[Frontiers in Bioscience, Elite, 8, 129-142, January 1, 2016]

Diet and sex hormones regulate hepatic *Synaptotagmin 1* mRNA in mice

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1. ABSTRACT

The expression of *Synaptotagmin* 1 (*Syt1*) has been found to be associated with the lipid droplets in liver. Here, we studied the expression of *Syt1* in *Apoe*-deficient mice receiving cholesterol, Western diet, squalene, and oleanolic acid. We also studied the influence of sex and impact of surgical castration. Dietary cholesterol increased hepatic *Syt1* expression, an effect that was enhanced when cholesterol was combined with saturated fat present in a Western diet. This potentiation was modified by the administration of 10 mg/kg oleanolic acid or 1 g/kg squalene. Females fed chow or Western diet showed higher levels of hepatic *Syt1* expression as compared to male mice on the same diet. Surgical castration of males did not modify the *Syt1* expression; however, ovariectomy led to decreased levels. The data show that hepatic *Syt1* expression is influenced by diet and hormonal milieu.

2. INTRODUCTION

Synaptotagmins (SYTs) belong to a family of membrane trafficking proteins characterized by

Table 1. Summary of experimental conditions using *Apoe*-deficient mice

Experiment	Genetic background	Diet	Sex	Influence
1	C57BL/6J X OLA 129	Commercial chow w/wo cholesterol	Males	Cholesterol
2	C57BL/6J	Purified chow and purified Western	Males	HFD
3	C57BL/6J	Purified Western w/wo oleanolic acid	Males	Oleanolic acid
4	C57BL/6J	Purified Western w/wo squalene	Males	Squalene
5	C57BL/6J	Purified chow	Both sexes	Sex in chow diet
6	C57BL/6J	Purified Western	Both sexes	Sex in Western diet
7	C57BL/6J	Purified Western	Castrated and non-castrated males	Castration in males
8	C57BL/6J	Purified Western	Castrated and non-castrated females	Castration in females
HFD, high fat diet; w/wo, with or without				

an N-terminal, a variable linker and two C-terminal C2-domains, C2A and C2B, designed to bind Ca^{2+} and phospholipids (1). SYTs are reported to participate in the docking and fusion of membrane vesicles in different cell types such as neurons, macrophages (2), pituitary cells (3), osteoblasts and osteoclasts (4), mucin-secreting airway and intestinal goblet cells (5) and muscle cells (6).

Synaptotagmin 1 (SYT1) is a 65-kDa integral membrane protein of synaptic vesicles and secretory granules in neuronal and neurosecretory tissues (7,8), where it serves as a Ca^{2+} sensor in synaptic exocytosis (9). Likewise, SYT1 has been proposed to have a role in endocytosis (10,11). In these situations, its Ca^{2+} binding domain plays an important role in the mechanisms of action (12). In line with the versatility of the SYT family described above, SYT1 is also expressed in other tissues. For instance, it binds to intestinal Na^+/H^+exchanger 3 and mediates cAMP- and Ca^{2+}-induced endocytosis in a process requiring phosphorylation of exchanger 3 in the Caco2BBE cell line (13,14). In addition, its intestinal mRNA expression has been reported to be modified by a mixture of conjugated linoleic acid isomers in rats (15). SYT1 has been localized in pancreatic acinar cells (16) and on the insulin secretory granules of pancreatic beta-cell lines, where it plays an essential role in insulin vesicle exocytosis through its Ca^{2+}-dependent phospholipid-binding activity (17,18). In fact, pancreatic and duodenal homeobox 1 stimulates insulin secretion in response to high glucose through the positive induction of *Syt1* expression (19). It also appears to participate in the regulation of podocyte homeostasis in kidneys (20). Not surprisingly, a significant association has been reported between SYT1 polymorphisms and levels of creatinine, the most important biomarker for noninvasive assessment of kidney function (21). Using microarray analysis, with confirmation by quantitative real time PCR (RT-qPCR), our group found differential hepatic *Syt1* gene expression in *Apoe*-deficient mice fed different conjugated linoleic acid isomers added to Western diets. Furthermore, significant associations have been observed between *Syt1* expression levels and hepatic steatosis (22). Recently, we characterized the mouse hepatic *Syt1* transcript as being 1807 bp in length and encoding a 421-amino acid protein with a predicted molecular mass of 47.4. kDa. However, immunoblotting of hepatic protein showed two isoforms with molecular masses that were higher than the theoretical prediction based on amino acid sequence, but lower than those found in brain. Subcellular distribution corresponded to plasma membrane, lysosomes and microsomes. Quantitative tissue distribution of *Syt1* mRNA showed that the highest values corresponded to the brain, followed by liver, spleen, abdominal fat, intestine and skeletal muscle. These findings were considered to indicate an extensive role of SYT1 in different tissues and that it undergoes a posttranslational modification specific for liver function (23). On the basis of these facts, we hypothesized that *Syt1* hepatic regulation might be complex. The present work was undertaken to characterize the influence of different dietary conditions and sex on *Syt1* gene expression in mouse liver.

3. MATERIAL AND METHODS

3.1. Mice

Apoe-deficient mice on the C57BL/6J genetic background were obtained from Charles River (Charles River Laboratories, Barcelona, Spain). *Apoe*-deficient mice on the C57BL/6JxOla129 genetic background were generously provided by Dr. Nobuyo Maeda from the University of North Carolina at Chapel Hill. Blood samples were taken from the facial veins of 2-month-old mice (after four-hour fasting) to determine plasma cholesterol and to establish groups with similar initial values. All animals were housed in sterile filter-top cages in rooms maintained under a 12-h light/12-h dark cycle in the *Servicio de Biomedicina y Biomateriales*, University of Zaragoza (Zaragoza, Spain). All had *ad libitum* access to food and water, and study protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza.

