Pgc1a is responsible for the sex differences in hepatic Cidec/Fsp27β mRNA expression in hepatic steatosis of mice fed a Western diet

Luis V. Herrera-Marcos¹², Sara Sancho-Knapik¹, Clara Gabás-Rivera¹⁰, Cristina Barranquero¹²⁹, Sonia Gascón¹²⁹, Eduardo Romanos³, Roberto Martínez-Beamonte¹²⁹, María A. Navarro¹²⁹, Joaquín C. Surra²⁴⁹, Carmen Arna¹²⁵⁹, José A. García-de-Jalón⁵, María J. Rodríguez-Yoldi²⁶⁹, Manuel Tena-Sempere⁷⁹, Cristina Sánchez-Ramos⁸, María Monsalve⁸ and Jesús Osada¹²⁹

¹ Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Instituto de Investigación Sanitaria de Aragón-Universidad de Zaragoza, Zaragoza, Spain
² Instituto Agroalimentario de Aragón, CITA-Universidad de Zaragoza, Spain
³ Instituto de Investigación Sanitaria de Aragón-Universidad de Zaragoza, Zaragoza, Spain
⁴ Departamento de Producción Animal y Ciencia de los Alimentos, Escuela Politécnica Superior de Huesca Facultad de Veterinaria, Instituto de Investigación Sanitaria de Aragón-Universidad de Zaragoza, Huesca, Spain
⁵ Departamento de Patología Animal, Facultad de Veterinaria, Instituto de Investigación Sanitaria de Aragón-Universidad de Zaragoza, Zaragoza, Spain
⁶ Departamento de Farmacología y Fisiología, Facultad de Veterinaria, Instituto de Investigación Sanitaria de Aragón-Universidad de Zaragoza, Spain
⁷ Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba e Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain
⁸ Instituto de Investigaciones Biomedicas 'Alberto Sols' (CSIC-UAM), Madrid, Spain
⁹ CIBER de Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Spain
Correspondence to: Prof. Dr. Jesús Osada
Department of Biochemistry and Molecular Biology,
Veterinary School, University of Zaragoza,
Miguel Servet, 177, E-50013 Zaragoza, Spain.
Fax number: 34-976-761612
Telephone number: 34-976-761644
E-mail: josada@unizar.es

Running title: Hepatic Cidec/Fsp27 gene expression

Abbreviations: Pgc1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Abstract

Hepatic fat-specific protein 27 (Cidec/Fsp27) mRNA levels have been associated with hepatic lipid droplet extent under certain circumstances. To address its hepatic expression under different dietary conditions and both sexes, Apoe-deficient mice were subjected to different experimental conditions for 11 weeks to test the influence of cholesterol, Western diet, squalene, oleanolic acid, sex and surgical castration on Cidec/Fsp27 mRNA expression. Dietary cholesterol increased hepatic Cidec/Fsp27β expression, an effect that was suppressed when cholesterol was combined with saturated fat as represented by Western-diet feeding. Using the latter diet, oleanolic acid or squalene did not modify its expression. Females showed lower levels of hepatic Cidec/Fsp27β expression than males when they were fed Western diets. A result that was translated into lesser amount of CIDEC/FSP27 protein in lipid droplets and microsomes. A result also confirmed in Ldlr-deficient mice and in AML12 cells incubated with estradiol. While male surgical castration did not modify the expression, ovariectomized females did show increased levels compared to control females. Females also showed increased expression of Pgc1a, suppressed by ovariectomy, and the values were significantly and inversely associated with those of Cidec/Fsp27β. When Pgc1a-deficient mice were used, the sex-differences on Cidec/Fsp27β expression disappeared. Therefore, hepatic Cidec/Fsp27β expression has a complex regulation influenced by diet and sex hormonal milieu. The mRNA sex differences are controlled by Pgc1a.

Keywords:

Lipids/liver, lipid droplets, animal models, gene expression, non-alcoholic fatty liver disease.

Cidec/Fsp27, Pgc1a, apolipoprotein E deficient mice, high-fat diet, sex.
Introduction

Fat-specific protein 27 (FSP27) gene encodes a protein of 27 kDa with 238 amino acids, belonging to the cell-death-inducing DNA fragmentation effector (CIDE) family, composed of CIDEA, CIDEB, and CIDEC/CIDE-3/FSP27, all of which contain a conserved CIDE N-domain and a unique C-terminal domain. Cidec/Fsp27 is expressed at high levels in white adipose tissue (26). By alternative splicing in HepG2, CIDE-3 gene displays two transcripts, CIDE-3 and CIDE-3alpha. While CIDE-3 comprises a full-length open reading frame, CIDE-3alpha encodes a truncated protein (29). In the liver, a third transcript, FSP27β, which contains 10 additional amino acids at the N-terminus of the original protein and is activated through the liver-enriched transcription factor cyclic-AMP-responsive-element-binding protein H (CREBH) but not by peroxisome proliferator-activated receptor gamma, has been described (11, 63). In this organ, CIDEC/CIDE-3/FSP27 contributes to triglyceride accumulation both in humans and pigs (28) and to the regulation of lipidation and maturation of very low-density lipoproteins (62). It is localized to lipid droplets (LD) and endoplasmic reticulum (55). The latter participates in the regulation of LD formation, expansion, and morphology under lipid-deficient conditions (25). To promote the formation of a unilocular droplet, the formation a ternary complex of AS160, the GTPase activating protein for RAB8a, FSP27 and RAB8a is required (59).

Fsp27-deficient mice show increased energy expenditure and lower levels of plasma triglycerides and free fatty acids (39). Only when they are crossed with leptin-deficient mice or BATless mice, or are fed them a high-fat diet, hepatic steatosis and insulin resistance are observed. Therefore, Fsp27 deficiency requires further implication of genes to display hepatic insulin resistance (57, 68). In contrast, mice with adipocyte-
specific disruption of the Fsp27 gene upon high-fat diet feeding are resistant to weight gain and fat-storing. This results in a lipid overflow from adipose tissue that generates hepatosteatosis, dyslipidemia, and systemic insulin resistance pointing out a role for this adipocyte protein to prevent lipodystrophies (56). An increased expression of Cidec has been found in a number of experimental or pathological conditions, such as in endoplasmic reticulum stress (24), spontaneous mouse insulin resistance (51) and hepatocellular carcinoma cells (37). Similar effects have been described in liver steatosis and in obese humans (13), being the latter increase reduced by weight loss (16). A homozygous human mutation of CIDEC has been reported to induce lipodystrophy and insulin-resistant diabetes (40, 48). Reduced expression of hepatic Fsp27 abolished fasting-induced liver steatosis (23) and the former condition in combination with a PPARalpha agonist was also found to reduce hepatic steatosis (45) and even atherosclerosis (46) in Ldlr-deficient mice, a model of atherosclerosis and hepatosteatosis (49).

