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RESEARCH ARTICLE | *Gut Hormones, Metabolism, Appetite, and Obesity*

High-fat diet overfeeding promotes nondetrimental liver steatosis in female mice

Lino Arisqueta,[1] Hiart Navarro-Imaz,[2] Ibone Labiano,[3] Yuri Rueda,[2] and Olatz Fresnedo[2]

[1]*Facultad de Ciencias Naturales y Ambientales, Universidad Internacional SEK, Quito, Ecuador;* [2]*Lipids and Liver Research Group, Department of Physiology, Faculty of Medicine and Nursing, University of the Basque Country UPV/EHU, Leioa, Spain; and* [3]*Department of Liver and Gastrointestinal Diseases, Health Research Institute, Biodonostia, Spain*

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Arisqueta L, Navarro-Imaz H, Labiano I, Rueda Y, Fresnedo O. High-fat diet overfeeding promotes nondetrimental liver steatosis in female mice. *Am J Physiol Gastrointest Liver Physiol* 315: G772–G780, 2018. First published August 10, 2018; doi:10.1152/ajpgi.00022.2018.—High-fat diet (HFD) feeding or leptin-deficient mice are extensively used as models resembling features of human nonalcoholic fatty liver disease (NAFLD). The concurrence of experimental factors as fat content and source or total caloric intake leads to prominent differences in the development of the hepatic steatosis and related disturbances. In this work, we characterized the hepatic lipid accumulation induced by HFD in wild-type (WT) and *ob*/*ob* mice with the purpose of differentiating adaptations to HFD from those specific of increased overfeeding due to leptin deficiency-associated hyperphagia. Given that most published works have been done in male models, we used female mice with the aim of increasing the body of evidence regarding NAFLD in female subjects. HFD promoted liver lipid accumulation only in the hyperphagic strain. Nevertheless, a decrease of lipid droplet-associated cholesteryl ester (CE) in both WT and obese animals was observed. These changes were accompanied by an improvement in the profile of lipoproteins that transport cholesterol and liver function markers in plasma from *ob*/*ob* mice and a lower hepatic index. Using primary hepatocytes from female mice, overaccumulation of CE induced by 0.4 mM oleic acid reversed in the presence of a specific Takeda G protein-coupled bile acid receptor agonist. Nevertheless, hepatocytes from male mice were not responsive. This study suggests that enterohepatic circulation of bile acids might be one of the factors that can affect sex dimorphism in NAFLD development, which underlines the importance of including female models in the NAFLD research field.

NEW & NOTEWORTHY This work provides new insight into the use of high-fat diet as a model to induce nonalcoholic fatty liver disease in wild-type and *ob*/*ob* female mice. We show that high-fat diet induces steatosis only in *ob*/*ob* mice while, surprisingly, several health indicators improve. Noteworthy, experiments with primary hepatocytes from male and female mice show that they express Takeda G protein-coupled bile acid receptor and that it and bile acid enterohepatic circulation might be accountable for sex dimorphism in nonalcoholic fatty liver disease development.

cholesterol; high-fat diet; lipid droplets; nonalcoholic fatty liver disease; sex dimorphism

INTRODUCTION

One of the main organs modulating the whole body lipid homeostasis is the liver. Hepatocytes can accumulate and mobilize high quantities of lipids as a physiological response to changes in nutrient provision and in pathological states as inflammation (1–4, 24, 26). Cytosolic lipid droplets (LD) are the cellular structures specialized in lipid storage and mobilization. Accumulation of LDs, revealed by histochemical or biochemical analysis, is the main characteristic of liver steatosis, considered the first step in the progression of nonalcoholic fatty liver disease (NAFLD).

Obesity is a well-established risk factor for health that may lead to different metabolic disorders, including NAFLD. This disease, often associated with metabolic syndrome, ranges from the mentioned steatosis to nonalcoholic steatohepatitis with or without fibrosis and cirrhosis, and can progress to hepatocarcinoma (15). NAFLD has become one of the most frequent causes of hepatopathy and trends disturbingly upward (16). Understanding the factors that cause or affect the metabolic disturbances linked to obesity has a potential applicability in health care.

To address mechanistic studies, several animal models are used. Strains with food intake disturbances like mice with altered satiety mechanisms (e.g., *ob*/*ob* mice) or administration of diets with high percentage of energy in fat, combined or not with extra sugar in water, are usual. There are a lot of recent examples of the use of such models (6, 8, 25, 28).

Nevertheless, high-fat diet (HFD) feeding works as a model of NAFLD depending on the duration of the treatment and the composition of the diet (reviewed in Refs. 12 and 20). Moreover, although long-term HFD induces extra body weight gain and associated alterations like impaired insulin resistance in mice, liver steatosis degree and inflammatory state are higher in male mice compared with females (17).

Plenty of works describe the effects of different types of HFD feeding during varying time periods in male mice (for example, Refs. 8, 23, 25, 27, 28, and 31). Several parameters that can impact NAFLD show sex dimorphism in humans. Males tend to deposit more fat in abdominal adipose tissue, a condition correlated with intrahepatic fat accumulation and inflammatory biomarkers (43); thus, abdominal adiposity is considered a predictor of NAFLD progression (40). Liver metabolic deregulation in overweight populations seems to be a differential feature between men and women (35, 39) that might impact NAFLD.

Address for reprint requests and other correspondence: O. Fresnedo, Dept. of Physiology, Faculty of Medicine and Nursing, Univ. of the Basque Country UPV/EHU. B° Sarriena s/n, 48940 Leioa, Spain (e-mail: olatz.fresnedo@ehu.eus).

In this work we used wild-type (WT) and *ob/ob* female mice. We fed them a standard, well-defined control diet (CD) and a HFD that provides 60% of energy as fat for a period of 9 wk. This design allows differentiating between the effectiveness of the extra calorie intake due to the fat and hyperphagia in inducing steatosis. We also wanted to search for possible protective factors in early phases of NAFLD development that can lead to long-term differences between males and females.