Table 2. Composition of purified diets used in the different studies, based on AIN-93-recommended diets for laboratory rodents (35)

Ingredients	Chow (g/kg)	Western (g/kg)
Corn starch[1]	465.7.	371.9.
Casein[2]	140.0.	111.8.
Maltodextrin[1]	155.0.	123.8.
Saccharose[3]	100.0.	79.9.
Soybean oil[4]	40.0.	31.9.
Cellulose[5]	50.0.	39.9.
Mineral mix[6]	35.0.	27.9.
Vitamin mix[7]	10.0.	8.0.
Choline bitartrate[8]	2.5.	2.0.
L-Cystine[8]	1.8.	1.4.
Cholesterol[8]	-	1.5.
Palm oil[9]	-	200

[1]Cargill, Barcelona, Spain, [2]Lactalis Ingredients, Bourgbarré, France, [3]Azucarera Ibérica S.L., Madrid, Spain, [4]Aceites Muñoz, Toledo, Spain, [5]Vitacel R200, Rettenmaier Ibérica S.L, Barcelona, Spain, [6]AIN-93M Mineral Mix (MP Biomedicals, Illkirch, France). The salt mixture contains the following amounts (g/kg): calcium carbonate, 357; monopotassium phosphate, 250; potassium citrate monohydrate, 28; sodium chloride, 74; potassium sulphate, 46.6.; magnesium oxide, 24; ferric citrate, 6.0.6; zinc carbonate, 1.6.5; manganese carbonate, 0.6.3; copper carbonate, 0.3.; potassium iodate, 0.0.1; sodium selenate anhydrous, 0.0.1025; ammonium molybdate. $4H_2O$, 0.0.0795; sodium metasilicate. $9H_2O$, 1.4.5; chromium potassium sulfate. $12H_2O$, 0.2.75; lithium chloride, 0.0.174; boric acid, 0.0.815; sodium fluoride, 0.0.635; nickel carbonate, 0.0.318; ammonium vanadate, 0.0.066 and powdered sugar, 209.8.06., [7]AIN-93-VX Vitamin Mix (MP Biomedicals, Illkirch, France). Vitamin mixture contains the following amounts (mg/kg): nicotinic acid, 3; D-calcium pantothenate, 1.6.; pyridoxine HCl, 0.7.; thiamine HCl, 0.6.; riboflavin, 0.6.; folic acid, 0.2.; D-biotin, 0.0.2; vitamin B_{12} (0.1.% triturated in mannitol), 2.5.; α-tocopherol powder (250 U/g), 30; vitamin A palmitate (250,000 U/g), 1.6.; vitamin D_3 (400,000 U/g), 0.2.5; phylloquinone, 0.0.75 and powdered sucrose, 959.6.55., [8]Sigma-Aldrich Química, Madrid, Spain and [9]Gustav Heess, Barcelona, Spain. AIN: American Institute of Nutrition.

3.2. Experimental designs

A summary of all experiments undertaken is shown in Table 1.

3.2.1. Effect of dietary cholesterol on *Syt1* expression in male *Apoe*-deficient C57BL/6JxOla129 mice fed a commercial chow diet

Mice were assigned randomly to one of the following experimental groups: a) a control group (n=7) fed a standard chow diet (B & K Universal Ltd, Humberside, UK) and b) a cholesterol group (n=7) fed a diet supplemented with 0.1.% (w/w) cholesterol. The nutritional intervention lasted 10 weeks (24).

3.2.2. Effect of a Western diet on *Syt1* expression in male *Apoe*-deficient C57BL/6J mice

Two study groups were established: a) one (n=13) receiving a purified chow diet and b) the other (n=9) receiving a purified Western diet containing 0.1.5% cholesterol and 20% palm oil (Gustav Heess, S.L., Barcelona, Spain) (Table 2) (25).

3.2.3. Effect of dietary oleanolic acid on *Syt1* expression in male *Apoe*-deficient C57BL/6J mice fed a Western diet

Two study groups were established: a) one (n=8) receiving a purified Western diet and b) the other (n=9) receiving the same diet but supplemented with 0.0.1% oleanolic acid (Extrasynthese, Genay, France), equivalent to a dose of 10 mg/kg mouse, assuming a daily intake of 3 g per mouse (26).

3.2.4. Effect of dietary squalene on *Syt1* expression in male *Apoe*-deficient C57BL/6J mice fed a Western diet

Two study groups were established: a) a control group (n=9) receiving a purified Western diet and b) a treatment group (n=10) receiving the same diet but containing 1% squalene (Sigma, Madrid, Spain), equivalent to a dose of 1 g/kg mouse, assuming a daily intake of 3 g per mouse (27).

3.2.5. Effect of sex on *Syt1* expression in *Apoe*-deficient C57BL/6J mice fed a chow diet

Two study groups were established: a) male mice (n=13) and b) female mice (n=13), both of which received a purified chow diet (Table 2) for 11 weeks.

3.2.6. Effect of sex on *Syt1* expression in *Apoe*-deficient C57BL/6J mice fed a Western diet

Two study groups were established: a) male mice (n=9) and b) female mice (n=10), both of which received a purified Western diet for 11 weeks.

3.2.7. Effect of surgical castration on *Syt1* expression in male *Apoe*-deficient C57BL/6J mice fed a Western diet

Two study groups of males were established: a) a control group (n=9) that received a purified Western diet and b) a group of orchiectomized mice (n=9) that received the same diet, both for 11 weeks.

3.2.8. Effect of surgical castration on *Syt1* expression in female *Apoe*-deficient C57BL/6J mice fed a Western diet

Two study groups of females were established: a) a control group (n=9) that received a purified Western diet and b) a group of ovariectomized mice (n=9) that received the same diet, both for 11 weeks.

3.3. RNA isolation

At sacrifice, the livers were immediately removed and frozen in liquid nitrogen. RNA was isolated from each liver using Tri-reagent (AMBION, TX, USA). DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from AMBION. RNA was quantified by absorbance at $A_{260/280}$. The integrity of the 28 S and 18 S ribosomal RNAs was verified by agarose gel electrophoresis.

3.4. Quantification of mRNA

The potential changes in *Syt1* mRNA expression were determined by RT-qPCR analysis of individual samples using equal amounts of DNA-free RNA from each sample taken from each animal. First-strand cDNA synthesis was performed using the First Strand synthesis kit (Thermo Scientific, Madrid, Spain). RT-qPCR reactions were carried out using the Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primers were designed using Primer Express® (Applied Biosystems) and checked by BLAST analysis (NCBI) to verify gene specificity, as well as to ensure amplification of cDNA but not genomic DNA (28). RT-qPCR reactions were performed on a Step One Real Time PCR System (Applied Biosystems) following the standard procedure. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and normalized to the reference *Cyclophilin B* mRNA expression.

3.5. Hepatic lipid analysis

Tissues (10 mg) were homogenized in 1 mL of PBS. An aliquot was saved to determine protein concentration by the BioRad dye binding assay (BioRad, Madrid, Spain). The total lipids were extracted from one volume of the tissue homogenates according to Folch's method (29) using a chloroform-methanol (2:1, v/v) solvent system, evaporated under N_2 stream and dissolved in 100 µL of isopropanol. Cholesterol and triglycerides were measured by colorimetric assay with Infinity kits (Thermo Scientific).

3.6. Liver histology analysis

Aliquots of liver, stored in neutral formaldehyde, were embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin and observed under a Nikon microscope. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0. and expressed as percentage of total liver section (27).

