Sex Hormone-Binding Globulin Protects Against Non- Alcoholic Fatty Liver Disease

CRISTINA SAEZ-LOPEZ[1], ANNA BARBOSA-DESONGLES[1], CRISTINA HERNANDEZ[1], ROGER A DYER[2], SHEILA M INNIS[2], RAFAEL SIMÓ[1] AND DAVID M. SELVA[1]

[1]Diabetes and Metabolism Research Unit, Vall Hebron Institut de Recerca (VHIR). UAB and CIBERDEM (ISCIII), Barcelona, Spain
[2]Child and Family Research Institute, Department of Paediatrics, Faculty of Medicine, UBC, Vancouver, BC, Canada

Introduction and Aim

Non-alcoholic fatty liver disease (NAFLD) is one cause of fatty liver, occurring when fat is deposited (steatosis) in the liver not due to excessive alcohol issue. The increased prevalence of diabetes and obesity is considered to be the most common cause for NAFLD. Several studies have shown that obese subjects, type 2 diabetic patients and individuals with NAFLD have low SHBG levels, a protein produced by the liver that carries sex steroids and regulates their bioavailability at tissue level.

Apart from the increase in lipogenesis that drives fat accumulation in the liver, there are evidences supporting a central role of TNF-α in the development of NAFLD. We have previously demonstrated that an increase in both hepatic lipogenesis and TNF-α downregulated SHBG production. Therefore, lipid accumulation and low grade inflammation present in NAFLD could be a common link explaining the low circulating SHBG levels in this disease. The latter raises the intriguing question of whether low SHBG could contribute to the progression of NAFLD, rather than simply being a consequence and a surrogate biomarker.

Aim of this study is to address the importance of SHBG expression in NAFLD *in vivo*, we developed a unique mouse model by crossing the human *SHBG* transgenic mice with the C57BL/ksJ-db/db mice. The characterization of this SHBG-db/db mice allowed us to demonstrate for the first time that SHBG overexpression reduces hepatic steatosis by inhibiting lipogenesis. In addition, in a diet induced model of NAFLD using the human *SHBG* transgenic mice and their WT littermates, we demonstrated that SHBG overexpression protected against NAFLD development induced by a high fructose diet (HFrD) feeding. Moreover, to test the cell autonomous effect of SHBG on lipogenesis we performed *in vitro* experiments using the only human liver cell line (HepG2) that expresses and secretes SHBG. We analyzed the effects of under or overexpression of SHBG on cellular lipogenesis and triglyceride (TG) accumulation. Finally, studies with human liver biopsies demonstrated a negative correlation between SHBG mRNA expression and both TG content and acetyl-CoA carboxylase (ACC) mRNA expression.

Overall, our results suggest that SHBG protects against NAFLD development. More specifically, rather than being merely a biomarker, SHBG downregulation is associated with NAFLD through an increase of lipogenesis. Further research should address whether SHBG could be a new therapeutic target for preventing or arresting NAFLD.

