Modulation of SHBG binding to testosterone and estradiol by sex and morbid obesity

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<td>Grasa, Maria del Mar; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine; Centro de Investigación Biomédica en Red de Obesidad y Nutrición, CIBER-OBN; Gulfo, José; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Camps, Núria; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Alcalá, Rosa; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Monserrat, Laura; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Moreno-Navarrete, José María; Girona Institute of Biomedical Research and Hospital of Girona &quot;Dr. Trueta&quot;, Endocrinology and Diabetes; Centro de Investigación Biomédica en Red de Obesidad y Nutrición, CIBER-OBN; University Hospital &quot;Dr. Josep Trueta&quot;, Internal Medicine Ortega, F; Girona Institute of Biomedical Research and Hospital of Girona &quot;Dr. Trueta&quot;, Endocrinology and Diabetes; Centro de Investigación Biomédica en Red de Obesidad y Nutrición, CIBER-OBN; University Hospital &quot;Dr. Josep Trueta&quot;, Internal Medicine Esteve, Montserrat; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Remesar, Xavier; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Fernández-López, José Antonio; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine Fernández-Real, José Manuel; Girona Institute of Biomedical Research and Hospital of Girona &quot;Dr. Trueta&quot;, Endocrinology and Diabetes; Centro de Investigación Biomédica en Red de Obesidad y Nutrición, CIBER-OBN; University Hospital &quot;Dr. Josep Trueta&quot;, Internal Medicine Alemany, Marià; University of Barcelona, Faculty of Biology, Department of Biochemistry and Molecular Biomedicine</td>
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Modulation of SHBG binding to testosterone and estradiol by sex and morbid obesity

María del Mar Grasa 1,4,5, José Gulfo 1, Núria Camps 1, Rosa Alcalá 1, Laura Monserrat 1,
José María Moreno-Navarrete 2,3,5, Francisco José Ortega 2,3,5, Montserrat Esteve 1,4,5,
Xavier, Remesar 1,4,5, José Antonio Fernández-López 1,4,5, José Manuel Fernández-Real 2,3,5
and Marià Alemany 1,4,5

1Department of Biochemistry and Molecular Biomedicine; Faculty of Biology, University of Barcelona, Barcelona, Spain
2University Hospital "Dr. Josep Trueta", Girona, Spain
3Girona Institute of Biomedical Research, and Hospital of Girona "Dr. Josep Trueta", Spain
4Institute of Biomedicine, University of Barcelona, Barcelona, Spain
5CIBER Obesity and Nutrition, Barcelona/Girona, Spain

Corresponding Author:
Dr. Marià Alemany; Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona. Av. Diagonal, 643; 08028 Barcelona, Spain; tel. +34934034606; fax +34934037064; e-mail: malemany@ub.edu

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ABSTRACT

Objective: Sex hormone-binding globulin (SHBG) binds and transports testosterone and estradiol in plasma. The possibility that SHBG is a mixture of transporting proteins has been postulated. We analyzed in parallel the effects of obesity status on the levels and binding capacity of circulating SHBG and their relationship with testosterone and estradiol.

Design: Anthropometric measures and plasma were obtained from apparently healthy young (i.e. 35 ± 7 years) premenopausal women (n=32) and men (n=30), with normal weight and obesity (BMI>30 kg·m⁻²).

Methods: SHBG protein (Western blot), as well as the plasma levels of testosterone, estradiol, cortisol and insulin (ELISA) were measured. Specific binding of estradiol and testosterone to plasma SHBG was analyzed using tritium-labelled hormones.

Results: Significant differences in SHBG were observed within the obesity status and gender, with discordant patterns of change in testosterone and estradiol. In men, testosterone occupied most of the binding sites. Estrogen binding was much lower in all subjects. Lower SHBG of morbidly obese (BMI>40 kg·m⁻²) subjects affected testosterone but not estradiol. The ratio of binding sites to SHBG protein levels, was constant for testosterone, but not for estradiol. The impact of gender was maximal in morbid obesity, with men showing the highest binding/SHBG ratios.

