SHBG-C57BL/ksJ-db/db: a new mouse model to study the link between SHBG regulation and obesity development

Vall d'Hebron Institut de Recerca VHIR

Instituto de Salud Carlos III

CRISTINA SÁEZ-LÓPEZ, MARTA RIVERA GIMENEZ, CRISTINA HERNÁNDEZ, RAFAEL SIMÓ AND DAVID M. SELVA

Diabetes and Metabolism Research Unit, Vall Hebron Institut de Recerca (VHIR). Universitat Autònoma de Barcelona and CIBERDEM (ISCIII), Barcelona, Spain

Introduction

Sex hormone-binding globulin (SHBG) is produced by the human liver under control of hormones and nutritional factors. The human liver secretes SHBG to the blood where it binds sex steroids with high affinity regulating their bioavailability. Low serum SHBG levels in overweight individuals are a biomarker for the metabolic syndrome and are predictive of type 2 diabetes and cardiovascular disease risk.

There are no *in vivo* models to study SHBG expression and regulation during obesity development. The main reason for this is that the obesity-prone rodent models can not be used to study this issue since rodents unlike humans do not express the *SHBG* gene in their livers; instead they express the SHBG in the Sertoli cells of the testis.

To circumvent this issue, we have taken advantage of having the human *SHBG* transgenic mice and have developed a unique mouse model that expresses the human SHBG and develops obesity, by crossing the human *SHBG* transgenic mice with the C57BL/ksJ-db/db mice.

The aim of the study has been the characterization of this new SHBG-C57BL/ksJ-db/db mouse model, which has allowed us to determine the molecular mechanisms and transcription factors involved in the downregulation of SHBG during obesity development. These mechanisms of SHBG downregulation involve changes in hepatic HNF-4α and PPARγ mRNA and protein levels. Furthermore, these results were confirmed using a small set of human liver biopsies. Importantly, we also showed that this model resembles what occurs in human obese subjects since plasma SHBG levels were reduced together with total and free testosterone levels in obese SHBG-db/db mice when compared to lean SHBG-db/+ mice. Future research includes using this unique mouse model to design strategies to increase SHBG plasma levels and to determine the role of the SHBG reduction in the development and progression of obesity.

Methods

Animals

The human *SHBG* transgenic mice were backcrossed onto C57BL/ksJ-db/db background in order to obtain mice expressing human *SHBG* and developing obesity and NAFLD. Mice were maintained under standard conditions with food (Global Diet 2018, Harlan Interfauna Iberica, Barcelona, Spain) 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 Universitat Autonoma Barcelona (45/13 CEEA).

In vivo experiments

One set of lean SHBG-db/+ and obese SHBG-db/db male mice (n=5 each) were followed up to 12 weeks of age. Total body weight and blood glucose levels were assessed every two weeks. Blood samples were taken by saphenous vein for measurements of plasma SHBG levels every two weeks. Another set of lean SHBG-db/+ and obese SHBG-db/db male and female mice (n=5 each) were sacrificed at 6 weeks of age and blood and tissues were collected and weighted for RNA and protein isolation.

SHBG and testosterone measurements

Human SHBG levels and total and free testosterone levels from mice plasma were measured using an ELISA (Demeditec Diagnostics GmbH).

RNA analysis

Total RNA was extracted from mouse livers and adipose tissue and human liver samples using TRIzol reagent (Invitrogen SA). 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 (Invitrogen SA) with appropriate oligonucleotide primer pairs corresponding to mouse HNF-4α, mouse PPARγ, mouse TNFα, mouse IL6, mouse IL1β, mouse 18S and human SHBG, human HNF-4α, human PPARγ2 and human 18S. Results were analyzed using the 7000 SDS program.

Western Blot Analysis

Mouse livers were homogenized in RIPA buffer with Complete™ protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain). Protein extracts were used for western blotting with antibodies against HNF-4α (C-19; catalog sc-6556; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), PPARγ (H-100; catalog sc-7196; Santa Cruz Biotechnology Inc.) and PPIA (SA-296; BIOMOL Int., Madrid, Spain). Specific antibody-antigen complexes were identified using the corresponding HRP-labeled rabbit anti-goat IgG or goat anti-rabbit IgG and chemiluminescent substrates (Millipore) by exposure to x-ray film.