Methods

- ***Subjects and samples.*** We recruited 36 obese subjects [body mass index median 42.27 kg/m2 of Caucasian origin who underwent bariatric surgery at the University Hospital Vall d'Hebron (UHVH). In order to have a homogeneous population, only non-diabetic subjects without evidence of metabolic disease were included. Liver biopsies were obtained using a fine needle. Informed written consent was obtained from all participants, and the study was approved by the human ethics committee from the UHVH. Liver steatosis, inflammation and ballooning were analyzed by the pathologist on the hematoxylin-eosin preparations.
- ***Animals.*** The human *SHBG* transgenic mice were backcrossed onto C57BL/ksJ-db/db background. Mice were maintained under standard conditions with food and water provided *ad libitum* and a 12h light/dark cycle. Experimental procedures were approved by the Institutional Animal Use Subcommittees of UHVH Research Institute and the UAB (45/13 CEEA).
- ***In vivo experiments.*** Male and female mice of the four genotypes (db/+, db/db, SHBG-db/+ and SHBG-db/db) n=5 each were sacrificed at 6 weeks of age and blood and tissues were collected and weighted for RNA and protein isolation. Human *SHBG* transgenic and wild-type mice (n=5) were fed during 8 weeks with a semisynthetic diet containing 20% protein, 4% soybean fat and 76% fructose. At the end of the study mice were sacrificed and blood and tissues were collected for RNA and protein isolation.
- ***Cell culture experiments.*** HepG2 cells were maintained in DMEM (10% FBS and antibiotics). HepG2 overexpressing or underexpressing SHBG were achieved by stable transfection using an SHBG expression vector (pCMV-SHBG) or a vector expressing siRNA against SHBG (pLKO.1-SHBG). An empty vector (pCMV) and a pLKO.1 containing random sequences (pLKO.1-Control) were used as controls, respectively. The pCMV and pCMV-SHBG vectors were kindly provided by Dr. Geoffrey Hammond, UBC, Canada) while pLKO.1-Control was kindly provided by Dr. Josep Villena, VHIR, Spain). All transfections were performed using Lipofectamine 2000.
- ***Histology.*** For morphological studies, 3 animals of each genotype (db/+, db/db, SHBG-db/+ and SHBG-db/db) were used. From the diet study, wild-type and human *SHBG* transgenic mice (n=3 each) were also analyzed. Livers were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Serial 5-µm thick sections were used for histological examination and stained with hematoxylin-eosin (H&E).
- ***Testosterone, estradiol and SHBG measurements.*** Total testosterone, estradiol and human SHBG plasma levels from mice were measured using an ELISA (Demeditec Diagnostics GmbH).
- ***Liver triglycerides.*** Mouse and human liver TG were measured using a triglyceride assay kit (Cat.#K622-100 BioVision Inc., CA, US) following the manufacturer's instructions.
-- ***RNA analysis.*** Total RNA was extracted from HepG2, mouse livers and human liver samples using TRIzol reagent. Reverse transcription (RT) was performed at 42 °C, for 50 min using 3 µg of total RNA and 200 U of Superscript II together with an oligo-dT primer and reagents provided by Invitrogen. An aliquot of the RT product was amplified in a 25-µl reaction using SYBRGreen with appropriate oligonucleotide primer pairs corresponding to human SHBG, ACC and 18S, and mouse ACC, FAS, ACLY and 18S. Results were analyzed using the 7000 SDS program. For microarrays analysis mRNA liver samples from db/db and SHBG-db/db mice were isolated using the RNeasy Mini Kit (Cat. No. 74104, QIAGEN) following the manufacturer instructions.
- ***Western Blot Analysis.*** HepG2 cells and mouse liver samples were homogenized in RIPA buffer with Complete™ protease inhibitor cocktail. Protein extracts were used for western blotting with antibodies against FAS, ACC, ACLY and PPIA. Specific antibody-antigen complexes were identified using the corresponding HRP-labeled rabbit anti-goat IgG, rabbit anti-mouse IgG or goat anti-rabbit IgG and chemiluminescent substrates (Millipore) by exposure to x-ray film.
- ***Microarray hybridization and analysis.*** The goal of the study was to compare hepatic gene expression patterns between db/db and SHBG-db/db mice. Microarrays were carried out using the Affymetrix microarray platform and the Genechip Mouse Gene 2.0 ST Array. The arrays were performed at the High Technology Unit of our Research Institute as described elsewhere (www.affymetrix.com). Data obtained from the microarrays were analyzed by the Statistics and Bioinformatics Unit of our research institute. All the statistical analysis was done using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org).
- ***Statistical analyses.*** Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Comparison of quantitative variables was performed by either the Student's *t* test or Mann–Whitney test according to the data distribution. All data are presented as means ± standard deviation. Spearman's correlation coefficients were used to establish the association between SHBG levels and the other parameters. For graphics a linear regression test was applied. Significance was accepted at the level of $p < 0.05$. Statistical analyses were performed with the SPSS statistical package (SPSS Inc, Chicago, Illinois).