3.7. Statistical analysis

Data are expressed as means ± standard deviation of the values. The data were analyzed using the Statistical Package for Social Sciences (SPSS) program (SPSS, Chicago, IL, USA) or Instat 3.0.2 software for Windows (GraphPad, S. Diego, CA, USA). For parametric distributions, a Student's t test was employed. When the variables did not show normal distribution (according to the Shapiro-Wilk test), or failed to show homology of variance, data were analyzed with the Mann-Whitney U test. Correlations between variables were sought using the Spearman correlation test. The statistical significance was set at $p < 0.0.5$.

4. RESULTS

4.1. Nutritional regulation of hepatic *Syt1* expression in *Apoe*-deficient mice

For the initial characterization of the dietary regulation of this gene expression in mice, historical hepatic RNA samples from males that had consumed a chow diet with and without cholesterol (24) were analyzed for *Syt1* expression. Cholesterol supplementation increased the liver surface area occupied by lipid droplets, as well as hepatic cholesterol and triglyceride contents (Figure 1A, B and C). As shown in Figure 1D, the presence of dietary cholesterol induced a modest increase in the hepatic expression of this gene. Hepatic cholesterol content was associated with hepatic *Syt1* expression (Figure 1E).

A second study focused on the combination of cholesterol and a high-fat diet (HFD) containing palm oil as source of long-chain saturated fatty acids, provided as a purified Western diet to male C57BL/6J mice (Figure 2). The consumption of a Western diet significantly increased the liver surface area occupied by lipid droplets (Figure 2A, B and C), as well as hepatic cholesterol and triglyceride contents. In this experimental setting, there was a significant elevation of hepatic *Syt1* expression (Figure 2D).

Two experiments were set up to investigate whether the increased *Syt1* expression induced by HFD was modulated by other dietary components. In the first (Figure 3), male mice receiving an oleanolic acid-supplemented Western diet were compared to those receiving the unsupplemented HFD. Oleanolic acid administration induced a significant reduction of hepatic *Syt1* expression (Figure 3D), with no significant changes in hepatic cholesterol or triglyceride contents. However, the liver surface area occupied by lipid droplets was found to be significantly increased (Figure 3C) and negatively associated with hepatic *Syt1* expression (Figure 3E). In the second experiment (Figure 4), the effect of a squalene-enriched Western diet was explored, again in males. The addition of this compound increased the hepatic *Syt1* expression while it reduced hepatic cholesterol and triglyceride contents. No significant changes were noted in relation to the percentage of liver surface occupied by lipid droplets and there were no significant associations between hepatic *Syt1* expression and these liver parameters.

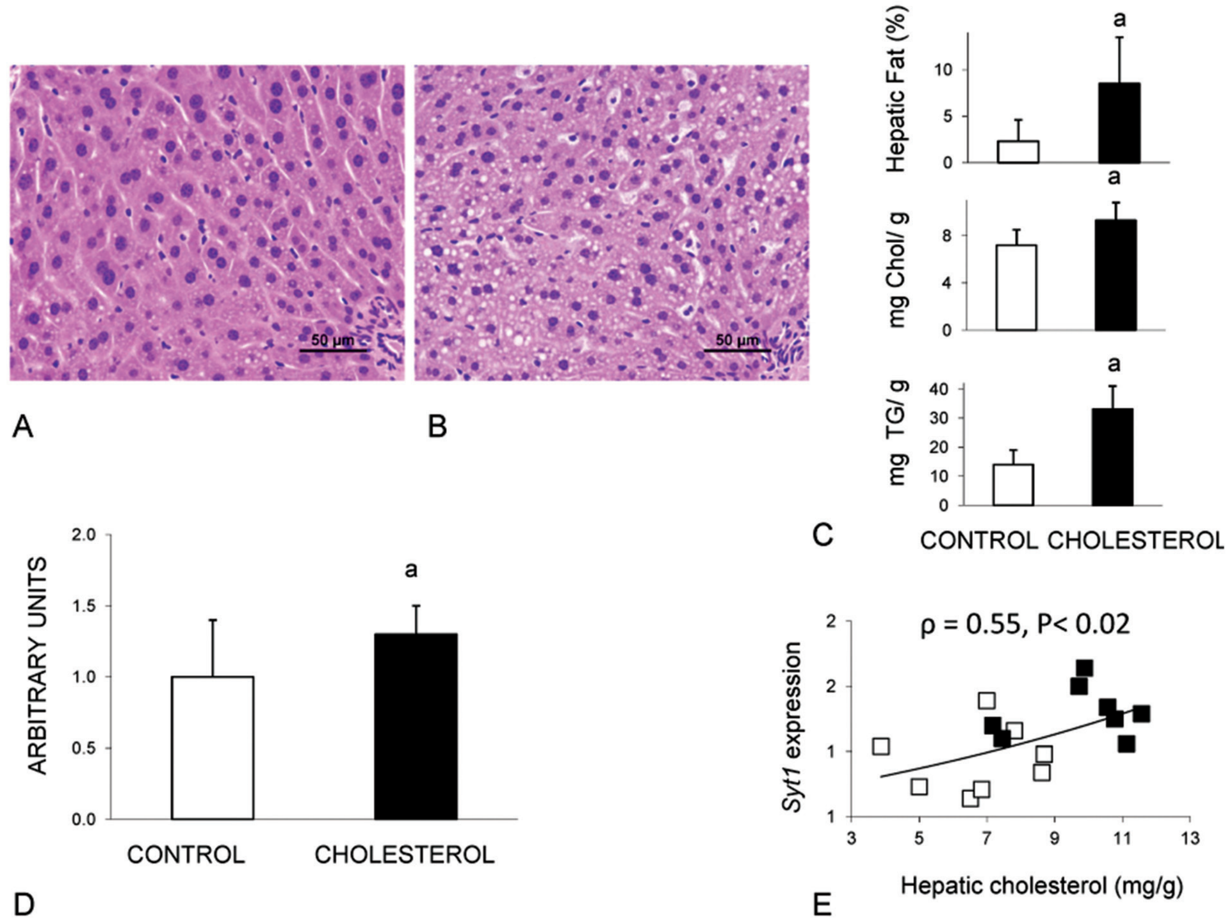

Figure 1. Effect of dietary cholesterol on hepatic steatosis and *Syt1* expression in *Apoe*-deficient mice. Representative liver micrographs (x400 magnification) from *Apoe*-deficient mice consuming commercial chow (control) (A) and cholesterol-enriched (B) diets. Morphometric evaluation of surface of liver section occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test. [a]P < 0.0.5 vs control. Association between hepatic cholesterol content and *Syt1* expression (E). Open squares correspond to controls and black squares to cholesterol-fed mice. Spearman's correlation is shown.