The expression of CIDEC is controlled at both transcriptional and posttranslational levels (5, 13). Different molecules seem to be involved in its expression, such as CD44 (17) or osteopontin, whose absences decrease its levels (22), while leptin absence displays the opposite (35). Ceramide (27) and TNF-alpha reduced its expression while insulin upregulated it. In the latter response, the activity of phosphatidylinositol 3-kinase was involved (21), so was the phosphatase and tensin homologue, an enzyme involved in degradation of phosphorylated phosphatidylinositol (50). Final effectors of these signaling pathways seem to be nuclear receptors such as TAK1/TR4/NR2C2, RORalpha and PPARalpha, nuclear proteins (CAAT-enhancer-binding proteins), LXRα and SREBP-1c (3, 7, 8, 18, 19, 23). Peroxisome proliferator-activated receptor gamma2 (PPARgamma2) also plays a role (34). Posttranslational regulation of FSP27 involves
stability through the proteasomal ubiquitin-dependent protein catabolic process (67),
glycosylation (65) and acetylation (44).

A complex physiological regulation of CIDE-C seems to exist, in which fasting
and diet composition play important roles. In this regard, during the initial stages of
fasting, Fsp27 expression has been found dramatically increased by involvement of the
PKA-CREB-CRTC2 signaling pathway (58). The fasting effect was not present in
PPARalpha-deficient mice (66). However, after a long period of fasting, a decrease in
Fsp27 expression was observed (58). Despite the observed hepatic steatosis after a
choline-deficient diet, no changes were observed for Fsp27 (66). Nevertheless, a marked
induction of its expression was found in the high-fat- or methionine- and choline-deficient
diet-induced fatty liver, but not in alcohol-induced fatty liver. The induction of Fsp27
mRNA was independent of peroxisome proliferator-activated receptor gamma
(PPARgamma) levels and completely absent in the liver from PPARgamma-deficient
mice (2). In less extreme dietary conditions, it has been reported that a high fat diet
increased Fsp27 expression through activation of PPARgamma (41). In vitro, a high
supply of fatty acids stimulated hepatic expression (25). Using Apoe-deficient mice as a
model of spontaneous hepatosteatosis, nature of fatty acids was important to increase its
expression in these mice fed a Western-type diet enriched with linoleic acid isomers since
only those mice receiving trans-10, cis-12-conjugated linoleic acid showed this effect.
Furthermore, consuming olive oil-enriched diet reduced Fsp27 expression (15). In
addition, only one study has addressed the influence of sex on its expression in young
mice (12). Growth hormone has also found to regulate this protein (52, 53). Therefore,
influence of sex may be different in adult mice. Based on these facts, it was hypothesized
that Fsp27 hepatic regulation might be the result of complex interactions of dietary
components and sex. To this end, the present work was undertaken to characterize the
influence of different dietary conditions and sex on $Fsp27$ gene expression in adult liver of several animal models.
Material and methods

Animals

Charles River (Charles River Laboratories, Barcelona, Spain) was the source of Apoe-deficient mice on the C57BL/6J genetic background. Dr. Nobuyo Maeda (University of North Carolina at Chapel Hill, NC, USA) generously provided these mice on the C57BL/6JxOla129 genetic background. Ldlr-deficient mice on the C57BL/6J.SJL genetic background were obtained from Dr. Vicente Andrés from CNIC, (Madrid, Spain). C57BL/6J wild-type and Pgc1a-deficient mice were part of a colony established at the IIB animal facility (Madrid) and originally derived from mice provided by Dr. Bruce Spiegelman (DFCI, Boston, USA). Wistar rats were obtained from Charles River (Charles River Laboratories, Barcelona, Spain).

For all experiments, two-month-old mice were used. Blood samples were taken (after four-hour fasting) from the facial vein to determine plasma cholesterol and accordingly establish groups with similar initial values. Animals, housed in sterile filter-top cages, were maintained under a 12-h light/12-h dark cycle at the CIBA, Universidad de Zaragoza. Pgc1a-deficient mice were maintained at Autónoma Universidad de Madrid. Wistar rats were maintained at Universidad de Córdoba. Animals were handled and killed observing guidelines (Directive 2010/63/UE) from the European Union for care and use of laboratory animals in research. All had ad libitum access to food and water and study protocols were approved by the Ethics Committees for Animal Research of the Universities of Zaragoza, Madrid and Córdoba. After the diet intervention, and four-hour fast, the animals were killed by suffocation with CO2. The livers were removed, weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis.

Experimental design

Table 1 provides detailed information of all experiments regarding characteristic
of animals, type of diets, number of animals and length of intervention. Since C57BL/6J mice do not express hepatic Cidec/Fsp27 (35, 58), we decided to use Apoe-deficient mice which showed hepatic expression of this gene influenced by some dietary components (15). Using this model, we tested the effects on Cidec/Fsp27 expression of dietary cholesterol, Western diet and sex. On Western diet, the influence of two modifiers of lipid droplet surface, oleanolic acid (10) and squalene (14) were tested. Likewise, this diet was used to analyze sex differences and its inhibition by surgical castration. A confirmation of the effects of ovariectomy on Cidec/Fsp27 expression was carried out in female Wistar rats fed a Western diet. Since sex differences emerged on Western diet, this was also tested in another model of hepatic steatosis, Ldlr-deficient mice on C57BL/6J genetic background. All previous experiments were suggestive of an involvement of PGC1a, to confirm such issue; Pgc1-deficient mice on C57BL/6J genetic background fed a purified Western diet were used to analyze the sex differences on hepatic Cidec/Fsp27 expression. Detailed compositions of purified diets are shown in supplementary Table 1.

