose supplementation reduced liver IRS2 expression probably through an mTORC1-related mechanism, as previously reported in rats [24,25]. Liver *cd36* expression correlates with reduced whole-body insulin sensitivity, and its liver ablation restores and improves insulin sensitivity [32]. Thus, the combined effects of liquid fructose supplementation and Western diet consumption are sufficient to clearly impair not only liver but also whole-body insulin sensitivity in mice, even with unchanged calorie intake.

In conclusion, through a mechanism that probably involves liver mTORC1 activation, liquid fructose supplementation in metabolically unfit mice consuming a Western diet potentiates liver cholesterol and triglyceride deposition, impairs liver insulin signaling and reduces whole-body insulin sensitivity, without increasing total calorie intake and thus visceral adipose tissue deposition and body weight. Whether metabolic disease of polygenic origin (such as human obesity or insulin resistance) confers a high susceptibility to fructose-related metabolic derangement deserves further research.

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Conflict of interest

No potential conflict of interest, including related consultancies, shareholdings and funding grants, exists for any of the authors of the present work.

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