Overall, these experiments underline the exquisite nutritional regulatory role of hepatic *Syt1* expression at the transcriptional level in *Apoe*-deficient mice, where cholesterol and saturated fat increase its levels, and the latter can be modulated by minor dietary components such as oleanolic acid or squalene.

4.2. Female hormones are involved in hepatic *Syt1* expression in *Apoe*-deficient mice

To explore the influence of sex, two different experiments were carried out in *Apoe*-deficient mice. In one, hepatic *Syt1* expression was analyzed in males and females being fed a purified chow diet. As shown in Figure 5, females had higher levels of gene expression and significantly higher hepatic cholesterol content than males, whereas no differences in triglyceride content were observed. There was a significant positive association between hepatic *Syt1* values and hepatic cholesterol concentrations. In view of the effects of HFD described above, the second experiment was designed to assess the influence of sex in mice fed a purified Western diet. As shown in Figure 6, this experimental approach revealed significant differences between sexes in terms of hepatic *Syt1* expression. However, no significant difference was observed in the percentage of liver surface occupied by lipid droplets, and the levels of hepatic cholesterol and triglycerides were significantly lower in females than in males. A significant inverse association was also found between hepatic *Syt1* values and hepatic triglyceride levels. These data indicate that, irrespective of diet, sex plays an important role in hepatic *Syt1* expression.

To analyze whether sex differences were due to hormonal changes, mice of both sexes underwent surgical castration and were fed a purified Western

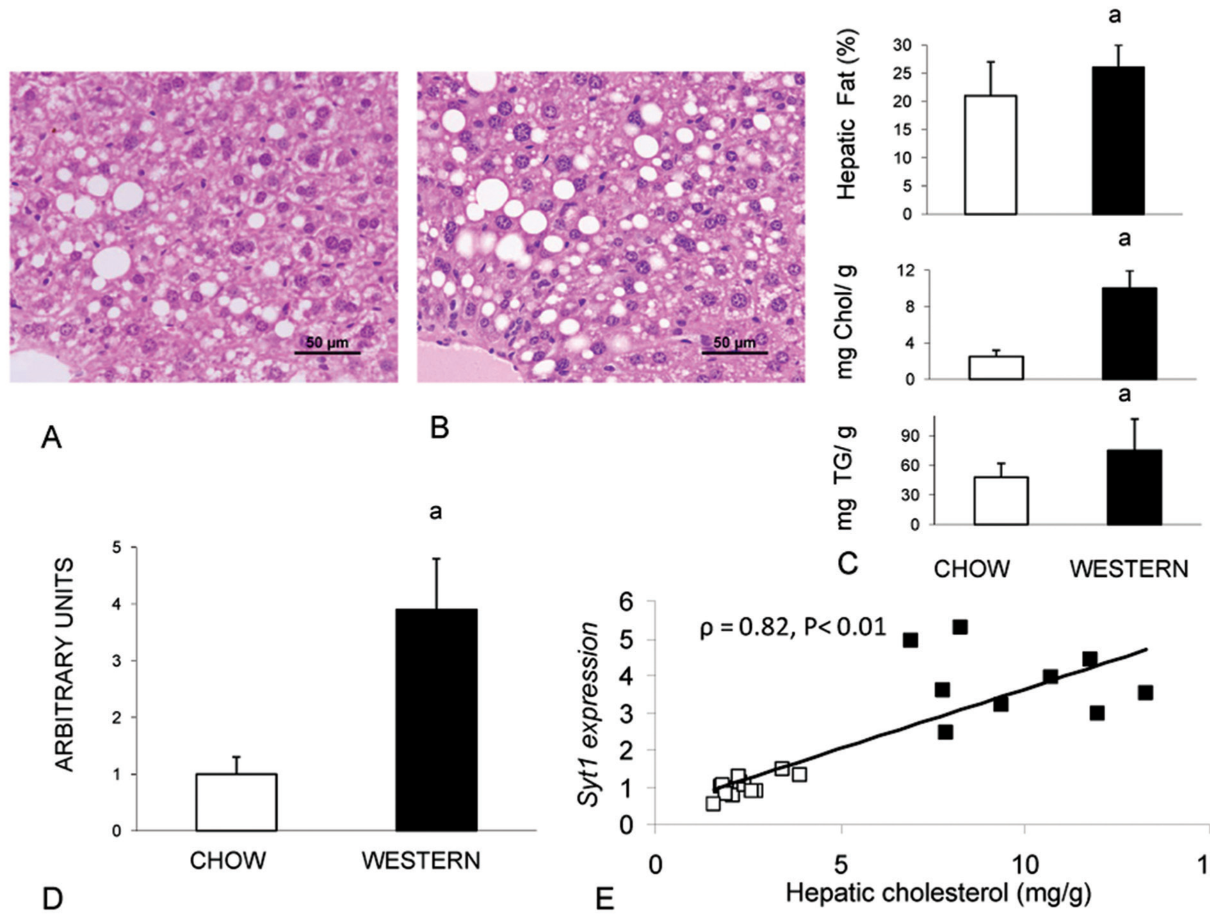

Figure 2. Effect of Western diet on hepatic steatosis and *Syt1* expression in *Apoe*-deficient mice. Representative liver micrographs (x400 magnification) from *Apoe*-deficient mice consuming purified chow (A) and Western (B) diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test. ^{a}P < 0.0.1 vs control (chow). Association between hepatic cholesterol content and *Syt1* expression (E). Open squares correspond to controls and black squares to Western diet-fed mice. Spearman's correlation is shown.

diet. As shown in Figure 7, no significant change in *Syt1* expression was observed in orchiectomized males; nor was there any significant change in hepatic cholesterol or in hepatic triglycerides. However, there was a significant increase in the liver surface occupied by lipid droplets in orchiectomized males. No significant association was found between *Syt1* expression and lipid parameters (data not shown). In contrast, ovariectomized females (Figure 8) showed a significant decrease in *Syt1* expression compared to control females. Ovariectomy resulted in significant increases in hepatic cholesterol and triglycerides, and in the surface area occupied by lipid droplets. A significant inverse association was also found between hepatic *Syt1* values and those of hepatic triglycerides (Figure 8E). These results indicate that the release of ovarian hormones is necessary to induce the increased hepatic *Syt1* expression observed in females.

5. DISCUSSION

This experimental approach was designed to evaluate putative hepatic *Syt1* transcriptional changes induced by dietary components and sex. We found that, in *Apoe*-deficient mice, dietary cholesterol produced an increase that was potentiated by its combination with saturated fat. The latter effect was seen to be modulated by minor dietary components such as oleanolic acid or squalene, administered at pharmacological doses. Likewise, our study revealed a sex-related regulatory influence irrespective of the diet provided, with female sex acting as a positive regulator. In an attempt to establish the role of sex hormones, both males and females were subjected to castration. This experiment revealed that ovarian hormones are crucial for the increased hepatic *Syt1* expression observed in females.