**Isolation and quantification of hepatic RNA**

RNA was isolated using Tri-reagent (Ambion, Austin, TX, USA). Contaminant DNA was removed using the DNA removal kit from Ambion. Absorbance at A$_{260/280}$ served to quantify RNA concentrations and the ratio 28S/18S ribosomal RNAs used to estimate their quality. Changes in mRNA expression were determined by RT-qPCR. cDNA synthesis was carried out using the First Strand synthesis kit (Thermo Scientific, Madrid, Spain). The Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used to analyze gene expression by qPCR. Specific primers, designed and checked as previously described (33) were purchased from Applied Biosystems. Sequences are shown in supplementary Table 2. RT-qPCR reactions were performed on a Step One Real Time PCR System (Applied Biosystems) following the standard procedure and using
equal amounts of DNA-free RNA from each animal. The relative amount of all mRNAs was calculated using the comparative $2^{-\Delta\Delta C_q}$ method and Cyclophilin B (Pipb) mRNA expression as the reference gene.

Liver histology analyses

Aliquots of liver, stored in neutral formaldehyde, were used and processed as described (14).

Hepatic homogenate and lipid extraction

A piece of liver was homogenized in homogenization buffer (phosphate buffered solution with protease inhibitor cocktail (Roche, Mannheim, Germany) and used to assay protein concentration by the BioRad dye binding assay (BioRad, Madrid, Spain). Extracted lipids according to Folch’s method (9) were evaporated under N₂ stream and dissolved in 100 µL of isopropanol. Infinity kits (Thermo Scientific) were used to measure total cholesterol and triglycerides.

Preparation of microsomal fractions

This fraction was prepared according to Osada et al. (43). Basically, 600 mg of pooled hepatic tissue of each group were homogenized in 2 mL of 0.25 M sucrose containing the Roche protease inhibitor cocktail at 4°C and centrifuged at 280g for 5 min. Supernatants were centrifuged at 1500g for 10 min followed by another centrifugation at 19000g for 10 min to collect the supernatants containing cytosolic and microsomal proteins. After a centrifugation at 100000g for 60 min, the obtained pellets containing the microsomal fractions were resuspended in PBS containing 0.2% Triton X-100 and 10% glycerol and centrifuged at 12000 rpm 10 min in order to remove insoluble proteins. Protein concentrations were determined by BioRad dye binding assay.

Preparation of lipid droplets

They were prepared following the protocol of Ontko et al. (42). Briefly, pooled
hepatic tissue (600 mg) of each group were homogenized in 3 ml of 65% sucrose solution with protease inhibitor cocktail (Roche, Mannheim, Germany) at 4ºC. Discontinuous sucrose gradients were prepared as follows: 3 ml of liver homogenates in 65% sucrose were pipetted at the bottom of the centrifuge tubes kept in an ice bath. Then 2 ml of 60% sucrose solution were slowly added, followed by 2 ml of 52% sucrose, 2 ml of 44% sucrose and 2 ml of distilled water. The tubes with the gradients were centrifuged at 25000g for 30 min at 4ºC and the bands containing the different lipid droplets were collected. They were mixed with 3 volumes of acetone and kept at -80ºC for 10 min and then at -20ºC overnight. The tubes were centrifuged at 15000g for 15 min at 4ºC. The pellets were washed three times, firstly with acetone: diethyl ether 1:1 and then twice with diethyl ether. Dry pellets were resuspended in PBS containing 0.2% Triton X-100 and 10% glycerol and centrifuged at 12000 rpm 10 min in order to remove insoluble proteins. Protein concentrations were determined by BioRad dye binding assay.

**Western blot**

20 μg of protein were loaded onto a 10% SDS-polyacrylamide gel and electrophoresed for 120 min at 90V in a Bio-Rad Miniprotein cell (Hercules, CA). Proteins were transferred to PVDF membranes (GE Healthcare, Madrid, Spain). Membranes were blocked with PBS buffer containing 5% BSA for 1 h at room temperature. The primary antibodies, diluted in PBS buffer containing 2.5% BSA and 1% Tween 20, were added and the membranes were incubated 2 h at room temperature and then overnight at 4ºC. FSP27 protein expression was evidenced by using a rabbit polyclonal antibody (NB100-430 diluted 1/1,000, Novus Biologicals, Centennial, Colorado, USA). Equal loadings were confirmed by using a goat polyclonal anti-HSC70 (TA302666 diluted 1/500, OriGene, Rockville, MD, USA). Membranes were washed with PBS buffer containing 0.1% Tween 20. Conjugated goat anti-rabbit IgG (H&L)
DyLight 800 secondary antibody (SA5-35571, diluted 1/15,000, Thermo-scientific, Waltham, MA, USA) for FSP27 detection and a donkey anti-goat IRDye 680RD (926-68074, diluted 1/5,000, LI-COR Biosciences, Lincoln, NE, USA) for HSC70 detection were used and incubated for 1 h at room temperature in PBS buffer containing 2.5% BSA and 1% Tween 20. Images were captured using an Odyssey® Clx (LI-COR).

**AML12 cell culture**

The murine hepatocyte cell line was grown in a humidified atmosphere of 5% CO₂ at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) (ThermoFisher Scientific, Waltham, MA, USA): F12-Ham’s medium (GE Healthcare Life Science, South Logan, Utah) in 1:1 ratio supplemented with 10% foetal bovine serum (ThermoFisher Scientific), 1:500 insulin/transferrin/selenium (Corning, Bedford, MA, USA), 40 ng/ml dexamethasone (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) 1% nonessential amino acids (ThermoFisher Scientific), 1% penicillin (1000 U/ml) (ThermoFisher Scientific), 1% streptomycin (1000 mg/ml) (ThermoFisher Scientific) and 4 mM L-glutamine (ThermoFisher Scientific) in a 6 multiwell plate (in triplicate). Medium was changed every two days. After one week of growth, this medium was removed, and cells were washed twice with phosphate buffered saline (PBS) prior to the addition of the serum-free media supplemented with 200 µm stearic acid for 24 hours or 200 µM stearic acid for 24 hours and 50 nM estradiol dissolved in ethanol for 6 hours. Then, media were removed and cells collected with Tri-reagent solution (Ambion). RNA isolation and cDNA synthesis were performed as above described.