ChIP Assays

Mouse livers were used to perform ChIP assays with a ChIP-IT kit (Active Motif Inc.) with HNF-4α, PPARγ, rabbit IgG or goat IgG. The purified DNA was subjected to PCR amplification (35 cycles) using specific primers designed to amplify a 262-bp region of the human *SHBG* promoter.

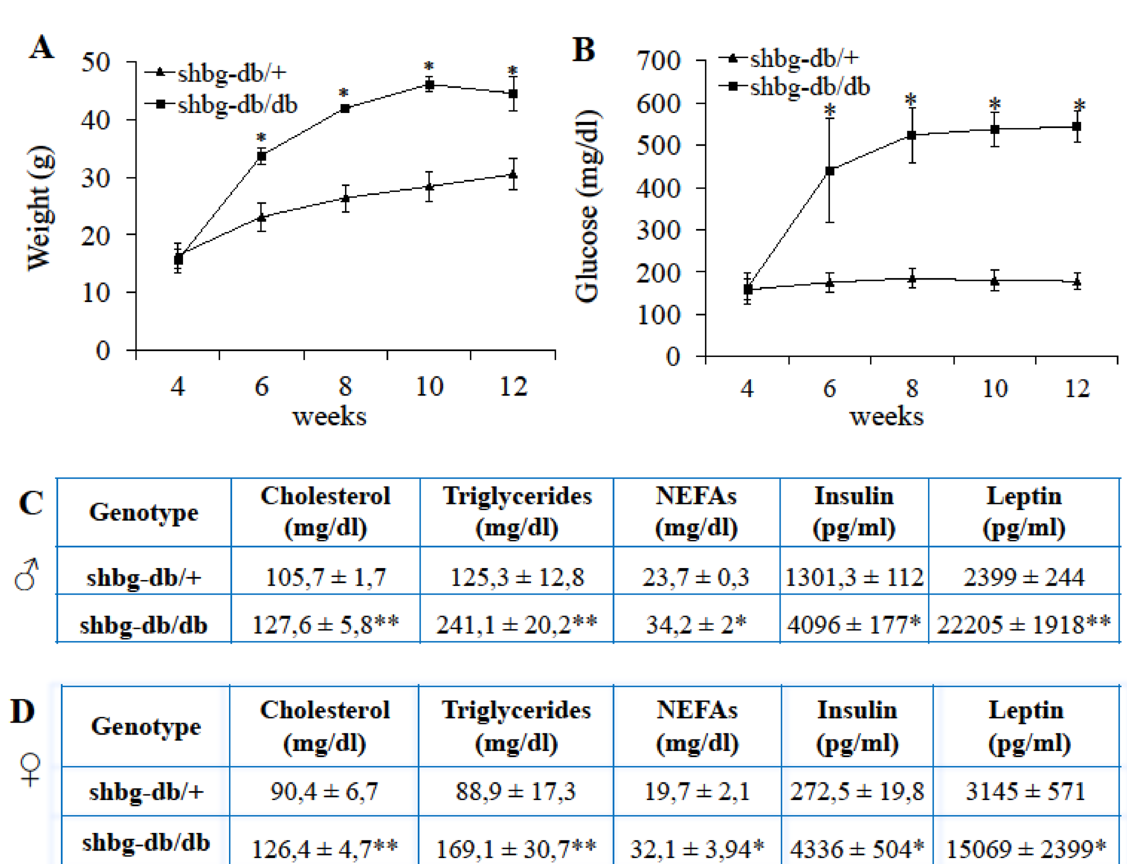
Subjects and samples

We recruited 15 obese subjects [body mass index median 42.27 kg/m2 (range 32.61–52.31 kg/m2)] of Caucasian origin who underwent bariatric surgery at the University Hospital Vall d'Hebron (UHVH) (Barcelona, Spain). None of the subjects had evidence of metabolic disease other than obesity, and patients with type 2 diabetes were excluded. Liver biopsies were obtained using a fine needle. All biopsies were at least 2 cm in length and contained at least eight portal tracts. Samples were frozen at -80°C for subsequent analysis. Informed written consent was obtained from all participants, and the study was approved by the human ethics committee from the UHVH.