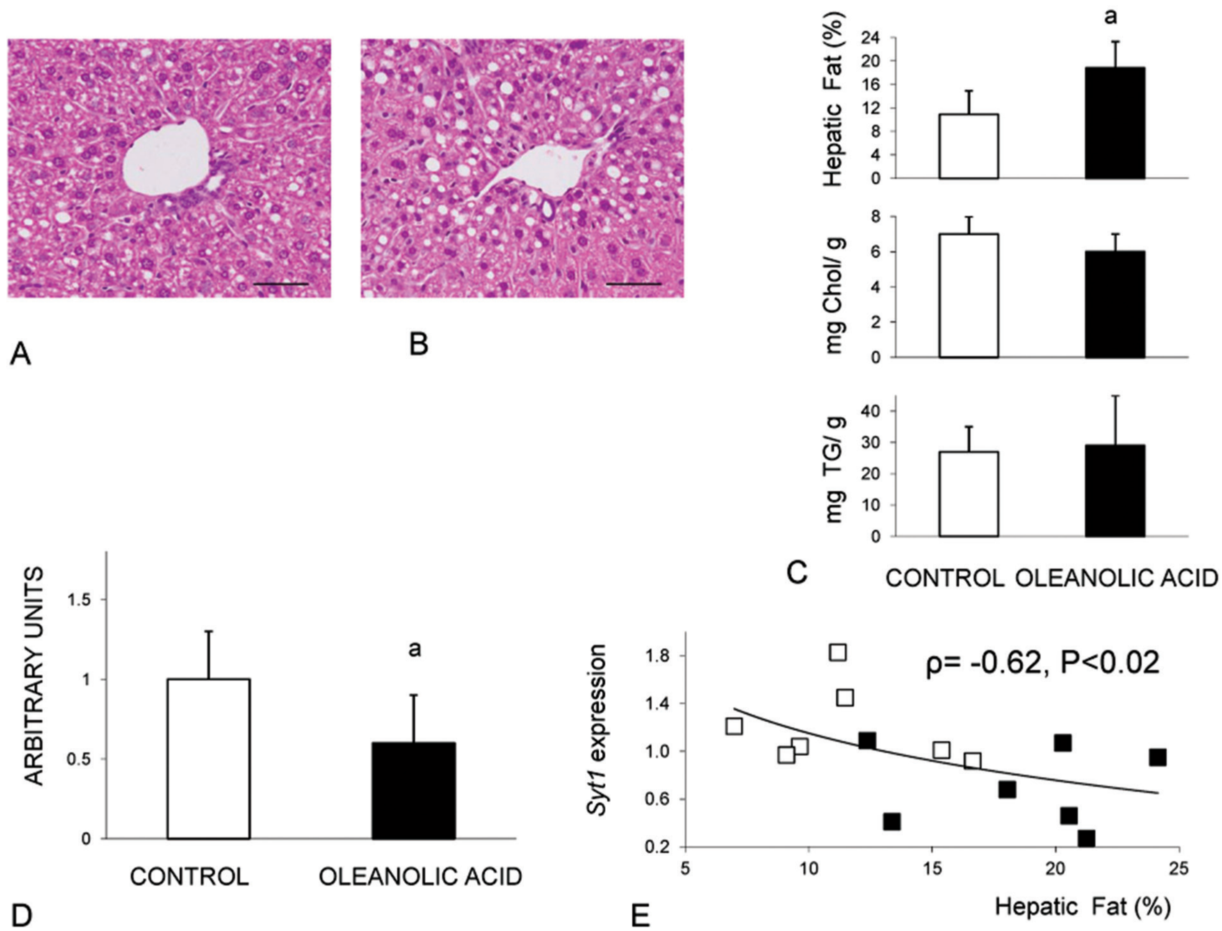

Figure 3. Effect of dietary oleanolic acid on hepatic steatosis and *Syt1* expression in *Apoe*-deficient mice fed a purified Western diet. Representative liver micrographs (x400 magnification) from *Apoe*-deficient mice consuming Western (control) (A) and oleanolic acid-enriched (B) diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test. [a]P < 0.0.5 vs control. Relationship between percentage of hepatic surface occupied by fat and *Syt1* gene expression (E). Open squares correspond to controls and black squares to oleanolic acid-fed mice. Correlations were calculated according to Spearman's test.

In a previous study using extreme dietary conditions with conjugated linoleic acid isomers and *Apoe*-deficient C57BL/6JxOla129 mice on a mixed genetic background, we identified hepatic *Syt1* as an important gene whose expression is susceptible to modulation in response to diet or dramatic changes in hepatic fat content (22). Recently, our group characterized mouse hepatic *Syt1* mRNA in detail, finding an important difference in length compared to the brain transcript (23). The present work explored the influence of several dietary components, especially cholesterol and saturated fat, on its hepatic expression. This would be in agreement with the results found in mice receiving a n-3 polyunsaturated fatty acid-depleted diet and in those deficient in stearoyl-CoA desaturase 1 (Table 3), in which saturated fatty acids tend to accumulate in liver. However, the discrepancy between the findings in mice overexpressing lipin 1-beta and those lacking medium-chain acyl-coenzyme A dehydrogenase (Table 3) may suggest that the nature of fatty acids is important. In this regard, the differences in the results observed in mice receiving different conjugated linoleic acid isomers (22) reinforce this notion. Overall, nutritional components are critical modulators of hepatic *Syt1* expression.

In this study, the intake of oleanolic acid, a pentacyclic triterpene, decreased *Syt1* expression, as did dietary supplementation with *Boswellia serrata*, an extract rich in particular derivatives of boswellic acid, also a pentacyclic triterpene-based compound (30). However, squalene, a linear triterpene, showed the opposite effect. These data indicate that the chemical structure of triterpenes may be crucial in the final outcome of this gene expression. Due to the anti-inflammatory properties of oleanolic and boswellic acids, its decreasing action would be in agreement with absence of c-Jun N-terminal