**Reporter assays**

The genomic region -2042 bp to 0 bp at 5’ side of the starting transcription site of CIDE/CSP/27β (XM_024453700.1) from human genomic DNA was amplified by PCR using direct (5’-agaaccagatgtgCAAGTGATCCACCTGACCT-3) and reverse (5’-
gatatctcagaattGAGCAGATAACCCAACTCAGGGC -3’) primers. The 2-kb PCR product was cloned upstream a secreted Gaussia luciferase (GLUC) reporter gene using linearized pEZX-GAO1 (Genecopeia Rockville, Maryland, USA) according to In-Fusion® cloning protocol from Takara-Clontech (Cat No 638909, Kusatsu, Shiga, Japan). Restriction enzymes and DNA sequencing confirmed the resulting plasmid. This latter was transfected to AML12 cells alone or in combination with a plasmid containing Pgc1a (MN_008904) under the control of CMV promoter (MC204789, Origene) using lipofectamine 3000 (ThermoFisher) following manufacturer’ instructions. Two days after, media were taken and secreted GLUC and alkaline phosphatase, also present in pEZX-GAO1, activities were evaluated. The ratio of GLUC/alkaline phosphatase was calculated.

Statistical analysis

The Statistical Package for Social Sciences version 15 (SPSS, Chicago, IL, USA) or Prism 5 for windows software for Windows (GraphPad, S. Diego, CA, USA) were used for statistical analyses. Variables, not showing normal distribution (according to the Shapiro-Wilk’s test), or homology of variance, were analyzed with the Mann-Whitney’s U test. Data are shown as medians and 10-90 percentile range of the values. Correlations between variables were tested using the Spearman’s correlation test. The statistical significance was considered when p < 0.05.
Results

Dietary fat and hepatic Cidec/Fsp27β expression in Apoe-deficient mice.

To characterize the dietary regulation of the expression of this gene in mice, the supplement of dietary cholesterol to male mice was tested. Increased hepatic surface occupied by lipid droplets as well as hepatic total cholesterol and triglyceride contents (Fig 1a, b and c) were observed following dietary cholesterol supplementation. The latter induced a significant increase in the hepatic Cidec/Fsp27β expression as shown in Fig 1d. Hepatic cholesterol content was associated with hepatic Cidec/Fsp27β expression (Fig 1e).

In a second study, the influence of a Western diet (WD), containing cholesterol and palm oil as source of saturated fat, was explored in male Apoe-deficient mice on C57BL/6J genetic background (Fig 2). Significant increased hepatic areas occupied by lipid droplets (Fig 2a, b and c) as well as hepatic total cholesterol and triglyceride contents were also observed in mice on the Western diet. Unexpectedly, a significant decrease of hepatic Cidec/Fsp27β expression was found (Fig 2d). These expression changes were inversely associated with hepatic cholesterol (Fig 2e).

To further explore this dissociation between hepatic Cidec/Fsp27β and Western diet, its expression was tested in two dietary components that had been shown to influence dietary droplets without altering lipid content (oleanolic acid) or viceversa (squalene). In the first experiment and as expected, male mice receiving an oleanolic acid-enriched WD showed an increase in the hepatic area occupied by lipid droplets (Supplementary Fig 1a, b and c) without changes in hepatic cholesterol and triglyceride contents (Supplementary Fig 1c). In these conditions, no significant change was observed for hepatic Cidec/Fsp27β expression (Supplementary Fig 1d). In the second experiment, the effect of a squalene-enriched WD was explored, again in males. No significant changes were noted for the
percentage of hepatic surface occupied by lipid droplets despite the decreased liver cholesterol and triglyceride contents (Supplementary Fig 2a, b and c). Nor was there any significant change in the hepatic Cidec/Fsp27β expression by squalene (Supplementary Fig 2d). Overall, these nutritional experiments emphasize that hepatic Cidec/Fsp27β expression possesses a fine nutritional regulation at transcriptional level in Apoe-deficient mice, where cholesterol increased its levels and saturated fat reverted this finding, being the latter not influenced by minor dietary components, such as oleanolic acid or squalene, despite the changes in hepatic lipids.

Hepatic Cidec/Fsp27β expression is influenced by sex hormones in Apoe-deficient mice and in vitro.

The influence of sex on Cidec/Fsp27β expression was explored in Apoe-deficient mice on a chow diet of low fat content. As shown in Supplementary Fig 3, panels a,b,c, females showed lower surface occupied by lipid droplets despite a significant increase in hepatic cholesterol content and no changes in triglycerides. In this experiment, no significant changes were observed in hepatic Cidec/Fsp27β between sexes. In the second experiment, the differences between sexes were explored when both groups received a WD. As shown in Fig 3a, b and c, no significant change was observed in the percentage of liver surface occupied by lipid droplets. However, the levels of hepatic total cholesterol and triglycerides were significantly lower in females than in males. In this experimental approach, females showed significantly decreased hepatic Fsp27β expression (Fig 3d). The latter was significantly associated with hepatic triglyceride contents (Fig 3e). This mRNA decrease was translated in decreased amounts of CIDEC/FSP27 protein in lipid droplets and microsomes (Fig 3f and g). These data indicate that sex is playing an important role in hepatic Cidec/Fsp27β expression in the presence of WD and these
changes are reflected in a lesser amount of CIDE in hepatic lipid droplets of female livers.

The involvement of hormonal changes on sex-differences was characterized in Apoe-deficient mice of both sexes that underwent surgical removal of gonads and were fed a purified Western diet. As shown in Supplementary Fig 4, no significant change in Cidec/Fsp27β expression was observed in orchiectomized males; nor was there any significant change in hepatic total cholesterol, or in hepatic triglycerides. However, there was a significant increase in the liver surface occupied by lipid droplets in orchiectomized males. In contrast, ovariectomy resulted in significant increases in hepatic cholesterol, triglycerides, and in the surface occupied by lipid droplets (Fig 4c). Ovariectomized females showed a significant increase in Cidec/Fsp27β expression compared to control females (Fig 4d). A positive significant association was also found between hepatic Cidec/Fsp27β values and those of hepatic triglycerides (Fig 4e). The increase in mRNA expression was translated into increased contents of CIDE proteins in lipid droplets and microsomes (Fig 4f and g). These results indicate that ovarian hormones are responsible for the decreased hepatic Cidec/Fsp27β expression observed in females consuming WD.

In fact, incubation of stearic-stimulated hepatic AML12 cells with estradiol resulted in a significant decrease in Cidec/Fsp27β expression (Supplementary Fig 5a).

Pgc1a is involved in hepatic Cidec/Fsp27β expression sex differences in vivo.