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 or means ± standard error of the mean. 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.

Results



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Genotype	Cholesterol (mg/dl)	Triglycerides (mg/dl)	NEFAs (mg/dl)	Insulin (pg/ml)	Leptin (pg/ml)
shbg-db/+	105,7 ± 1,7	125,3 ± 12,8	23,7 ± 0,3	1301,3 ± 112	2399 ± 244
shbg-db/db	127,6 ± 5,8**	241,1 ± 20,2**	34,2 ± 2*	4096 ± 177*	22205 ± 1918**

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Genotype	Cholesterol (mg/dl)	Triglycerides (mg/dl)	NEFAs (mg/dl)	Insulin (pg/ml)	Leptin (pg/ml)
shbg-db/+	90,4 ± 6,7	88,9 ± 17,3	19,7 ± 2,1	272,5 ± 19,8	3145 ± 571
shbg-db/db	126,4 ± 4,7**	169,1 ± 30,7**	32,1 ± 3,94*	4336 ± 504*	15069 ± 2399*

Figure 1. Obesity development alters total body weight, glycemia and metabolic profile in obese SHBG-db/db mice when compared with lean SHBG-db/+ mice.

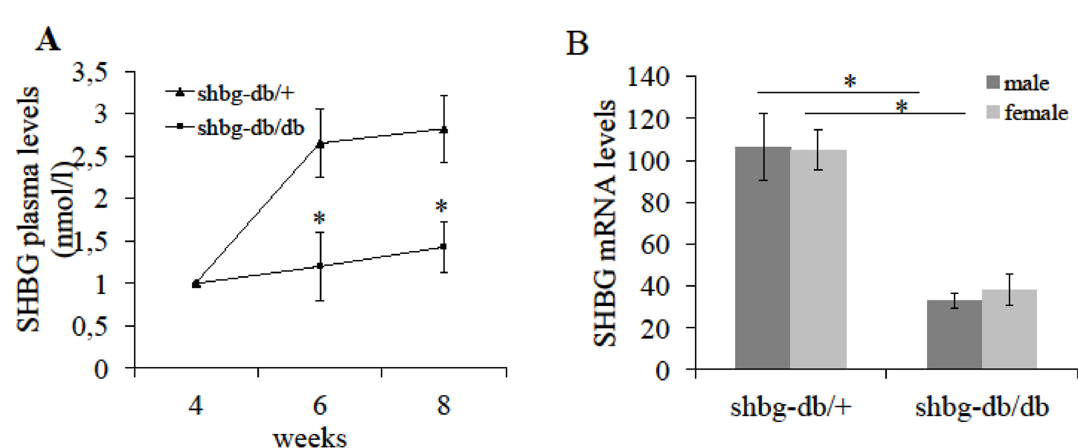

Figure 2. Hepatic SHBG production is reduced in obese SHBG-db/db mice when compared with lean SHBG-db/+ mice.