Results

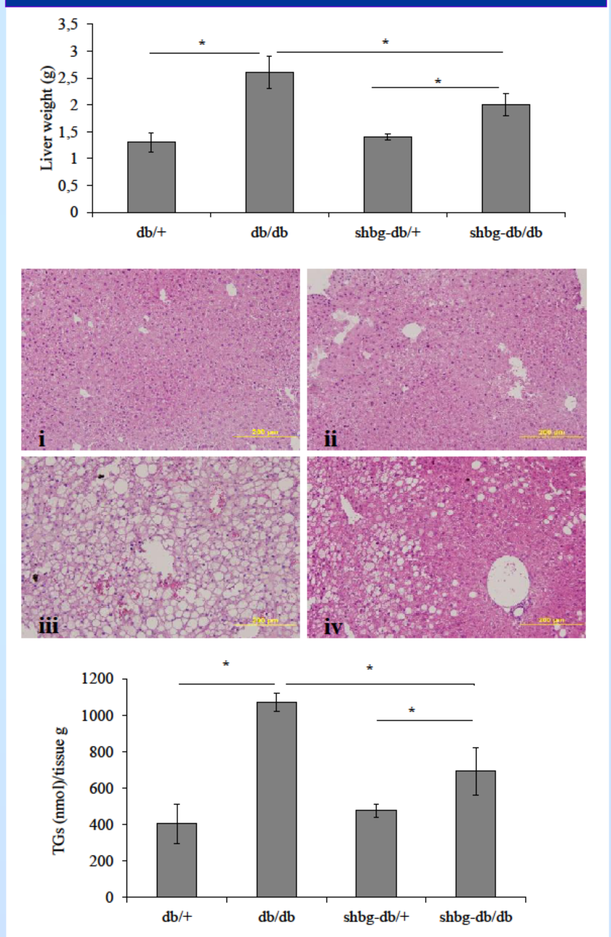
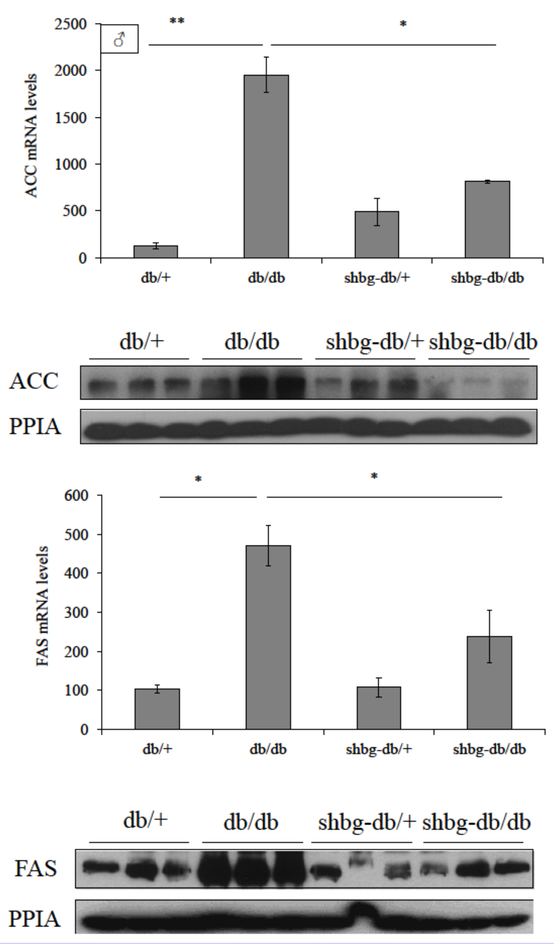


Figure 1. SHBG overexpression reduces liver weight and fat accumulation in SHBG-db/db mice.