Conclusions: The results reported here are compatible with SHBG being a mixture of at least two functionally different hormone-binding globulins, being affected by obesity and gender, and showing different structure, affinities for testosterone and estradiol, and also different immunoreactivity.
INTRODUCTION

Hormone-binding globulins are serpins, whose main role is to specifically bind and transport steroid and thyroid hormones in plasma. Sex hormone-binding globulin (SHBG) essentially binds / transports testosterone and estradiol 1. The finding of its possible use as marker of a number of metabolic 2-3 or psychological 4 disorders has contributed to enhance the study of SHBG role in the control of gene expression 5 and sex hormone levels 6, 7, as well as its more than probable implication in metabolic syndrome (MS) 8.

The function of SHBG is far from being fully understood. In fact, although it binds the hormone ligands in a high-affinity specific way, SHBG levels in plasma are low in comparison with albumin and other plasma proteins. The latter bind non-specifically these same hormones with high capacity and low affinity. The equilibrium of free hormone levels, thus, is not solely dependent on SHBG binding ability or levels, but also on its interaction with tissues and their complex and variably modulated compartmentation 9. The absence of SHBG in rodents, in contrast with CBG, present in them and in humans, may hint at additional functions for this serpin, but the presence of a specific testosterone-binding protein in rodent testicles 10, with a marked structural and gene parallelism with SHBG 11, 12 suggests that at least for this organ, a specific protein binding testosterone is required.

The high-affinity binding of sex hormones to SHBG competes with cell receptors, altering its direct availability, which prompts the question whether SHBG main role is to transport the hormones, or retain them to modulate their binding to cell membranes, including the specific binding of SHBG itself 13. This sequestering role has been postulated for tissues, where membrane-related hormone-binding globulins may act barrier-like to limit or control the access of active hormones to cells, as is the case for CBG 14. However, hormone-binding globulins are required as vector for transfer of hormones to cell ligands 15, to elicit effects on gene expression and the activation of other cell functions 16, 17.

SHBG is a glycoprotein 18, which appears in plasma as a dimer, containing two subunits, each one having a site able to bind testosterone, dihydrotestosterone or estradiol 19. The affinity for testosterone has been found to be higher than for estradiol 20 in line with the function of the precursor testosterone-binding protein of rodents compared with that of plasma and testicles in rabbits 21. In human plasma, SHBG has been found in several forms of variable molecular weight, distributed in markedly different proportions 22, 23; however, they shared the peptide size and immuno-reacting properties, albeit only partially 22, 24. The difference in size has been largely attributed to the varying proportion of oligosaccharide chains 23, 25. SHBG hormone-binding affinity can be changed by molecular modulation of the serpin structure, such as localized proteolysis 26, proteolytic cleavage resulting in increased free hormone levels because of loss of affinity of the protein to its ligand 2. Other possible modifications of SHBG structure / binding are the formation of O- or N-glycans 27, 28. An important factor of variability / regulation is the complex control 29, 30 of the expression of the single SHBG gene described 12, able to generate different products 12. In addition, a number of genetic variants, showing different hormone-binding affinities, has been described 31, 32.

Plasma concentrations of free testosterone and estradiol are related to the levels of SHBG 33. Sex affects markedly the levels 34, 35 and function of SHBG, essentially altering the levels of testosterone 56. The important modulatory effects of estradiol and testosterone, confronting (or allied to) the effects of glucocorticoids 37 and insulin 38, 39 have been clearly established to show sex-related differences 40, 41 especially under conditions of predominance of insulin resistance and glucocorticoid action as in
metabolic syndrome (MS)\textsuperscript{37, 41}. SHBG has been associated to the risk of MS in men\textsuperscript{38, 42}, independently of testosterone levels\textsuperscript{43}, whereas the relation of this syndrome with total or free testosterone is—at most—weak\textsuperscript{44, 45}. Women with MS showed increased testosterone and lower SHBG independently of menopausal status\textsuperscript{46}.