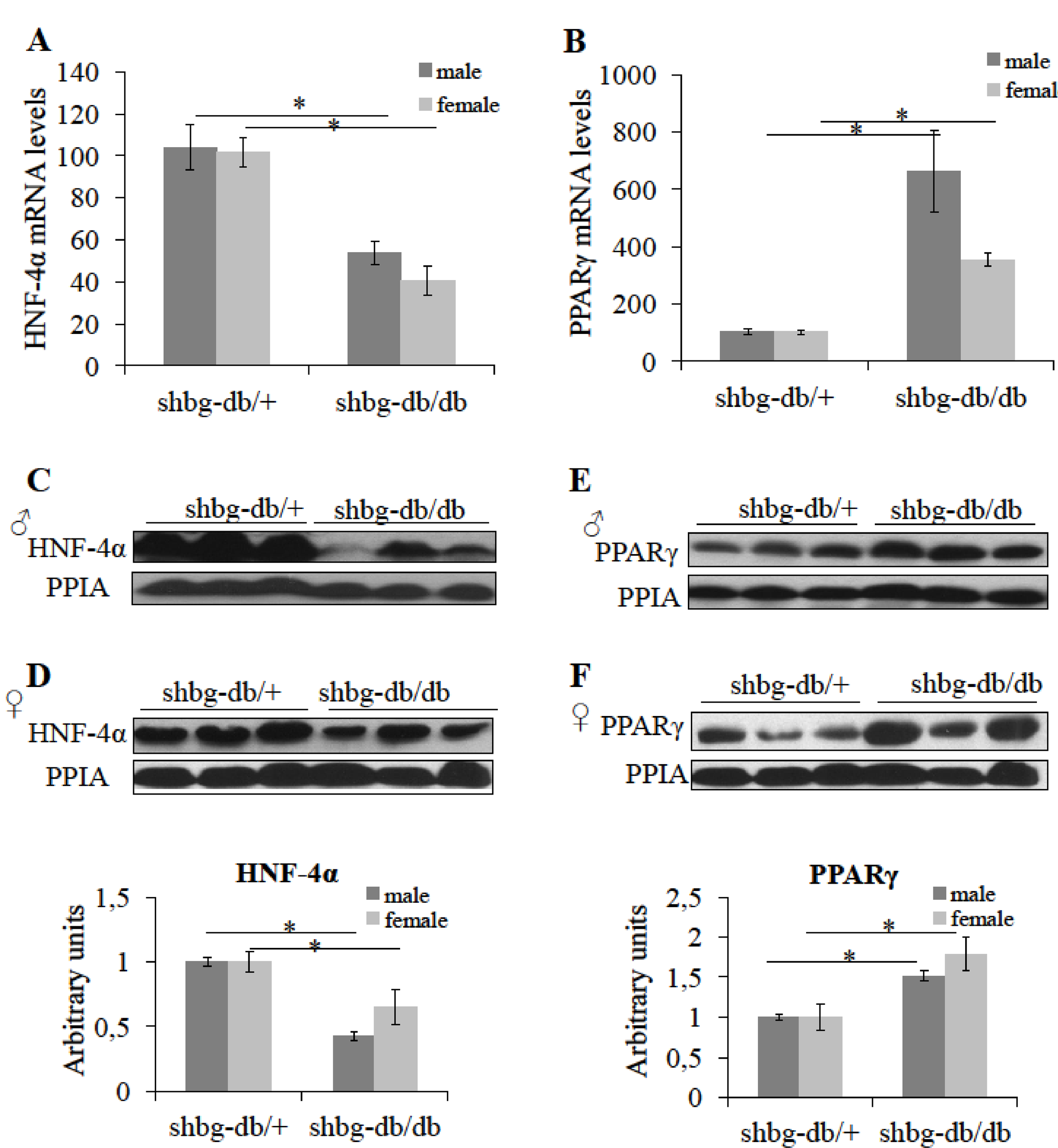

Figure 3. Hepatic SHBG production is reduced by the decrease of HNF-4α and the increase of PPARγ levels in obese SHBG-db/db transgenic mice when compared with SHBG-db/+ mice.