MATERIALS AND METHODS

Animals and dietary treatment. C57BL/6J (WT) and B6.V-Lepob/J (*ob/ob*) female mice (8 wk old; Jackson Laboratory) were housed in a temperature-controlled room with a 12-h:12-h light-dark cycle. Animals of each genotype were randomly distributed in two groups, and each of them had ad libitum access to water and CD (F4031; BioServ) or HFD (F3282; BioServ) with 60% of calories in fat for 9 wk.

After that period mice were weighed and sacrificed, and blood samples and liver were collected. Liver was cut into pieces adequate for the preparation of homogenates, immediately frozen in liquid nitrogen, and stored at −80°C until use. Blood was allowed to clot and centrifuged (2,000 *g*, 30 min, 4°C), and the supernatant was collected and further centrifuged (10,000 *g*, 10 min, 4°C) to obtain serum, which was stored at −20°C until use. Serum biochemical markers were determined using an automatic Cobas clinical analyzer (Laboratorio Lafita).

All of the procedures that involved animal handling were approved by the Ethics Committee for Animal Welfare of the University of the Basque Country UPV/EHU.

Hepatocyte isolation, purification, and culture. Hepatocytes were isolated from mice and purified in Percoll gradient as described elsewhere (7). Primary cell culture was performed essentially as described by Arisqueta et al. (2). Purified hepatocytes plated in six-well plates (6×10^5 cells/well) were incubated for 1 h for adhesion, and just afterward culture medium was supplemented with 0.4 mM oleic acid complexed with fatty acid-free BSA (1.33%), 10 μM of the Takeda G protein-coupled bile acid receptor (TGR5) agonist 3-(2-chlorophenyl)-*N*-(4-chlorophenyl)-*N*-5-dimethyl-4-isoxazolecarboxamide dissolved in N_2-saturated ethanol, or BSA and ethanol for the control conditions. The compounds were added from concentrated stocks. Simultaneously, a trace of 60 nCi of [^{14}C]cholesterol (Perkin Elmer) dissolved in ethanol (0.5% final concentration) was added. After the pulse period (4 h), cultures were washed two times with PBS and harvested or incubated for 2 h in the same conditions but without radioactive cholesterol to chase the incorporated radioactivity. After the chase period, cells were also washed and processed for radioactivity incorporation analysis as described elsewhere (3).

Hepatic lipid droplet isolation. Liver pieces were homogenized in 4 vol of 10 mM Tris·HCl, pH 7.4, containing 2 mM Na_2-EDTA and 0.25 M sucrose using a Potter-Elvehjem homogenizer (10 strokes, 700 revolutions/min, 4°C). Cytosolic LDs were obtained from homogenates by ultracentrifugation in a sucrose gradient in the presence of EDTA as described elsewhere (2). Only the upper fraction, which contained the lightest LDs, was used in this study.

Protein measurement and Western blotting. Protein concentration of liver homogenates was determined by the bicinchoninic acid method (Thermo Fisher Scientific) including 2% SDS in all samples to avoid erroneous measures due to the presence of lipid. For protein determination in LD samples the Lowry protein assay modified by Peterson (34) was used as previously described (2) due to the high amount of lipid present in the sample.

For Western blotting analysis, protein fractionation by SDS-PAGE and immunodetection was performed as described elsewhere (32). Guinea pig anti-mouse perilipin (PLIN) 2 and guinea pig anti-mouse PLIN3 (Progen) primary antibodies and anti-guinea pig IgG (Sigma) secondary antibody were used as described by Arisqueta et al. (2).

For TGR5 detection, lysates of purified primary hepatocytes were denatured with the classical Laemmli buffer with doubled concentrations of denaturing agents. SDS-PAGE was done in an 11% acrylamide/bis-acrylamide gel (pH 9.5). All incubations and washes of the semidry-transferred nitrocellulose membrane were done at 4°C. After blocking with 5% BSA for at least 24 h, the membrane was incubated with primary antibody (Abcam) for at least 48 h. Secondary goat anti-rabbit IgG conjugated with peroxidase from Cell Signaling was used for detection with Clarity ECL substrate (Bio-Rad). A mouse spleen extract was used as positive control, and glyceraldehyde 3-phosphate dehydrogenase was immunoblotted (Abcam) for normalization.

Table 1. *Body and serum parameters of HFD-fed WT and ob/ob mice*

	WT		*ob/ob*	
	CD	HFD	CD	HFD
Body parameters				
Body wt, g	22.8 ± 0.3	30.0 ± 1.4[c]	52.9 ± 0.6[f]	70.4 ± 4.2[c,f]
Liver wt, g	1.0 ± 0.02	1.2 ± 0.1	3.5 ± 0.1[f]	2.7 ± 0.1[c,f]
Hepatic index, %	4.5 ± 0.05	3.9 ± 0.2[c]	6.6 ± 0.3[f]	3.9 ± 0.3[e]
Serum parameters				
Glucose, g/dl	200 ± 6	285 ± 19[c]	268 ± 8[f]	367 ± 28[b,e]
Triacylglycerol, mg/dl	40.4 ± 1.9	53.0 ± 12.1	57.4 ± 7.3	68.4 ± 6.1
Cholesterol, mg/dl	89 ± 1	117 ± 8[c]	302 ± 11[f]	198 ± 28[b,c]
HDL-cholesterol, mg/dl	76 ± 2	96 ± 7[a]	193 ± 20[f]	167 ± 23[b]
Cholesterol in HDL, %	86 ± 1	82 ± 2	63 ± 5[d]	84 ± 4[a]
GGT, U/l	1.6 ± 0.2	0.4 ± 0.2[c]	0.6 ± 0.2[b]	0.4 ± 0.2
GPT, U/l	24.4 ± 3.5	28.2 ± 4.2	672 ± 67[f]	201 ± 57[b,c]
GOT, U/l	61 ± 5	57 ± 3	643 ± 82[f]	212 ± 65[b,c]
ALP, U/l	115 ± 3	76 ± 7[e]	358 ± 14[f]	96 ± 17[e]
Bilirubin, mg/dl	0.10 ± 0.01	0.03 ± 0.04	0.16 ± 0.01[d]	0.04 ± 0.01[e]