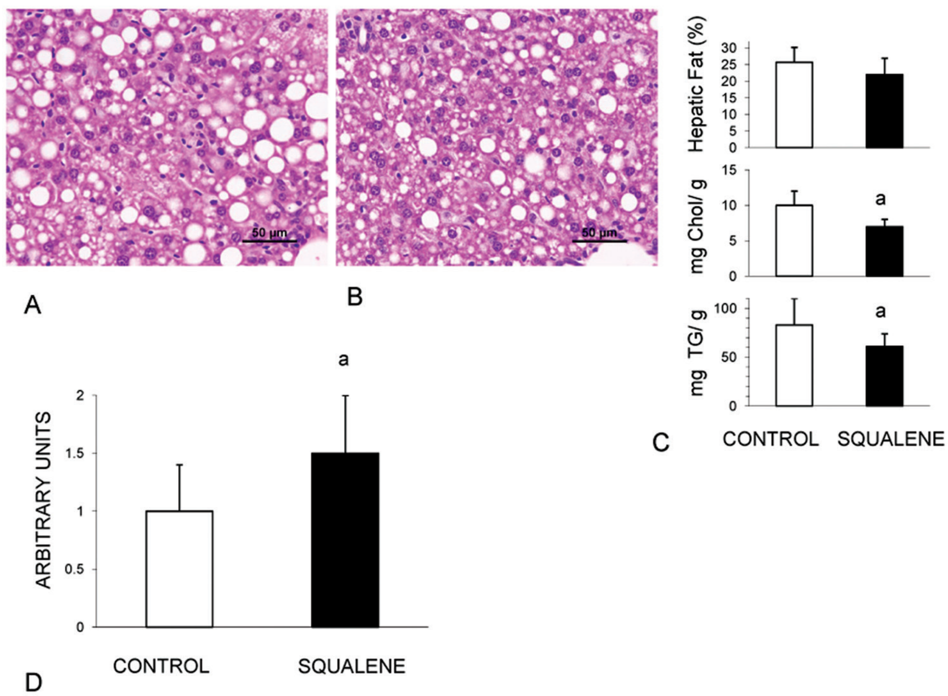

Figure 4. Effect of dietary squalene on hepatic steatosis and *Syt1* expression in *Apoe*-deficient mice fed a purified Western diet. Representative liver micrographs (x400 magnification) from *Apoe*-deficient mice consuming Western (control) (A) and squalene-enriched (B) diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test. [a]P < 0.0.5 vs control.

Table 3. Changes in hepatic *Syt1* expression according to the Genome Expressed Omnibus data bank

Experimental condition	Type of change	Accession number
Steroid receptor coactivator-2-deficient female mice	Decreased	GDS4785
SIRT1-deficient mice	Decreased	GDS3666
c-Jun N-terminal kinase 1-deficient livers of diet-induced obese mice	Decreased	GDS3001
Dietary supplement of Boswellia serrata	Decreased	GDS1227
NADPH-cytochrome P450 reductase-deficient mice	Decreased	GSE2362
Medium-chain acyl-coenzyme A dehydrogenase-deficient mice	Decreased	GDS4546
Concanavalin A-induced fulminant hepatitis model	Decreased	GDS3752
SIRT4-deficient mice	Increased	GDS4823
Senescent activated hepatic stellate cells	Increased	GDS3492
Mice overexpressing lipin 1beta	Increased	GDS1349
n-3 polyunsaturated fatty acid-depleted diet	Increased	GDS4796
Stearoyl-CoA desaturase 1-deficient mice	Increased	GDS1517
Kupffer cell depletion in high-fat-diet-induced steatosis	Increased	GDS4166
Hepatitis B virus (HBV)-associated acute liver failure in humans	Increased	GDS4387
Glycosylphosphatidylinositol-specific phospholipase D overexpression effect in hepatoma cells	No change	GDS2049

kinase 1. This kinase is activated by various stimuli, including UV light, interleukin-1, tumor necrosis factor-α (TNF-α), and CD28 co-stimulation (31), some of which also play a role in hepatitis (32). For this reason, an

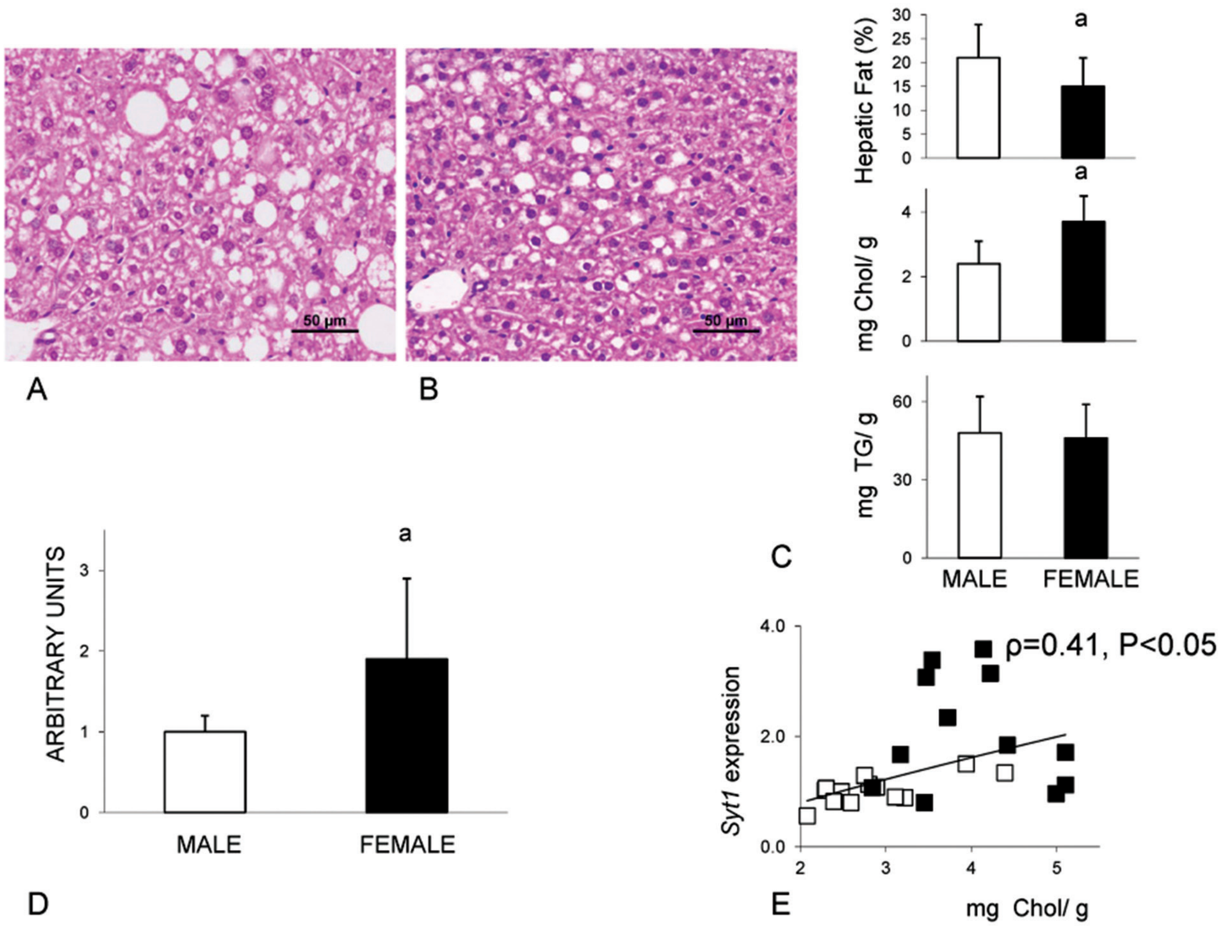

Figure 5. Effect of sex on hepatic steatosis and *Syt1* expression in *Apoe*-deficient mice fed a purified chow diet. Representative liver micrographs (x400 magnification) from male (A) and female (B) *Apoe*-deficient mice consuming chow diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test.[a]P < 0.0.5 vs male. Relationship between percentage of hepatic cholesterol content and *Syt1* gene expression (E). Open squares correspond to males and black squares to females. Correlations were calculated according to Spearman's test.