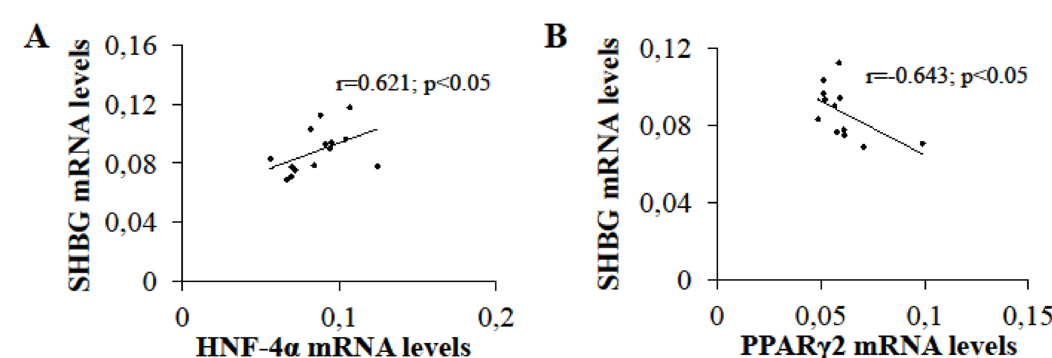

Figure 4. Correlations between SHBG mRNA levels and HNF-4α and PPARγ mRNA levels in human liver biopsies.