Values are means ± SE; $n = 5$ animals in each group. ALP, alkaline phosphatase; CD, control diet; GGT, γ-glutamyl transpeptidase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; HDL, high-density lipoprotein; HFD, high-fat diet; WT, wild type. Student's *t*-test: [a]$P < 0.05$, comparison between CD- and HFD-fed animals; [b]$P < 0.05$, comparison between two genotypes in the same condition; [c]$P < 0.01$, comparison between CD- and HFD-fed animals; [d]$P < 0.01$, comparison between two genotypes in the same condition; [e]$P < 0.001$, comparison between CD- and HFD-fed animals, and; [f]$P < 0.001$, comparison between two genotypes in the same condition.

Enzymatic activities. Enzymatic activities of postnuclear supernatants from liver homogenates were assayed using radioactive substrates as described by Arisqueta et al. (2).

Lipid extraction and quantification. Lipids from liver homogenates (postnuclear supernatants) and hepatic LDs were exhaustively extracted following the method described by Bligh and Dyer (9). Lipid quantification was performed by thin-layer chromatography and image analysis as described elsewhere (37). All data were normalized with protein content.

Relative mRNA quantification by real-time RT-PCR. RNA extraction and retrotranscription and mRNA quantification by RT-PCR were performed as described previously (1). All primers used have been previously described (1, 2) except for the following: Cyp7a1 forward: cgcatgtttctcaacgatacactc, reverse: cttgagatgcccagaggatcac; Hmgcr forward: aacatgttcaccggcaacaac, reverse: cgcgttatcgtcaggatgatg.

Statistics. Statistical analysis was performed using GraphPad Prism version 5.02 (GraphPad Software). Comparisons were performed by the unpaired Student's *t*-test. A $P \leq 0.05$ was considered statistically significant.

RESULTS

Fat overfeeding ameliorates liver damage parameters. To analyze the effect that fat overfeeding has on body and serum parameters, *ob*/*ob* and WT (control) mice were fed on a HFD for 9 wk. Control groups were fed on a CD. As expected, animals from both genotypes gained body weight (30%) in response to HFD (Table 1). Serum glucose levels also increased in good correlation with overweight. Nevertheless, serum triacylglycerol (TG) showed no significant modifications in response to HFD. Because the increase in body mass was not accompanied by an equivalent increase in liver weight, hepatic indexes decreased, especially in *ob*/*ob* mice.

In *ob*/*ob* mice the reduction in hepatic index was associated to a clear improvement of liver function markers; serum glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) levels in *ob*/*ob*-CD were higher than in WT-CD, and HFD led to a marked decrease in both transaminases. Altogether, these results suggest a reduction in hepatic inflammation. Notably, in parallel to those modifications, serum total cholesterol levels were lower in *ob*/*ob*-HFD due mainly to the decrease in non-HDL cholesterol (i.e., low-density lipoprotein, very-low-density lipoprotein). Actually, the percentage of cholesterol transported in HDL increased from 63 to 84% (Table 1).

HFD promotes TG accumulation and modulates lipid composition of hepatic lipid droplets in ob/ob mice. We analyzed the TG, cholesteryl ester (CE), unesterified free cholesterol (FC), and phospholipid (PL) content of extracts from liver homogenates and LDs isolated by ultracentrifugation in sucrose gradient. Significantly higher levels of TG were detected in *ob*/*ob*-CD than in WT-CD in liver homogenates, and only obese mice accumulated TG after HFD treatment, particularly in LD (Fig. 1 and Table 2). Figure 2 shows no differences in the molar percentage of TG, CE, FC, phosphatidylethanolamine, or phosphatidylcholine between LDs of WT-CD and *ob*/*ob*-CD. Besides the increase of LD-associated TG in *ob*/*ob* (Table 2), the most consistent change induced by HFD was the decrease in almost the 50% of LD-associated CE in both WT and obese animals. The decrease of LD-associated CE was reflected in its molar percentage, which is indicative of cholesterol-depleted LDs (Fig. 2).