PKA and PPAR have been described in the regulation of hepatic Cidec/Fsp27 expression (3, 7, 8, 18, 19, 23). To verify whether or not those agents were involved in the observed sex-dependent responses, hepatic Prka2 expression was determined and no significant changes were observed (data not shown). Regarding PPARgamma, the hepatic expression of its regulator, Pgc1a, was significantly increased in females compared to males consuming the Western diet (Fig 5a) and an inverse significant relationship was
found between Cidec/Fsp27β expression and that of Pgc1a in both sexes (Fig 5b). While orchiectomy had no effect on Pgc1a expression (Fig 5c), ovariectomy induced a significant decrease in its expression in Apoe-deficient females (Fig 5d). Likewise, ovariectomized female rats also showed a trend to increase Cidec/Fsp27β expression (Supplementary Fig 6e) and decreased hepatic Pgc1a expression (Supplementary Fig 6f). Both effects were even more pronounced in rats neonatally androgenized by testosterone administration and then ovariectomized. In this model, hepatic fat, cholesterol and TG contents followed a similar pattern (Supplementary Fig 6d) and Cidec/Fsp27β expression was associated with hepatic TG content (data not shown). The sex differences in Cidec/Fsp27β expression were observed in Ldlr-deficient fed on WD as well (Supplementary Fig 7d). Concomitantly, a significant increase in Pgc1a expression was observed in these female mice (Supplementary Fig 7e). Decreased Cidec/Fsp27β gene expression in females was translated into lower amounts of CIDEC/FSP27 protein in lipid droplets and microsomes (Supplementary Fig 7f and g). Overall, these findings are suggestive of an inverse association between Cidec/Fsp27β and Pgc1a expressions as a general response, independent of absence of APOE. These mRNA changes are reflected in CIDEC/FSP27 protein present in lipid droplets from female livers.

According to this association, it was hypothesized that Pgc1a would reduce the transcriptional activity of a reporter gene under the control of CIDEC promoter. This was the case, as shown in Supplemental Fig 5b. The opposite hypothesis would be that sex-differences in hepatic Cidec/Fsp27β would be abolished in the absence of Pgc1a. To test this, Pgc1a-deficient mice from both sexes were fed WD. In this model, female mice increased hepatic fat area, total cholesterol and TG contents (Fig 6a, b, c). As shown in Fig 6d, no differences were observed in hepatic Cidec/Fsp27β expression between sexes when using homozygous Pgc1a-deficient mice. However, the sex differences at the
CIDEC protein levels in lipid droplets and microsomes remained in absence of PGC1a (Fig 6e and f). These results suggest that absence of PGC1A abolishes the sex-induced mRNA changes of hepatic $Fsp27\beta$ expression in response to WD, being the transcription factor a transcriptional repressor. However, the sex-induced differences in CIDEC present in lipid droplets and microsomes are independent of PGC1A.
Discussion

The present work explores the putative hepatic Cidec/Fsp27β transcriptional changes induced by dietary components and sex. Using Apoe-deficient mouse as a model of hepatic steatosis, dietary cholesterol increased hepatic Cidec/Fsp27β, which was repressed when combined with saturated fat. The latter was not influenced by dietary minor components such as oleanolic acid or squalene administered at pharmacological doses. Moreover, our study revealed a previously unnoticed sex regulation dependent on the prevailing diet, being the female sex a negative regulator. An effect observed in two models of genetic hepatic steatosis (Apoe- and Ldlr-deficient mice) and reflected in CIDE/C/FSP27 content of lipid droplets. Using ovariectomized females, it was shown that ovarian hormones are crucial for the observed decrease in Cidec/Fsp27β expression noted in Apoe-deficient mice. This effect was also observed in Wistar female rats. An increased expression of Pgc1a inversely associated with that of Cidec/Fsp27 and the lack of such effect after ovariectomy in Apoe-deficient mice allow us to infer that ovarian hormones are executing their action through Pgc1a. This was confirmed in mice lacking Pgc1a where the sex differences on hepatic Cidec/Fsp27β were erased providing further in vivo support for this role. However, the sex differences at the CIDE/C/FSP27 content of lipid droplets and microsomes are independent of PGC1a.

As shown in Supplemental Figure 8, four set of primers were used to study hepatic Cidec/Fsp27 mRNA expression in mice. With the exception of primers, named α, corresponding to exon 1, which showed no expression in the liver (data not shown), the remaining three sets gave concordant results in all experimental conditions. None of the selected primers amplified the truncated form. Thus, the observed changes corresponded to Fsp27β, a recently described isoform of the protein regulated by CREBH (63).

The present work has explored the influence of two main components of Western diet, cholesterol and saturated fat. Using the first dietary component, an increase in the
Cidec/Fsp27β expression was noted. Using information from ENCODE, it was observed that both SREBP1 and 2 bind to this gene (6). Recently, the involvement of SREBP-1c has been proved (7). Surprisingly, the combination of cholesterol and saturated diet decreased hepatic Cidec/Fsp27β expression. In this regard, variable effects of high fat diets have been described depending on the length of fat administration (36). While a short-term administration (3 weeks) increased the expression, a long-term administration of 12 weeks had the opposite effect. In this sense, our study lasted 11 weeks and would be in agreement with the latter finding. Similar results were observed in Apoe-deficient mouse males receiving an olive oil-enriched diet (15). Likewise, a decreased expression was found in a postprandial regimen after a virgin olive oil bolus in male Wistar rats and this decrease was inversely associated with hepatic triglyceride and cholesterol contents (32). In the latter case, the hepatic mRNA changes occurred just 4 hours after fat intake. In fasting rats, a rapid increase was equally observed four hours after its start (58). Elevations of Cidec/Fsp27 mRNA expression by high fat diets required additional dietary deficiencies such those of methionine and choline (Table 2) or under certain metabolic derangements such as those posed by Db mice, PPAR-alpha-deficient mice (Table 2). In a previous study, using Apoe-deficient mice with C57BL/6JxOla129 genetic background and fed Western diets with different conjugated linoleic acid (CLA) isomers, we observed high hepatic Cidec/Fsp27 mRNA expression in those mice receiving the trans-10,cis-12 CLA isomer and the levels were associated with the hepatic surface occupied by lipid droplets. In contrast, when the cis-9, trans-11 CLA isomer was provided resulted in decreased Cidec/Fsp27 mRNA expression (15). Overall, regimen of administration and nutritional components are critical modulators of hepatic Cidec/Fsp27 expression and this mRNA undergoes a rapid metabolic variation in few hours.