increased expression was found in hepatitis B virus and in Kupffer cell depletion (Table 3), whereas, fulminant hepatitis induced a decrease. These results also suggest that the regulation of this gene may be influenced by several inflammatory compounds.

One finding of this study that proved striking was the increased hepatic *Syt1* expression in female mice and its suppression following ovariectomy. Interestingly, female mice lacking steroid receptor coactivator-2 also showed decreased hepatic expression of this gene (Table 3). Steroid receptor coactivator-2 promotes the transcriptional activation of estrogen receptor in some tissues (33). These results indicate the influence of estradiol or other female sex hormones on a positive regulation of this gene.

In our initial description of hepatic *Syt1* gene expression (22), we found significant associations with hepatic steatosis in *Apoe*-deficient mice fed different conjugated linoleic acid isomers, in *Cbs*-deficient mice and in olive oil-fed *Apoe*-deficient mice. In the present study, this association was observed only when oleanolic acid was administered (Figure 3). Interestingly, in three experiments, *Syt1* expression was associated with hepatic triglycerides (Figures 6 and 8) or cholesterol (Figures 1, 2 and 5). There are two major differences between the earlier study and the present report: genetic background and diet composition. The present study was conducted using C57BL/6J mice, whereas the previous one involved Ola129xC57BL/6J hybrids. These strains have been shown to differ considerably in terms of hepatic fat content (34), a circumstance that may have influenced the outcome. The second important difference is the use of an American Institute of Nutrition (AIN)-93 purified diet (35) in the present study, rather than the commercial chow employed in the earlier one. We were obliged to make this change because of the high variability among

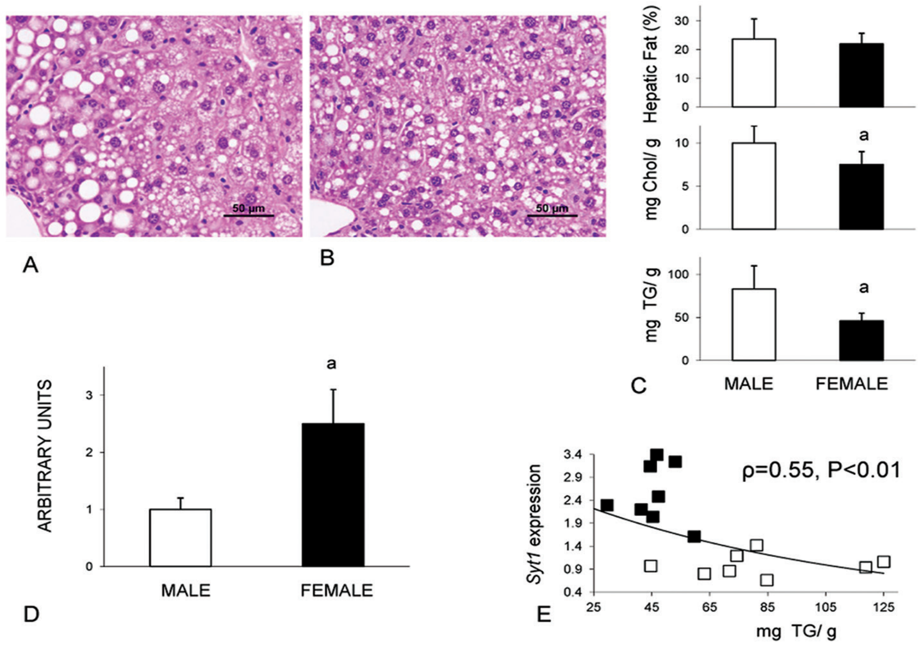

Figure 6. Effect of sex on hepatic steatosis and *Syt1* expression in *Apoe*-deficient mice fed a purified Western diet. Representative liver micrographs (x400 magnification) from male (A) and female (B) *Apoe*-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression was determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test. $^{a}P < 0.0.5$ vs male. Relationship between percentage of hepatic triglyceride content and *Syt1* gene expression (E). Open squares correspond to males and black squares to females. Correlations were calculated according to Spearman's test.

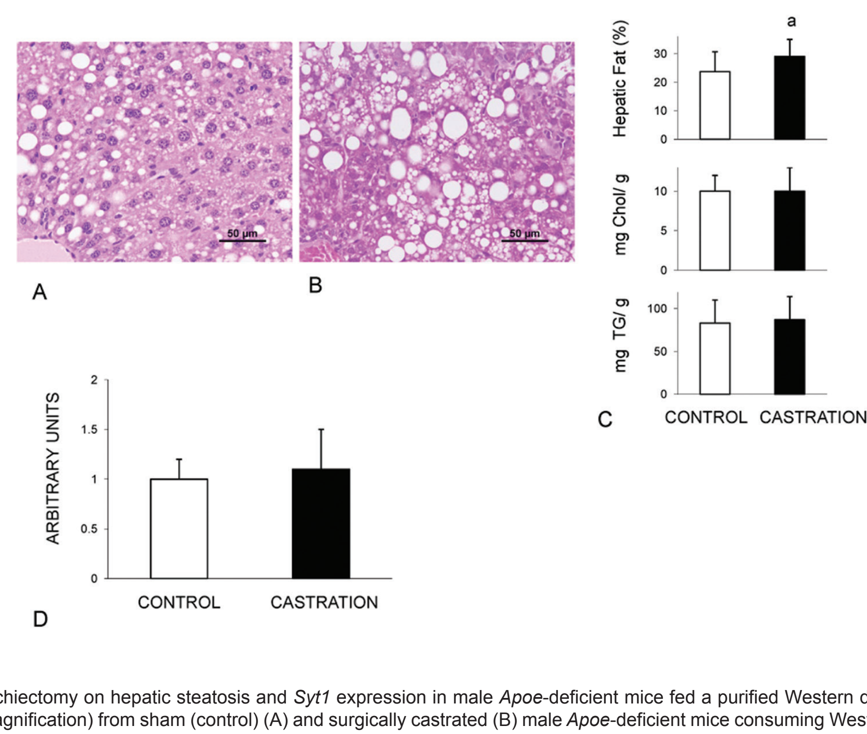

Figure 7. Effect of orchiectomy on hepatic steatosis and *Syt1* expression in male *Apoe*-deficient mice fed a purified Western diet. Representative liver micrographs (x400 magnification) from sham (control) (A) and surgically castrated (B) male *Apoe*-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test.$^{a}P < 0.0.5$ vs control.