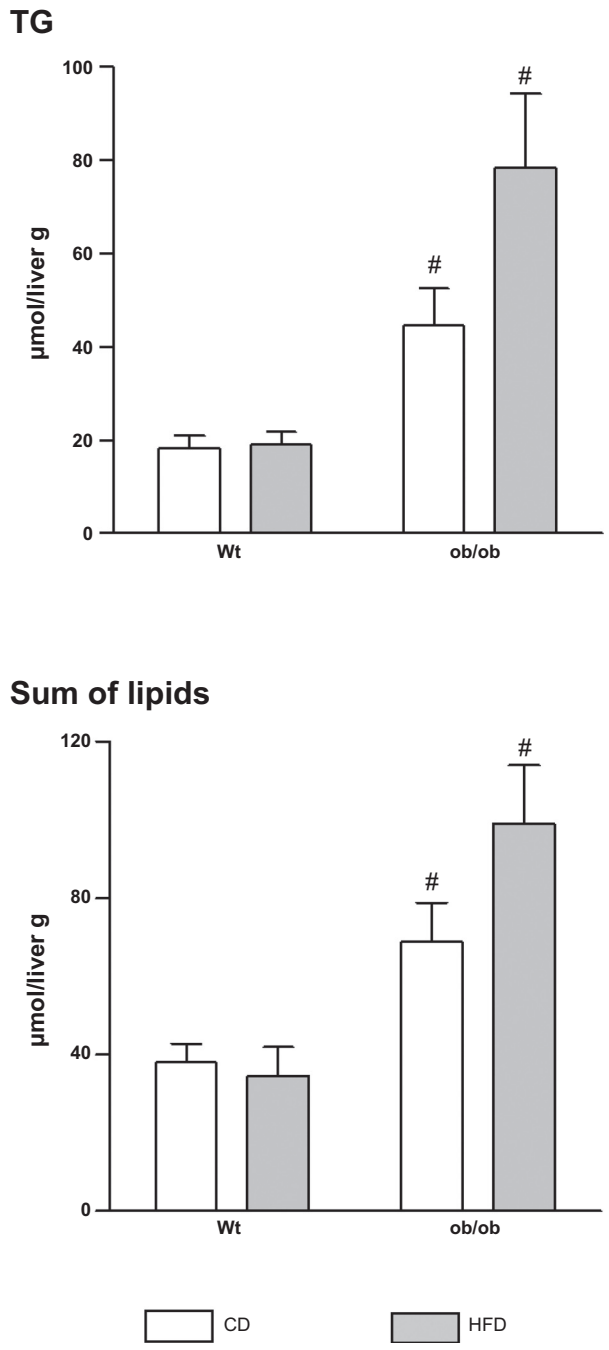

Fig. 1. Liver total lipid and triacylglycerol (TG). Hepatic lipids were extracted, and TG, cholesterol, cholesteryl ester, and phospholipids were separated and measured by thin-layer chromatography. WT, wild type; CD, control diet; HFD, high-fat diet. Data represent means ± SE of $n = 5$. Student's *t*-test: $\#P < 0.05$ when comparing the two genotypes in the same condition.

The expression of the main hepatic LD marker protein PLIN2, and the related protein PLIN3, was analyzed by Western blot analysis. LDs of *ob*/*ob*-HFD were enriched in PLIN2 (Fig. 3), the main structural protein of LDs in tissues other than adipose (10), whereas LDs of WT-HFD did not show any significant change. On the contrary, there was a marked decrease of PLIN3 in *ob*/*ob* upon HFD while LDs of WT mice showed the same trend but without statistical significance.

Table 2. *Lipid content of liver lipid droplets in WT and ob/ob mice fed CD and HFD*

	WT		*ob/ob*	
Lipids, nmol/g liver	CD	HFD	CD	HFD
TG	11,656 ± 1,927	10,270 ± 722	14,799 ± 2,456	35,498 ± 7,544[a,b]
CE	1,614 ± 451	967 ± 233[a]	2,137 ± 207	1,161 ± 179[a]
FC	243 ± 38	226 ± 52	394 ± 108	237 ± 90
PC	364 ± 66	397 ± 147	485 ± 122	666 ± 188
PE	252 ± 64	216 ± 86	256 ± 62	203 ± 86
Total lipids	14,129 ± 1,866	12,007 ± 739	18,071 ± 2,842	37,825 ± 7,916[b]
Total cholesterol	1,857 ± 210	1,124 ± 85[a]	2,531 ± 314	1,498 ± 221[a]
Phospholipids	616 ± 126	613 ± 231	741 ± 184	829 ± 219
Hydrophobic lipids	13,270 ± 1,811	11,168 ± 694	16,937 ± 2,617	36,659 ± 7,679[b]
Amphipathic lipids	859 ± 152	839 ± 281	1,135 ± 289	1,166 ± 271

Values are means ± SE; $n = 5$ animals in each group. CD, control diet; CE, cholesteryl ester; FC, free cholesterol; HFD, high-fat diet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol; WT, wild type. Student's *t*-test: [a]$P < 0.05$, comparison between CD- and HFD-fed animals and [b]$P < 0.05$, comparison between two genotypes in the same condition.

Adaptations in liver lipid metabolic profile. We analyzed the hepatic levels of mRNA corresponding to genes implicated in LD biogenesis and lipid mobilization from LDs (Fig. 4, *A* and *B*). The decrease in LD cholesterol percentage and the increase in HDL-associated cholesterol percentage in serum observed in *ob*/*ob*-HFD led us to hypothesize that reverse cholesterol transport and hepatic secretion of biliary cholesterol might be enhanced. To further test this hypothesis, we also measured the hepatic expression of several key genes involved in cholesterol and bile lipid metabolism (Figs. 4, *C* and *D*). We also measured the expression of other genes involved in hepatic lipoprotein biogenesis and uptake (data not shown).

The terminal enzymes in the synthesis of TG and CE, diacylglyceride acyltransferase (DGAT) and acyl-coenzyme A-cholesterol acyltransferase (ACAT), respectively, are metabolic drivers of LD biogenesis (42). Data shown in Fig. 4*A* indicate that fat feeding did not promote the expression of either Dgat1 or Soat1. Regarding hydrolytic enzymes (Fig. 4*B*), Pnpla2 (which encodes TG hydrolase A) and Hsl (which encodes hormone-sensitive lipase, a diacylglycerol and CE hydrolase) expression in *ob*/*ob*-HFD was markedly decreased.

To assess the cellular impact of these modifications of gene expression, we quantified the neutral TG hydrolysis and the DGAT activity in postnuclear supernatants from liver homogenates (Fig. 5). The overall ability for fatty acid release from TG was similar in all conditions. DGAT activity in *ob*/*ob* liver doubled that of WT in both conditions even though HFD promoted a significant drop in the obese mice (Fig. 5).

Regarding cholesterol homeostasis, HFD did not induce any significant change in the mRNA levels of Srebp2, Hmgcr, and Ldlr (Fig. 4*C*), which are central members of the protein network that maintains cholesterol homeostasis. The expression profile of Cyp7a1, which encodes the rate-limiting enzyme of the main bile acid (BA) synthesis pathway, cholesterol 7α-hydroxylase, is very interesting because it responded to HFD in WT mice and was constitutively activated in the obese strain (Fig. 4*D*). Finally, the mRNA levels of apical transporters of biliary lipids Mdr2 (Abcb4), Abcg5, and Abcg8 showed only small modifications in *ob*/*ob* mice.