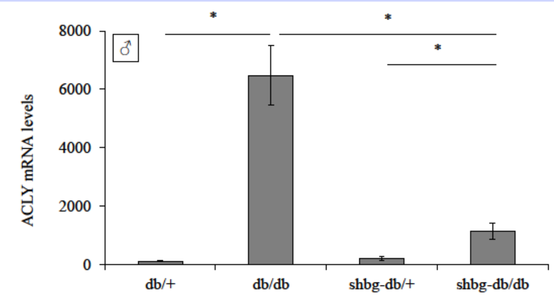


Figure 2. SHBG ameliorates hepatic steatosis by reducing mRNA and protein levels of key lipogenic enzymes (ACC, FAS and ACLY) in SHBG-db/db mice.

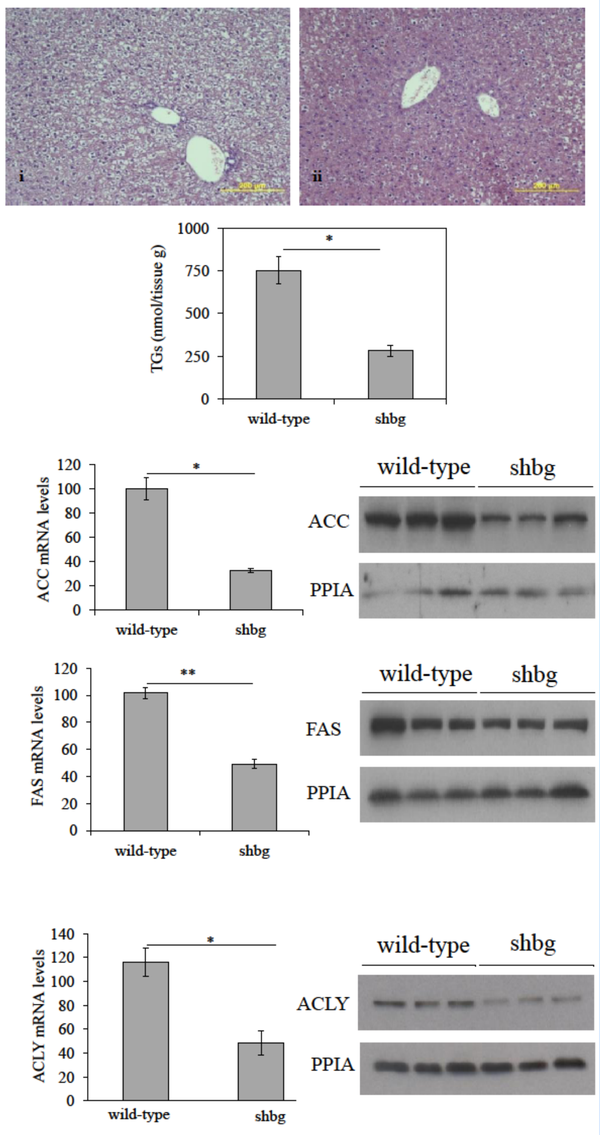
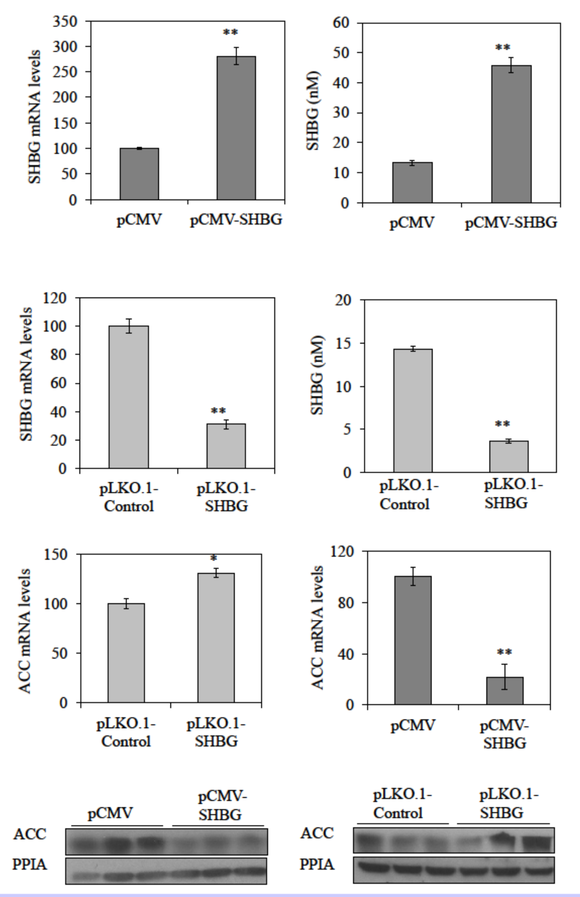