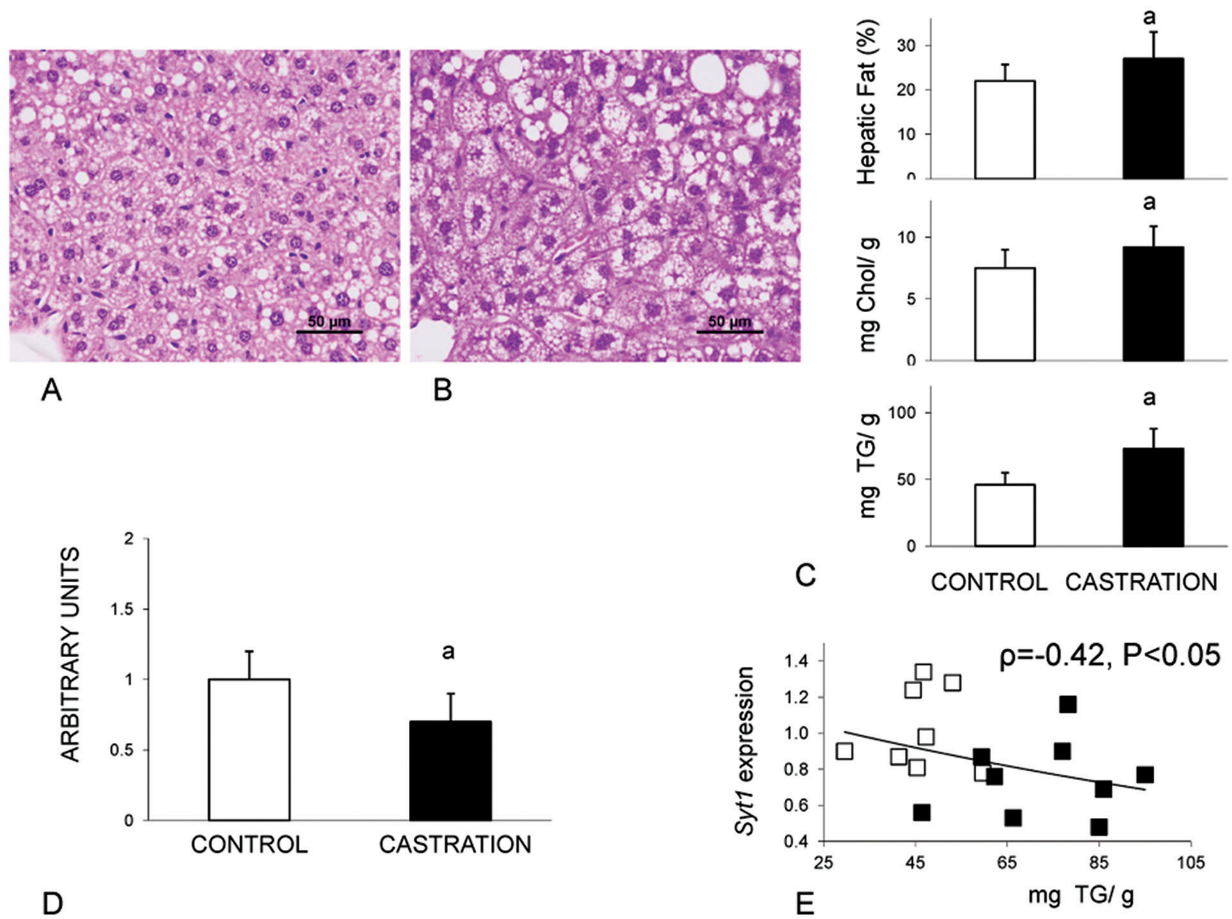

Figure 8. Effect of ovariectomy on hepatic steatosis and *Syt1* expression in female *Apoe*-deficient mice fed a purified Western diet. Representative liver micrographs (x400 magnification) from sham (control) (A) and surgically castrated (B) female *Apoe*-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat, cholesterol (Chol) and triglyceride (TG) contents (C). Analysis of hepatic *Syt1* expression determined by RT-qPCR normalized to *Cyclophilin B* (D). Data are expressed as mean ± SD for each group. Statistical analyses were done according to the Mann-Whitney U test.[a]P < 0.0.5 vs control. Relationship between percentage of hepatic triglyceride content and *Syt1* gene expression (E). Open squares correspond to controls and black squares to surgically castrated female mice. Correlations were calculated according to Spearman's test

control mice and their atherosclerotic lesions observed in our lab over the years when using commercial chows and the impossibility of obtaining the same batch from one year to another. Undoubtedly, dietary components are an important source of variation (36) and, in this respect, this study, performed under well-defined conditions of mouse strain and purified diets, maximizes the control of this variability.

In conclusion, the present report identifies three major factors in hepatic *Syt1* regulation defined by dietary components, inflammation and sex. Regarding diet, cholesterol and the nature of fatty acids are involved. Anti-inflammatory modulators such as oleanolic acids seem to play a role as well. Finally, the detection of higher levels of hepatic gene expression found in females, but not observed in ovariectomized mice, suggests that female sex hormones are involved in hepatic *Syt1* expression.

6. ACKNOWLEDGMENTS

We thank Martha Messman for her assistance in manuscript editing. We also thank Silvia Garcés and Mª Pilar Lierta for their help in caring for the animals. This research was supported by grants from CICYT-FEDER (SAF2010-14958 and SAF2013-41651-R) and Redes FSE-DGA (B-69). CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN) is an initiative of the ISCIII. S.S.K. and M.A.N. were recipients of a *Fundación Cuenca-Villoro* fellowship (BE 203/2009) and a Miguel Servet contract, respectively. No competing financial interests exist.

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Abbreviations: PCR, Polymerase Chain Reaction, RT, reverse transcriptase; qRT, Quantitative Real Time; Tg, Triglycerides

Key Words: Synaptotagmin 1, Cholesterol, Apolipoprotein E Deficient Mice, Squalene, High-Fat Diet, Syt1, sex

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