Sexual dimorphism in response to TGR5 activation. It has been shown that liver and plasma levels of cholesterol are modulated in response to HFD by TGR5 in a sex-specific way (41). TGR5 knockout female and male mice showed differential responses in fat accumulation and other features related to bile homeostasis under HFD (29). Therefore, we next wanted to explore whether the changes in cholesterol management in prosteatotic conditions are mediated by TGR5. We used purified hepatocytes from female and male mice maintained only 1 h for adhesion to minimize the loss of sex-specific phenotypic physiology during the assay. Figure 6*A* shows that primary hepatocytes from both female and male mice express TGR5 at similar levels. We analyzed the cholesterol accumulation in primary hepatocyte cultures treated with the selective TGR5 agonist 3-(2-chlorophenyl)-*N*-(4-chlorophenyl)-*N*-5-dimethyl-4-isoxazolecarboxamide. To model steatosis, cells were incubated with 0.4 mM oleate. To analyze cholesterol accumulation and storage capacity, together with treatment or vehicle addition, a trace of radioactive cholesterol was added to primary cultures. During the 4-h incubation, male hepatocytes accumulated lower quantities of cholesterol than female ones

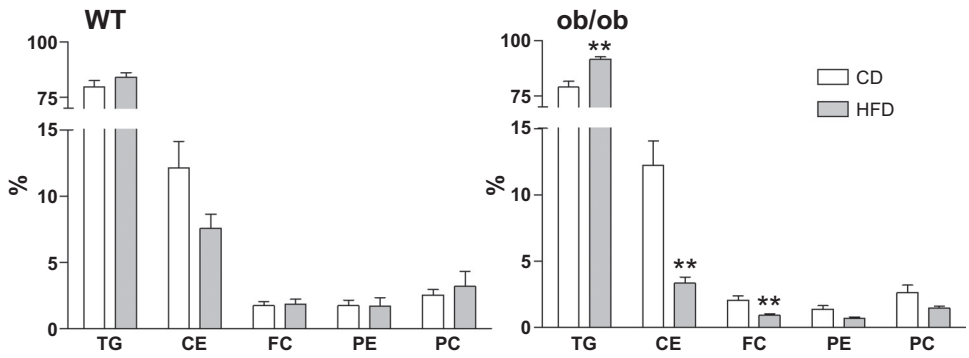

Fig. 2. Lipid composition of hepatic lipid droplets from wild-type (WT) and *ob*/*ob* mice. Lipid droplets were isolated from the liver, lipids were extracted, and triacylglycerol (TG), cholesteryl esters (CE), unesterified free cholesterol (FC), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) were separated and measured by thin-layer chromatography. The molar percentage of each lipid species is shown. Data represent means ± SE of $n = 5$. Student's *t*-test: $^{**}P < 0.01$ when comparing animals on a high-fat diet (HFD) with those on a control diet (CD).

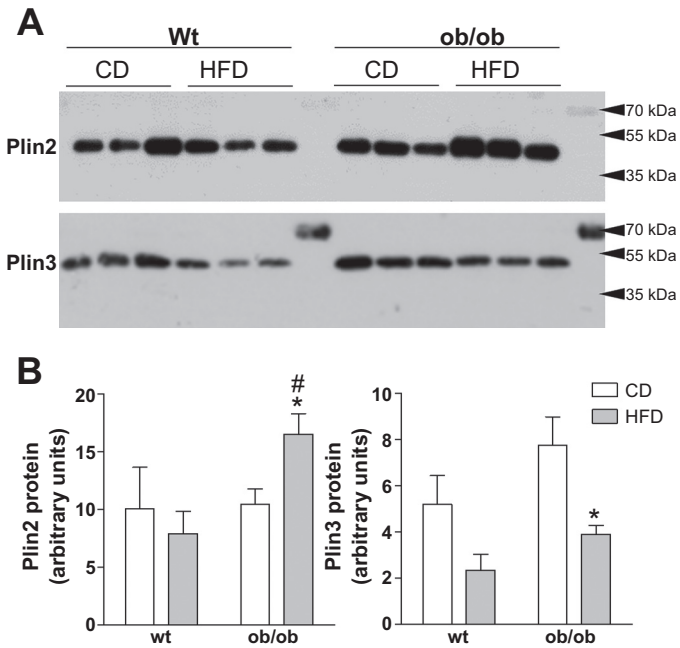

Fig. 3. Perilipin (Plin) expression in hepatic lipid droplets. *A*: lipid droplets were isolated from the liver, and Plin2 and Plin3 were immunodetected by Western blotting. The same amount of total protein was loaded in each lane. *B*: the signal in each band was measured by densitometry. WT, wild type. Data represent means ± SE of $n = 5$. Student's *t*-test: $^{*}P < 0.05$ when comparing animals on a high-fat diet (HFD) with those on a control diet (CD); $\#P < 0.05$ when comparing the two genotypes in the same condition.

(Fig. 6*B*). After elimination of [^{14}C]cholesterol from the medium (2 h), cells from female mice maintained higher CE levels and, unlike those from male mice, increased in response to steatosis, an effect that was reversed by TGR5 agonist treatment (Fig. 6*C*).