In the present study, the intake of oleanolic acid, a pentacyclic triterpene, and
squalene, a lineal triterpene, had no effect on Cidec/Fsp27β expression despite the changes induced in lipid droplet area (10). Similar finding was reported by the administration of a dietary supplement of Boswellia serrata, an extract rich in particular derivatives of boswellic acid, also a pentacyclic triterpene-based compound (20). As triterpenes tend to accumulate in the liver altering distribution of triglycerides in lipid droplets (30, 31), it could be hypothesized that those lipid droplets would not need changes in Cidec/Fsp27β expression or these are not executed at the mRNA level.

In a previous study, we observed that hepatic Cidec/Fsp27 gene expression was significantly associated with hepatic surface occupied by lipid droplets in Apoe-deficient mice fed different conjugated linoleic acid isomers, in Cbs-deficient mice and in olive oil-fed Apoe-deficient mice (15). This was not the case in the present study. Notably, Cidec/Fsp27β expression was associated with hepatic triglyceride (Figures 3 and 4) or cholesterol contents (Figures 1 and 2). The genetic background and the diet composition are main differences between the previous and the current study. The former one used Ola129xC57BL/6J mixed genetic background mice while the present study has been carried out using C57BL/6J mice. Due to both strains do have important differences in hepatic fat content (54), the experimental setting may have influenced the outcome. The second aspect is the use of AIN-93 purified diet (47) in the present study compared to commercial ones in the previous one. This choice was forced by the high variability noted in our lab among control mice for years in atherosclerotic lesions when using commercial chows and the failure of obtaining the same batch throughout years. Indeed, source of protein has also been shown to induce changes in Cidec/Fsp27 expression (60, 64). By and large, dietary components are an important source of variation (49), and our current study, in well-defined conditions of mouse strains and purified diets, adds further evidence supporting this contention.
A striking result observed in this work was the decreased hepatic Cidec/Fsp27β expression in female mice consuming WD in Apoe- and in Ldlr-deficient mice. As consequence of this decrease, the amount of CIDE/CSP27 protein in lipid droplets was decreased in females. This fact points to a sex-difference in hepatic regulation of lipid droplet enlargement considering the role of CIDE/CSP27 in this process. An effect that was abolished when ovariectomy was performed in Apoe-deficient mice and Wistar rats. Interestingly, female mice lacking steroid receptor coactivator-2 showed increased hepatic expression of this gene (Table 2). Steroid receptor coactivator-2 promotes the transcriptional activation of estrogen receptor in some tissues (61). These results are indicating a negative regulation of the gene by the influence of female hormones. This could be executed through Pgc1a as the significant inverse association noted between Pgc1a and Cidec/Fsp27β suggests. Further evidences to this suggestion comes from the binding of PGC1a to this gene as evidenced by ChIP assays reported by the ENCODE consortium (6). Indeed, estradiol action has been found to be modulated by Pgc1a (4) and Pgc1a decreased CIDE promoter activity. When we carried out ovariectomy, the decrease in Cidec/Fsp27β expression was lost. Deficiency of Pgc1a as the case of the experiment carried out in Pgc1a-deficient mice is also supporting the role of Pgc1a in the in vivo sex differences but only at the mRNA levels.

In conclusion, the present report evidences two axes of hepatic Cidec/Fsp27β regulation defined by diet and sex. Regarding the first one, cholesterol and the nature of fatty acids are a key component. On the other hand, the fact that the female decrease in hepatic gene expression was not observed in ovariectomized mice strongly suggests that ovarian hormones are involved in the control of hepatic Cidec/Fsp27β mRNA expression and this is modulated by Pgc1a. However, the sex-differences at the CIDE/CSP27 protein levels observed in lipid droplets and microsomes are independent of PGC1a.
Acknowledgments

We thank Silvia Garcés and Mª Pilar Lierta for their help in maintaining the animals. This research was supported by grants from Ministerio de Economía y Competitividad-Fondo Europeo de Desarrollo Regional (SAF2015-63904-R, SAF2016-75441-R), Fondo Social Europeo-Gobierno de Aragón (B16_17R), and from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement 721236-TREATMENT to M.M. CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN, CB06/03/1012) is an initiative of ISCIII. L.V.H.M. and S.S.K. were recipients of Fondo Social Europeo-Gobierno de Aragón and Fundación Cuenca-Villoro (BE 203/2009) fellowships, respectively.

No competing financial interests exist.
References


46. Rajamoorthi A, Lee RG, and Baldan A. Therapeutic silencing of FSP27 reduces the progression of atherosclerosis in Ldlr(-/-) mice. Atherosclerosis 275: 43-49,


54. Surra JC, Guillen N, Arbones-Mainar JM, Barranquero C, Navarro MA, Arnal


Table 1. Summary of experimental conditions

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Genetic background</th>
<th>Diet</th>
<th>Sex</th>
<th>Groups and sample size</th>
<th>Influence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6J x OLA 129</td>
<td>Commercial chow (B &amp; K Universal Ltd, Humberside, UK) w/wo 0.1% cholesterol for 10 weeks (1)</td>
<td>Males</td>
<td>Control (n=7) Cholesterol (n=7)</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>2</td>
<td>C57BL/6J</td>
<td>Purified chow and Western diets for 11 weeks</td>
<td>Males</td>
<td>Chow (n=13) Western (n=9)</td>
<td>Western diet</td>
</tr>
<tr>
<td>3</td>
<td>C57BL/6J</td>
<td>Purified Western w/wo 0.01% oleanolic acid (OA) (Extrasynthese, Genay, France) for 11 weeks (10)</td>
<td>Males</td>
<td>Western (n=8) Western + OA (n=9)</td>
<td>Oleanolic acid</td>
</tr>
<tr>
<td>4</td>
<td>C57BL/6J</td>
<td>Purified Western w/wo 1% squalene (Sigma, Madrid, Spain) for 10 weeks (14)</td>
<td>Males</td>
<td>Western (n=9) Western + Squalene (n=10)</td>
<td>Squalene</td>
</tr>
<tr>
<td>5</td>
<td>C57BL/6J</td>
<td>Purified chow for 11 weeks</td>
<td>Both sexes</td>
<td>Males (n=13) Females (n=13)</td>
<td>Sex in chow diet</td>
</tr>
<tr>
<td>6</td>
<td>C57BL/6J</td>
<td>Purified Western for 11 weeks</td>
<td>Both sexes</td>
<td>Males (n=9) Females (n=10)</td>
<td>Sex in Western diet</td>
</tr>
<tr>
<td>7</td>
<td>C57BL/6J</td>
<td>Purified Western for 11 weeks</td>
<td>Orchietomized and non-orchietomized males</td>
<td>Control (n=9) Orchietomized on postnatal day 30 (n=9)</td>
<td>Testicular contribution in males</td>
</tr>
<tr>
<td>8</td>
<td>C57BL/6J</td>
<td>Purified Western for 11 weeks</td>
<td>Orchietomized and non-orchietomized females</td>
<td>Control (n=9) Orchietomized on postnatal day 30 (n=9)</td>
<td>Ovarian contribution in females</td>
</tr>
<tr>
<td><strong>Ldlr-deficient mice</strong></td>
<td>C57BL/6J.S JL</td>
<td>Purified Western for 11 weeks</td>
<td>Both sexes</td>
<td>Males (n=17) Females (n=18)</td>
<td>Sex in Western diet</td>
</tr>
<tr>
<td>Species</td>
<td>Strain</td>
<td>Diet</td>
<td>Sexes</td>
<td>Males (n=8)</td>
<td>Females (n=8)</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>-------------------------------</td>
<td>----------------------</td>
<td>-------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Mice</td>
<td>C57BL/6J</td>
<td>Purified Western for 11 weeks</td>
<td>Both sexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td>Wistar</td>
<td>Purified Western for 100 post-weaning days (38)</td>
<td>Ovariectomized and non-ovariectomized females</td>
<td>Control (n=6)</td>
<td>Ovariectomized (n=6)</td>
</tr>
</tbody>
</table>