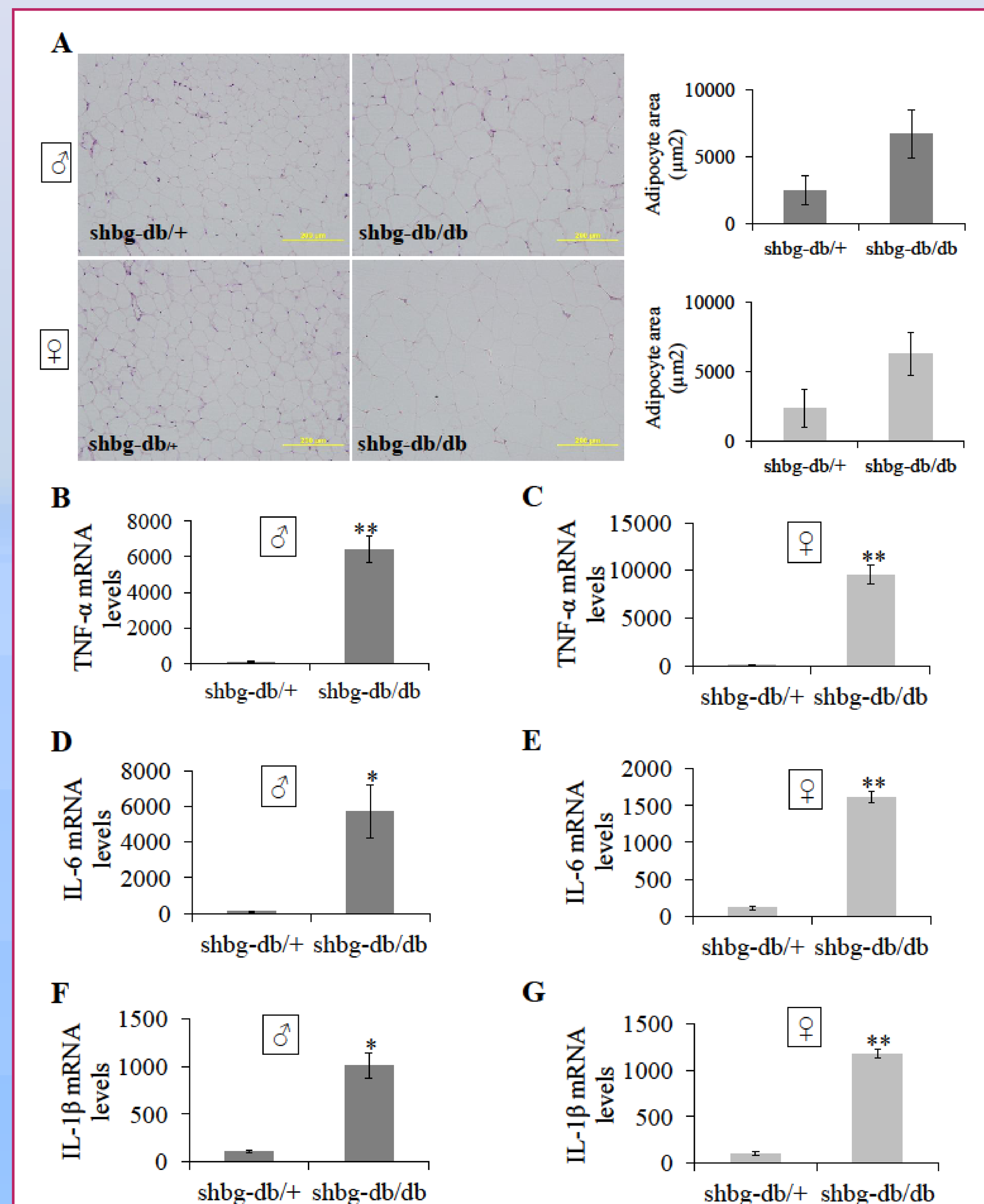

Figure 5. Obesity development increases adipocyte size and changes pro-inflammatory cytokine expression in obese SHBG-db/db transgenic mice when compared with SHBG-db/+ mice.

Genotype	Total testosterone (ng/ml)	Free testosterone (ng/ml)
shbg-db/+	42,5± 2,3	3,2± 0,75
shbg-db/db	14,6± 5,3*	0,5± 0,03*

Figure 6. Total and free testosterone levels are reduced in obese SHBG-db/db mice when compared with lean SHBG-db/+ mice.

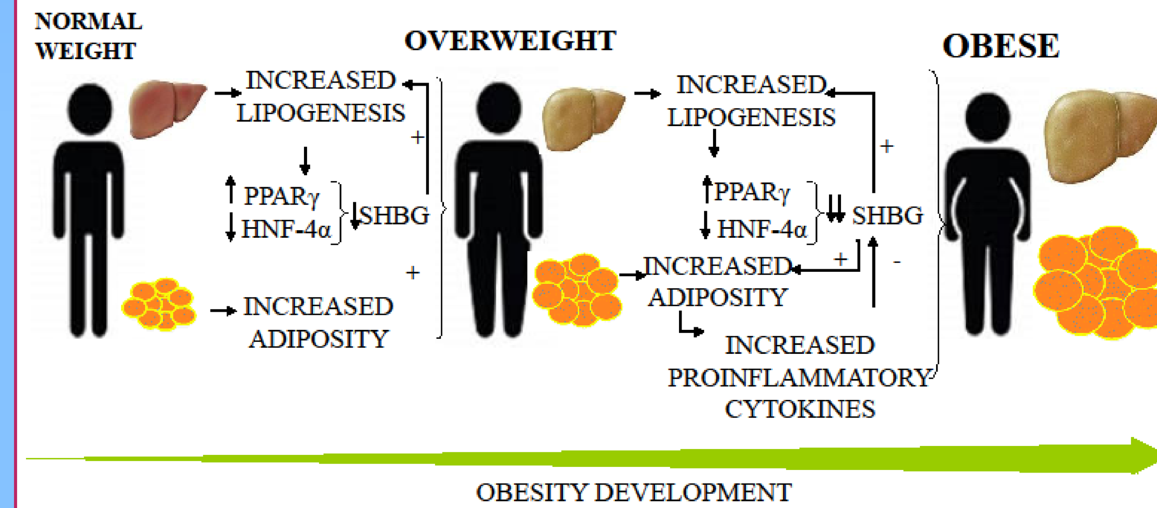

Figure 7. Diagram representing the molecular mechanisms of SHBG downregulation during obesity development.

Conclusions

➢SHBG production is downregulated in obese SHBG-db/db mice in comparison with lean SHBG-db/+ mice.

➢SHBG production is downregulated via a hepatic reduction in the levels of the transcription factor HNF-4α and upregulation of PPARγ.

➢In human liver biopsies, SHBG mRNA levels were positively correlated with HNF-4α and negatively correlated with PPARγ.

➢Cytokine production is increased in obese SHBG-db/db mice when compared to lean SHBG-db/+ mice, which may influence the low hepatic levels of HNF-4α.

We have developed a mouse model which resembles what occurs in human obesity in terms of SHBG regulation. Therefore, future research using this unique mouse model will allow us to further explore the link between SHBG, obesity and type 2 diabetes.

Acknowledgments

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