DISCUSSION

Animal models resembling features of NAFLD have been useful to investigate mechanisms of development and progression of the disease. Dietary and genetic mouse models like HFD or leptin deficiency are extensively used examples. Many works have shown that HFD feeding for variable times is an adequate model to develop features of early phases of NAFLD like liver simple steatosis (19, 27). Nevertheless, there are many examples in which similar HFD treatments did not achieve the development of hepatic steatosis (11, 36). It might be of interest to analyze which factors underlie those differences, as nutritional features of the diets other than fat content or total caloric intake (20). Additionally, given that male mice are more often used in NAFLD studies, many general concepts about NAFLD models could be prone to gender bias. In this work, we focused on the biochemical characterization of the hepatic lipid accumulation induced by HFD in female WT and *ob*/*ob* mice with the purpose of differentiating adaptations to HFD from those specific of overfeeding due to leptin deficiency-associated hyperphagia. Female mice were used with the aim of helping to clarify whether sex could be also a key factor in the development of liver steatosis.

As expected, weight gain of female WT and *ob*/*ob* mice fed on HFD (60% of calories in fat) during 9 wk is substantially higher than weight gain of those fed on CD. Glycemia increased in parallel to body mass possibly due to incipient glucose tolerance impairment. Nevertheless, the replacement of carbohydrates as the main source of calories of the CD by lipids did not promote detrimental changes in the hepatic index or the serum markers of liver function. A milder HFD treatment (45% calories in fat, 8 wk feeding) has been reported to promote a moderate rise of GPT in male mice (19); in our experimental model, female obese mice showed a marked improvement (75% mean decrease) in the levels of serum transaminases (GPT, GOT), alkaline phosphatase, and bilirubin (Table 1).

It has been discussed whether HFD is an effective treatment to induce NAFLD (20); factors like amount of dietary fat, the quality of fat, or the fatty acid composition seem to be important. Here we show that unexpected results can be obtained depending also on the strain and the sex. Treatments used in this work, CD and HFD provided by BioServ, did not promote hepatic steatosis in WT C57BL-6 female mice. That was revealed by the biochemical analysis of liver homogenates and isolated LDs using the procedure described by Ruiz and Ochoa (37), a method that enables the analysis of different PLs and neutral glycerolipids, and free and esterified forms of cholesterol (2, 37). In obese mice, the TG content of liver homogenates and isolated LDs increased after HFD (Fig. 1 and Table 2), a change that substantially modified LD lipid composition (Fig. 2) and that implies a larger LD volume.

HFD also promoted changes in the quantities of PLIN2 and PLIN3 in the obese mice (Fig. 3). This might be of importance in the development of liver steatosis, since the sequential expression of these two perilipins during liver LD maturation has been described by Pawella et al. (33); according to this model, microvesicular to macrovesicular steatosis transition in the liver occurs with the replacement of PLIN3 and PLIN5 by PLIN2 and PLIN1. Our results are in coherence with that, given that long-term LD accumulation in *ob*/*ob*-HFD is characterized by a higher content of PLIN2 and lower of PLIN3 than in *ob*/*ob*-CD.

Regarding lipid metabolism, the liver is one of the most complex organs. Accumulation and mobilization of lipids in hepatocytes depends on several factors affecting incorporation of extracellular lipids, de novo biosynthetic processes and the export through the basolateral or the apical membrane domains. In this work, the expression of genes representative of many processes was analyzed to depict a general overview of lipid metabolism in the liver. Although terminal acyltransferases (DGATs and ACATs) of the biosynthetic pathways of TG and CE stored in LDs and the hydrolases that mobilize them constitute a small part of the net that controls lipid accumulation, their activities modulate the grade of steatosis developed. Among the various mRNAs quantified, the most coherent adaptation attending LD accumulation due to HFD in the *ob*/*ob* strain was that of Pnpla2 and Hsl, which decreased (Fig. 4). That means that liver gene expression might limit the capacity of TG hydrolysis. Nevertheless, the ability of liver extracts to hydrolyze TG is similar in WT and obese mice. Thus, rather than TG hydrolases, it seems that DGAT activity is responsible for the extrahepatic TG accumulation in *ob*/*ob*-CD compared with WT-CD (Fig. 5). The extra TG in hepatic LDs from *ob*/*ob*-HFD is probably the result of the stabilization of the lipid accumulation due to Plin2 overexpression (Fig. 3).