w/wo, with or without
Table 2. Changes in hepatic *Cidec/Fsp27* expression according to Genome Expressed Omnibus data bank and Array express.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Type of change</th>
<th>Signal log₂ ratio</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 1 deficient mice</td>
<td>Increased</td>
<td>0.3</td>
<td>GDS4922</td>
</tr>
<tr>
<td>Glycerol kinase knockout</td>
<td>Increased</td>
<td>1.9</td>
<td>GDS1555</td>
</tr>
<tr>
<td>NADH-cytochrome P450 reductase deletion effect on liver</td>
<td>Increased</td>
<td>1.3</td>
<td>GDS1093</td>
</tr>
<tr>
<td>Stearoyl-CoA desaturase 1-deficient mutants on a very low-fat, high-carbohydrate diet</td>
<td>Increased</td>
<td>2.2</td>
<td>GDS1517</td>
</tr>
<tr>
<td>Steroid receptor coactivator-2-deficient female mice</td>
<td>Increased</td>
<td>0.8</td>
<td>GDS4785</td>
</tr>
<tr>
<td>Thioredoxin reductase 1-null liver</td>
<td>Increased</td>
<td>1.1</td>
<td>GDS4928</td>
</tr>
<tr>
<td>Fasting</td>
<td>Increased</td>
<td>0.5</td>
<td>GDS4918</td>
</tr>
<tr>
<td>Fasting and LPS in male BL6/SV129 mice</td>
<td>Increased</td>
<td>5.5</td>
<td>GDS4546</td>
</tr>
<tr>
<td>Alcoholic hepatitis</td>
<td>Increased</td>
<td>0.7</td>
<td>GDS4389</td>
</tr>
<tr>
<td>Sebacic acid supplemented diet effect on db/db liver</td>
<td>Increased</td>
<td>0.7</td>
<td>GDS3807</td>
</tr>
<tr>
<td>Ketogenic diet effect on the liver</td>
<td>Increased</td>
<td>5.7</td>
<td>GDS2738</td>
</tr>
<tr>
<td>High-fat high-calorie diet effect on liver</td>
<td>Increased</td>
<td>1.3</td>
<td>GDS2413</td>
</tr>
<tr>
<td>Liver response to a high fat diet deficient in methionine and choline</td>
<td>Increased</td>
<td>5.3</td>
<td>GDS4883</td>
</tr>
<tr>
<td>Liver response to a high fat diet: time course</td>
<td>Increased 12 h</td>
<td>2.4</td>
<td>GDS4783</td>
</tr>
<tr>
<td>Western diet induced changes in liver</td>
<td>Increased</td>
<td>4.9</td>
<td>GDS279</td>
</tr>
<tr>
<td>Perfluorooctanoic acid effect on livers lacking PPAR-alpha</td>
<td>Increased</td>
<td>7.1</td>
<td>GDS3407</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor subtype activation effect</td>
<td>Increased by PPARg2</td>
<td>0.6</td>
<td>GDS1373</td>
</tr>
<tr>
<td>Description</td>
<td>Effect</td>
<td>Value</td>
<td>Accession</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------</td>
<td>--------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>Female receiving dexamethasone on liver cell</td>
<td>Increased</td>
<td>1.7</td>
<td>GDS5036</td>
</tr>
<tr>
<td>Hepatocyte nuclear factor 4 alpha depletion on hepatocellular carcinoma cell line</td>
<td>Decreased</td>
<td>-0.6</td>
<td>GDS4798</td>
</tr>
<tr>
<td>Transcriptional coactivator PGC-1beta hypomorphic mutation effect on the liver</td>
<td>Decreased</td>
<td>-1.3</td>
<td>GDS3197</td>
</tr>
<tr>
<td>RORα-deficient staggerer mice fed high fat diet</td>
<td>Decreased</td>
<td>-5.7</td>
<td>GSE23736</td>
</tr>
<tr>
<td>SIRT3 deficient liver response to a high fat diet</td>
<td>Decreased</td>
<td>-0.1</td>
<td>GDS4817</td>
</tr>
<tr>
<td>GPR120-deficient liver response to a high fat diet</td>
<td>Decreased</td>
<td>-1.3</td>
<td>GDS4830</td>
</tr>
<tr>
<td>TAK1/TR4-deficient mice</td>
<td>Decreased</td>
<td>-18</td>
<td>GSE21903</td>
</tr>
<tr>
<td>Conditional GBA1 deletion model of Type 1 Gaucher disease</td>
<td>Decreased</td>
<td>-1.4</td>
<td>GDS4162</td>
</tr>
<tr>
<td>Atherogenic diet effect on the liver: time course</td>
<td>Decreased long term</td>
<td>-4.8</td>
<td>GDS2292</td>
</tr>
<tr>
<td>Streptozotocin induced type 1 diabetes</td>
<td>Decreased</td>
<td>-0.3</td>
<td>GDS4845</td>
</tr>
<tr>
<td>Adrenalectomized liver at light and dark periods of the circadian cycle</td>
<td>Variable</td>
<td>Dark 1.0</td>
<td>Light -1.4</td>
</tr>
<tr>
<td>Sex specific transcription in somatic and reproductive tissues</td>
<td>Decreased</td>
<td>-0.6</td>
<td>GDS565</td>
</tr>
</tbody>
</table>
Fig. 1. **Effect of dietary cholesterol on hepatic steatosis and Cidec/Fsp27β expression in male Apoe-deficient mice.** Representative liver micrographs at x600 magnification from Apoe-deficient mice consuming the chow (a) and cholesterol-enriched (b) diets. Morphometric evaluation of surface of liver section occupied by fat, total cholesterol and triglyceride contents (c). Hepatic Cidec/Fsp27β expressions determined by RT-qPCR normalized to Cyclophilin B (d). Data are medians and 10-90 percentile range for control (n=7) and cholesterol (n=7) groups. Statistical analyses were done according to Mann-Whitney’s U test. a, P< 0.05 vs control. Association between hepatic cholesterol content and Cidec/Fsp27β expression (f). Open squares correspond to control and striped squares to cholesterol-fed mice. Spearman’s correlation is shown.