Obesity is known to lead to insulin resistance\textsuperscript{47}, increase the risk of MS\textsuperscript{48}, and it is also associated with hypercortisolism\textsuperscript{49}, hypoandrogenism\textsuperscript{42} and increased estrogens\textsuperscript{50}. In the present study we studied whether SHBG function is modified by obesity status. The key question was to determine whether the changes that SHBG experiences with obesity are a direct consequence of the existence of different molecular species of this protein.

**SUBJECTS AND METHODS**

**Study population**

A sample of 62 subjects (32 of them women) of Caucasian origin, aged between 18 and 50 y (i.e. 35 ± 7 y) and including patients at the extremes of the weight continuum [16 participants with severe obesity (BMI≥40 kg.m\textsuperscript{-2}), 19 obese subjects, and 28 age and gender-matched healthy-weight controls (BMI<25 kg.m\textsuperscript{-2})], was enrolled for hormonal and circulating assessment of target proteins. Six groups of subjects were established and studied comparatively: 1) women with normal weight: BMI<25 kg.m\textsuperscript{-2}; 2) obese women: 30≤BMI<40 kg.m\textsuperscript{-2}; 3) morbid obese women: BMI>40 kg.m\textsuperscript{-2}; 4) men with normal weight: BMI<25 kg.m\textsuperscript{-2}; 5) obese men: 30≤BMI<40 kg.m\textsuperscript{-2}; 3) morbid obese men: BMI>40 kg.m\textsuperscript{-2}. Inclusion criteria, in addition to the BMI ranges described above, were the absence of any systemic disease, and the absence of any infections in the previous month. None of the subjects was taking medication (including glucocorticoids or estrogens) or showed evidence of metabolic disease other than obesity. All subjects reported that their body weight had been stable for at least 3 months before the study. Liver disease and thyroid dysfunction were specifically excluded by biochemical workup. All women had regular menstrual cycles and were studied on days 3–8 of the cycle. All participants were in apparently good health, and free of cardiovascular pathologies, clinically defined diabetes or hypertension.

The protocol was approved by the Hospital Ethics Committee, and informed consent was obtained from each subject.

Baseline studies included a standardized questionnaire, physical examination and common laboratory tests. Height and weight were measured with the participant in light clothing and barefoot. BMI was calculated, and blood pressure was measured in the supine position, using a sphygmomanometer of appropriate cuff size; the first and fifth phases were recorded.

**Analytical procedures**

Blood samples were collected, after a 12-hour fast, into blood collection tubes with a serum separator. After 15 minutes, tubes were centrifuged at 1500xg for 10 min at room temperature. The serum glucose was measured in duplicate by a glucose oxidase/peroxidase method (Glucose Analyzer 2; Beckman Coulter, Inc., Brea, CA USA). The coefficient of variation was 1.9%. Serum insulin levels were measured in duplicate by monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The lowest limit of detection was 28 pM. The intraassay coefficient of variation was 5.2% at a concentration 70 pM and 3.4% at a 900 pM. The interassay coefficients of variation were 6.9% and 4.5%.
at 100 pM and 620 pM, respectively. Blood glycosylated hemoglobin (HbA1c) proportions were analyzed by high pressure liquid chromatography (Merck Diagnostics, Darmstadt, Germany), with coefficients of variation below 4%. The range for HbA1c in glucose-tolerant subjects was 3.8 – 5.4%. Total serum cholesterol was measured through the reaction of cholesterol esterase/oxidase/ peroxidase, using a BM/Hitachi 747 analyzer (Hitachi, Tokyo, Japan). HDL cholesterol was quantified after precipitation with polyethylene glycol at room temperature. Total serum triacylglycerols were measured through lipolysis and the analysis of glycerol evolved with glycerol-phosphate-oxidase/ peroxidase. Whole blood hemoglobin levels (containing EDTA) were determined by a routine laboratory method (Coulter Electronics, Hialeah, FL USA).

Solid-phase enzyme-linked immunosorbent assays (ELISA) based on the sandwich principle were used for the quantitative determination of plasma estradiol (kit EIA-2693) and testosterone (kit EIA-1559, both from DRG International; Marburg, Germany). Plasma cortisol was determined by microparticle enzyme immunoassay (IMX system, Abbott Laboratories, North Chicago, IL USA), with intra and interassay coefficients of variation less than 8%.