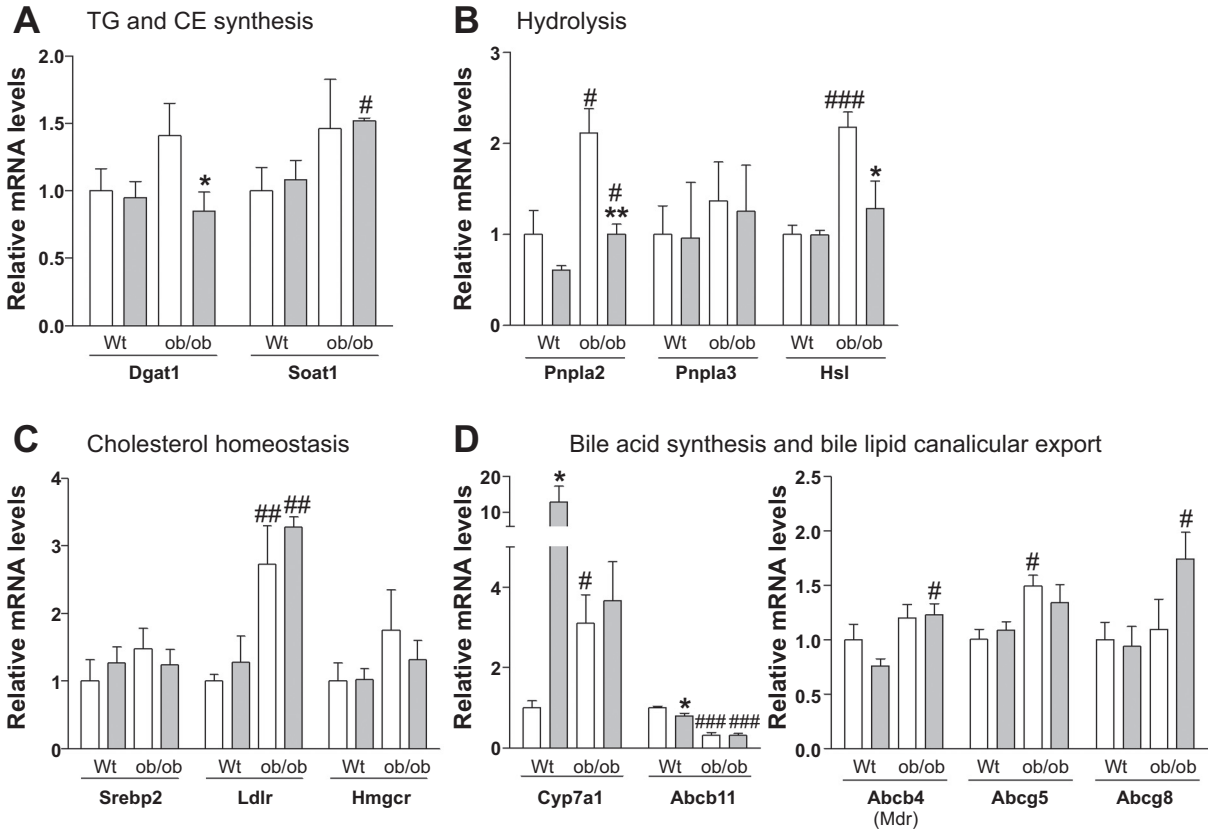

Fig. 4. Hepatic expression of genes related to triacylglycerol (TG) and cholesteryl ester (CE) synthesis (*A*) and hydrolysis (*B*), cholesterol homeostasis (*C*) and bile acid synthesis and bile lipid canalicular export (*D*). Total RNA was extracted and retrotranscribed, and mRNA levels of each gene were measured by RT-PCR. WT, wild type. Data represent means ± SE of $n = 5$. Student's *t*-test: $^{*}P < 0.05$ and $^{**}P < 0.01$ when comparing animals on a high-fat diet (HFD) with those on a control diet (CD); $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ when comparing the two genotypes in the same condition.

Besides TG, liver cholesterol quantities and percentages also changed after HFD, in this case in both strains. Total cholesterol in LDs (nmol/g liver) diminished 40% in WT and *ob*/*ob* mice fed on HFD (Table 2). Notably, these changes paralleled a net increase of serum HDL-cholesterol in WT and a relative increase in *ob*/*ob* mice. These modifications led us to hypothesize that reverse cholesterol transport and biliary secretion of cholesterol or its derivative bile salts might be enhanced. We analyzed the gene expression of relevant proteins implicated in these processes. It has been previously shown that HFD can enhance fecal loss of BAs (21, 31). The enterohepatic loss of BAs attenuates the feedback inhibition of hepatic Cyp7a1 (13), a mechanism that probably causes the marked increase in mRNA levels observed in WT-HFD compared with WT-CD.

One of the master regulators of Cyp7a1 gene expression in the liver is the farnesoid X receptor (FXR), the primary BA nuclear receptor, responsible of the enterohepatic feedback inhibition. FXR activates the expression of canalicular BA and PL transporters Abcb11 (Bsep) and Abcb4 (Mdr3), respectively, and that of Nr0b2 (Shp), responsible for inhibiting the expression of basolateral BA effluxer Slc10a1 (Ntcp) and Cyp7a1 (30). On the other hand, activation of FXR by BAs in the ileum promotes the secretion of the fibroblast growth factor 15 (18). Fibroblast growth factor 15 acts as a hormone that

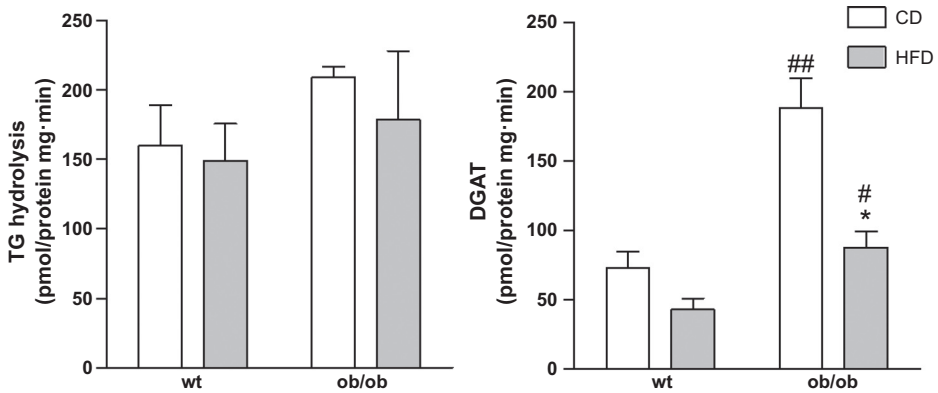

Fig. 5. Triacylglycerol (TG) hydrolysis and diacylglycerol acyltransferase (DGAT) activity in liver homogenates. Activities were measured using radioactive metabolites as described in MATERIALS AND METHODS. Data represent means ± SE of $n = 4$. Student's *t*-test: $^{*}P < 0.05$ when comparing animals on a high-fat diet (HFD) with those on a control diet (CD); $\#P < 0.05$ and $\#\#P < 0.01$ when comparing the two genotypes in the same condition.