Figure 4. SHBG protects against fatty liver disease induced by high fructose diet



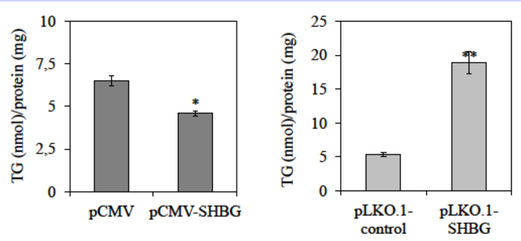


Figure 5. SHBG regulates hepatocyte lipid content by modulating ACC levels in HepG2 cells.

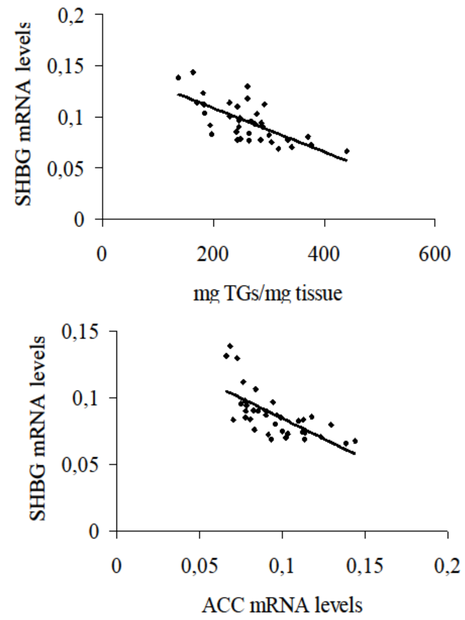


Figure 6. SHBG mRNA correlations with total TG content and ACC mRNA in 36 human liver biopsies.

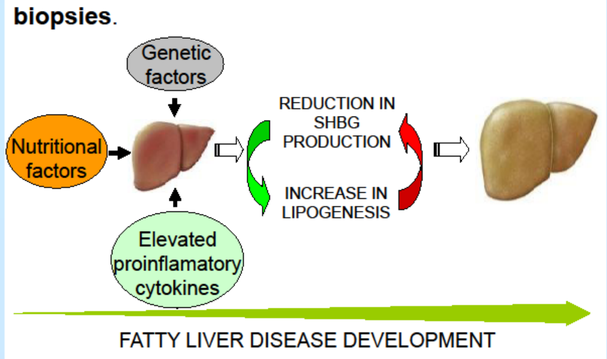


Figure 7. SHBG role in the development of NAFLD.

Conclusions

- Human SHBG overexpression protects against liver fat accumulation in a genetically-induced and a diet-induced mouse models of NAFLD.
- Human SHBG overexpression reduces hepatic fat accumulation by downregulating key lipogenic enzymes (ACC, FAS and ACLY).
- Using HepG2 cells we show a cell autonomous effect of SHBG expression levels on lipogenesis.
- SHBG mRNA levels correlates significantly with TG and ACC mRNA levels in human liver biopsies.
- Overall, our results suggest that SHBG protects against NAFLD development. Further research should address whether SHBG could be a new therapeutic target for preventing or arresting NAFLD.

Acknowledgement

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