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**Western blot of SHBG**

Plasma SHBG was estimated by Western blot using monoclonal anti-human SHBG (LS-C198189; LifeSpan Biosciences, Seattle, WA USA) as primary antibody, and polyclonal against albumin (sc-46293, Santa Cruz Biotechnology, Santa Cruz, CA USA) all of them raised against representative peptides. Proteins were run by SDS-PAGE (100 g·L⁻¹ resolving gel; 40 g·L⁻¹ stacking gel). A protein MW marker scale (kaleidoscope, BioRad, Berkeley, CA USA) was included in one of the wells. Samples of 1-2 µL of plasma were used. Proteins were transferred to PVDFImmobilon (Millipore, Billerica, MA USA) membranes, which were visualized with Ponceau red. The membranes were blocked with TBST and 50 g·L⁻¹ powdered skimmed milk; then they were incubated at 4 ºC overnight with the primary antibody diluted 1/2000. The membranes were, then, washed four times and then exposed (for 1 h at room temperature), diluted 1/3000, to the secondary antibody. After four washings, chemiluminiscence was analyzed using ECL (Millipore) for 5 min. Then, densitometric measurements were done using the Total-Lab program (Nonlinear USA, Durham, NC USA). This Western blot is an adaptation of that previously developed by our group for the measurement of rat plasma CBG.

**Hormone-binding assays**

The basic procedure for hormone-binding assays was the same described by us previously for CBG. In short: samples of just-thawed plasma (10 µL) were mixed with 1 mL of PBS (phosphate buffered-saline) pH7.4 containing 1 g/L gelatin. Total binding was estimated using 0.150 mL of diluted supernatant, 0.025 mL of PBS-gelatin, and 0.025 mL of ³H-labelled estradiol (NET317: (2,4,6,7⁻³H)−17β-estradiol, Perkin-Elmer, Boston MA USA; specific activity 2.8 GBq·mmol⁻¹) or testosterone (NET370: (1,2,6,7⁻³H)-testosterone, Perkin-Elmer, specific activity 2.6 GBq·mmol⁻¹). Hormones' final concentration was 30 nM, with a specific radioactivity of 12 kBq·nmol⁻¹). Non-specific binding was estimated under the same conditions, but now adding 0.025 mL of non-labelled hormone in PBS-gelatin (final concentration 15 µM). In both cases, the tubes were incubated under gentle shaking for 20 min at 37 ºC followed by 2 h at 4 ºC. Then 0.200 mL of chilled dextran-charcoal PBS-gelatin buffer were added, and the tubes were vortexed...
and kept 10 min in ice. Finally, the tubes were centrifuged 10 min at 4 °C and 2,000xg. Aliquots of the supernatants were used to measure the radioactivity bound to protein through liquid scintillation counting.

**Statistical methods**

Statistical comparisons were done using two-way ANOVA analyses (sex, obesity), with the Statgraphics Centurion XVI program package (Statpoint Technologies, Warrenton VA USA). Correlations were determined using the Prism 5 package (Graph-Pad Software Inc, La Jolla, CA USA). Before statistical analysis, normal distribution and homogeneity of the variables were tested. Levels of statistical significance were set at P <0.050.

**RESULTS**

Table 1 shows the basic plasma anthropometric and metabolic values of the six experimental groups. As expected, increased weight was concomitant with higher triacylglycerol levels in men and women, as well as with increased glycaemia, insulin and HOMA-IR.

Table 2 presents the levels of plasma hormones and ratios between these levels. Estradiol concentrations showed no significant differences between women (in the follicular phase) and men. Morbid obesity was associated with increased estradiol levels, especially in men. As expected, testosterone showed marked sex-related differences, increasing with the severity of obesity in women and decreasing in men. No significant differences were observed regarding basal cortisol.