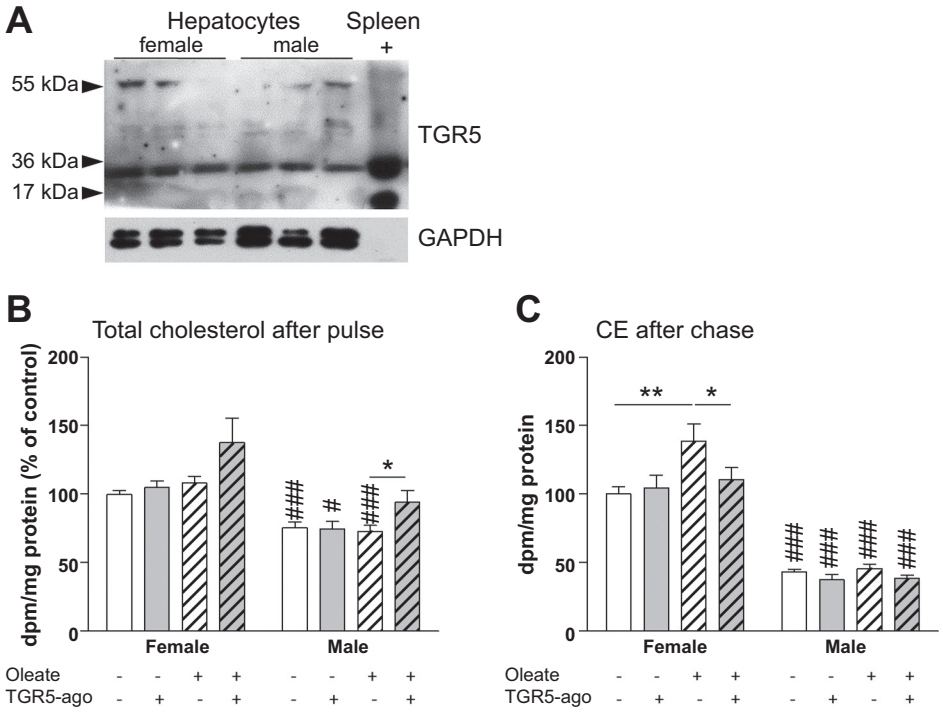

Fig. 6. Effect of Takeda G protein-coupled bile acid receptor (TGR5) agonist on the incorporation of cholesterol and storage as cholesteryl esters in cultured hepatocytes from female and male mice. *A*: TGR5 was immunodetected by Western blotting. A mouse spleen sample was included as positive control and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as loading control. *B*: primary cultures of purified mouse hepatocytes were incubated with a trace of [^{14}C]cholesterol during 4 h for pulse. *C*: afterward cultures were washed with PBS and incubated 2 h (chase). Oleic acid (0.4 mM) complexed with BSA and the TGR5 receptor agonist (ago; 10 μM final concentration) dissolved in ethanol was added to the culture medium, and fatty acid-free BSA and ethanol were added in control cultures. Lipids were extracted, and cholesteryl esters (CE) and unesterified cholesterol were separated by thin-layer chromatography and scraped for liquid scintillation. The radioactivity (dpm/mg protein) is expressed as the % of the control in female hepatocyte cultures. Data represent means ± SE of 12 plates from 3 independent experiments (female) or 8 plates from 2 independent experiments (male). Student's *t*-test: $*P \leq 0.05$ and $**P \leq 0.01$ when analyzing the effects of oleic acid and TGR5-agonist. $\#P \leq 0.05$ and $\#\#\#P \leq 0.001$ when comparing females and males.

controls, among others, BA synthesis by repressing CYP7A1 [reviewed by Cicione et al. (14)]. We here show that Cyp7a1 expression was enhanced by HFD in WT mice, paralleling a moderate repression of Abcb11 that is coherent with the inhibition of hepatic FXR. Nevertheless, HFD did not affect the mRNA levels of Nr0b2 or Slc10a1 (data not shown) in WT mice, suggesting that the enhanced capacity for BA synthesis might be a consequence of the combined effects of ileal and hepatic FXR downregulation. The fact that Cyp7a1 expression was not modified by HFD in *ob*/*ob* mice indicates that regulatory mechanism(s) other than that mediated by liver or ileal FXR activation might be involved in this adaptation.

BAs reabsorbed in the ileum have systemic effects on the energy balance and metabolism through the TGR5 receptor (5, 22, 29, 38). Interestingly, it has been shown that liver and plasma levels of cholesterol are modulated in response to HFD by TGR5 in a sex-specific way (41). TGR5 knockout female mice fed on HFD showed significant fat accumulation compared with HFD-fed WT mice. Besides this response, other features related to bile homeostasis (CYP8B1 and ABCB11 expression) differentiate female and male TGR5 knockout mice (29). Thus, given that modulation of liver cholesterol metabolism is the common response to HFD in WT and *ob*/*ob* mice regardless of liver steatosis development, we analyzed whether cholesterol accumulation capacity in hepatocytes and its responses to steatosis and TGR5 signaling present sexual dimorphism.

Using primary hepatocyte cultures, we show that both female and male hepatocytes express TGR5 (Fig. 6). Overaccumulation of CE induced by a prosteatotic condition (oleic acid in the medium) in female hepatocytes reversed in the presence of TGR5 agonist. These results confirm that a high pool of BAs due to HFD might act on the stores of CE of hepatocytes through basolateral cell membrane signaling. Additional research to explain the mechanisms implicated will clarify the potential and limitations of the TGR5 receptor signaling manipulations in the management of liver steatosis-associated diseases. The fact that accumulation of cholesterol in the esterified form in primary hepatocytes from males was not responsive to steatosis or TGR5 activation suggests that enterohepatic circulation of BAs might be one of the factors that can impact sex dimorphism in NAFLD development.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.A., H.N.-I., Y.R., and O.F. conceived and designed research; L.A., H.N.-I., I.L., Y.R., and O.F. performed experiments; L.A., H.N.-I., I.L., Y.R., and O.F. analyzed data; L.A., H.N.-I., I.L., Y.R., and O.F. interpreted results of experiments; L.A., H.N.-I., I.L., Y.R., and O.F. edited and revised manuscript; L.A., H.N.-I., I.L., Y.R., and O.F. approved final version of manuscript; H.N.-I., Y.R., and O.F. drafted manuscript; O.F. prepared figures.

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