Fig. 2. **Effect of Western diet on hepatic steatosis and Cidec/Fsp27β expression in male Apoe-deficient mice.** Representative liver micrographs at x600 magnification from Apoe-deficient mice consuming the chow (a) and Western (b) diets. Morphometric evaluation of surface of hepatocyte occupied by fat and hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic Cidec/Fsp27β expression determined by RT-qPCR normalized to Cyclophilin B (d). Data are medians and 10-90 percentile range for chow (n=13) and Western (n=9) groups. Statistical analyses were done according to Mann-Whitney’s U test. *, P< 0.05 vs chow. Association between hepatic cholesterol content and Cidec/Fsp27β expression (e). Spearman’s correlation is shown. Open squares correspond to control and striped squares to Western-fed mice.
Fig. 3. Effect of sex on hepatic steatosis, Cidec/Fsp27β expression and CIDE/CSP27 content in lipid droplets and microsomes in Apoe-deficient mice fed on a Western diet. Representative liver micrographs at x600 magnification from male (a) and female (b) Apoe-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat and hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic Cidec/Fsp27β expression was determined by RT-qPCR normalized to Cyclophilin B (d). Data are medians and 10-90 percentile range for male (n=9) and female (n=10) groups. Relationship between hepatic triglyceride content and Cidec/Fsp27β gene expression (e). Open squares correspond to males and striped squares to females. Correlations were calculated according to Spearman’s test. FSP27 protein levels normalized to HSC70 in lipid droplets (f) and microsomes (g), inserts show representative Western blots. Statistical analyses were done according to Mann-Whitney’s U test. a, P< 0.05 vs male.
Fig. 4. Effect of ovariectomy on hepatic steatosis, *Cidec/Fsp27β* expression and
*CIDEC/FSP27* content in lipid droplets and microsomes in female *Apoe*-deficient
mice fed on a Western diet. Representative liver micrographs at x600 magnification
from mock (a) and surgically castrated (b) female *Apoe*-deficient mice consuming
Western diets. Morphometric evaluation of surface of hepatocyte occupied by fat and
hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic *Cidec/Fsp27β*
expression determined by RT-qPCR normalized to *Cyclophilin B* (d). Data are medians
and 10-90 percentile range for control (n=9) and castrated (n=9) groups. Relationship
between hepatic triglyceride content and *Cidec/Fsp27* gene expression (e). Open squares
correspond to control and striped squares to ovariectomized females. Correlations were
calculated according to Spearman’s test. FSP27 protein levels normalized to HSC70 in
lipid droplets (f) and microsomes (g), inserts show representative Western blots.
Statistical analyses were done according to Mann-Whitney’s U test. a, P< 0.05 vs control.
Fig. 5. Effect of sex and castration on hepatic Ppargc1a/Pgc1a expression in Apoe-deficient mice fed on a Western diet. Influence of sex on hepatic Pgc1a expression in Apoe-deficient mice (a). Relationship between Cidec/Fsp27β and Pgc1a gene expression levels (b). Open squares correspond to males and striped squares to females. Correlations were calculated according to Spearman’s test. Effect of orchiectomy on hepatic Pgc1a expression in male Apoe-deficient mice (c). Effect of ovariectomy on Pgc1a expression in female Apoe-deficient mice (d). Analysis of hepatic Pgc1a expression was determined by RT-qPCR normalized to Cyclophilin B. Data are medians and 10-90 percentile range for each group. Statistical analyses were done according to Mann-Whitney’s U test. *, P<0.05 vs control.
Fig. 6. Effect of sex on hepatic steatosis, Cidec/Fsp27β expression and CIDE/CSP27 content in lipid droplets and microsomes in Pgc1a-deficient mice fed on a Western diet. Representative liver micrographs at x400 magnification from male (a) and female (b) Pgc1a-deficient mice consuming Western diets. Morphometric evaluation of surface of hepatocytes occupied by fat and hepatic total cholesterol and triglyceride contents (c). Analysis of hepatic Cidec/Fsp27β expression was determined by RT-qPCR normalized to Cyclophilin B (d). Data are medians and 10-90 percentile range for male (n=8) and female (n=8) groups. FSP27 protein levels normalized to HSC70 in lipid droplets (e) and microsomes (f), inserts show representative Western blots. Statistical analyses were done according to Mann-Whitney’s U test. a, P< 0.05 vs male.
Hepatic cholesterol (mg/g) \( \rho = 0.67, P < 0.01 \)

Arbitrary units

Fsp27 expression

Hepatic cholesterol (mg/g)
**Hepatic Fat (%)**

<table>
<thead>
<tr>
<th>Control</th>
<th>Castration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>

**mg Chol/g**

<table>
<thead>
<tr>
<th>Control</th>
<th>Castration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

**mg TG/g**

<table>
<thead>
<tr>
<th>Control</th>
<th>Castration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>45</td>
<td>40</td>
</tr>
</tbody>
</table>

**Fsp27β expression**

\[ \rho = 0.76, P < 0.001 \]

**FSP27/HSC70**

<table>
<thead>
<tr>
<th>Control</th>
<th>Castration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>2.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\[ a \] indicates statistical significance.
**Males**

**Females**

---

**Pgc1a expression**

---

**Pgc1a expression**

---

**Fsp27beta**

\[ \rho = -0.792, \ P < 0.000 \]

---

**Male**

**Orchidectomized males**

---

**Female**

**Ovariectomized females**

---

**Pgc1a expression**

---