The ratio of testosterone to estradiol concentrations showed significant differences according to sex and obesity. Cortisol/ testosterone ratios were significantly different in obese women, but not in obese men. In contrast, the cortisol/ estradiol ratio was significantly different in obese men. The estradiol/insulin ratios were similar according to sex and obesity status; however, the testosterone/insulin ratios were clearly different for sex and BMI, being the effects of obesity a consequence of the 6-fold difference between morbidly obese men and controls.

Plasma SHBG binding capacity for estradiol and testosterone is depicted in Figure 1. Estradiol binding showed no significant effect of sex, but obesity severity resulted in significant differences, solely attributable to males, since the morbid obese had much higher and significantly different binding values than those obese or with normal weight. The pattern for testosterone binding was quite different, with significant effects of both sex and obesity, the latter attributable, again to the group of men, with morbidly obese subjects showing the lowest testosterone binding values. These differences were magnified in the estradiol vs. testosterone binding ratio. Both sex and obesity showed significant differences, with rather uniform ratios for all groups except for the morbid obese men, with ratios several-fold higher than in all other groups.

Figure 2 shows the levels of testosterone and estradiol as a proportion of SHBG binding capacity. The only significant differences observed were for sex in the case of testosterone, since in men the levels accounted for 60-80% of maximal binding capacity of SHBG against 5-20% for women. These data contrast with the rather uniform 1-3% values for estradiol levels vs. binding.

Figure 3 presents the amount of SHBG protein in plasma, determined through Western blot. Normal-weight women had almost 2.5-fold higher levels of circulating SHBG than the corresponding group of men; these differences were observed again in the morbid obese groups, but with all values
practically halved. These differences were statistically significant according to sex (lower values in men) and obesity status (decreasing values with increasing obesity).

When estradiol or testosterone binding were related to the amount of SHBG protein (Figure 4), the resulting binding / Western blot ratios showed the actual capacity to bind the hormones with respect to the amount of protein present in plasma (i.e. the total binding capacity being a correlate of the amount of functional protein present). This value is ancillary to the number (or affinity) of the binding sites per molecule of protein. Estradiol binding was markedly different among morbidly obese men. Thus, the ability to bind was a uniform correlate of the amount of SHBG available, but in morbid obese men the ability to bind estradiol was much higher than that corresponding to the protein detected. In the case of testosterone, however, no significant differences for sex or obesity were found, with closely similar ratios of binding/ SHBG protein ratios for all groups.

DISCUSSION

SHBG binds both testosterone and estradiol, but not with the same affinity \(^1, ^{53}\) and may be deeply modulated by sex \(^{34}, ^{35}\), insulin resistance \(^{38}, ^{39}\), proinflammatory cytokines \(^{30}\) and other metabolic conditions or pathologies \(^{54}, ^{55}\). The physiologically functional (circulating) protein appears as a dimer \(^19\), but the conditions used for Western blot do separate the individual monomers. The main consequence is the loss, under laboratory conditions, of possible fine regulation factors such as allosteric modulation \(^{56}\) and the discrimination of dimers and heterodimers \(^{29}\). However, the conditions used to determine the binding value (i.e. specific binding calculated after discounting unspecific binding) facilitate the direct comparison of the functionality of the SHBG binding site (there is only one per glycoprotein monomer). That is, under the testing conditions, the affinity and capacity to bind a given hormone (testosterone or estradiol) should remain unchanged. However, the binding / Western blot ratios, experimentally obtained, clearly were not. This result proves that in one of the groups of individuals (e.g. all the morbidly obese men), the SHBG molecule was structurally different.

The existence of varying molecular weight SHBG proteins \(^{28}\), and the influence of the size of the monomers on binding has been already described \(^{53}\). The changes in the SHBG molecule have been related to trimming of carbohydrate chains \(^{23}\) and / or proteolytic shortening, in part attributed to regulatory serpin proteases such as kallikrein \(^{26}\). But changes were observed also in subjects with MS \(^{31}, ^{32}, ^{57}, ^{58}\), hepatic cirrhosis \(^{54}\) or just population variability \(^{31}, ^{32}\). However, the marked change in SHBG ability to bind estradiol in the group of morbidly obese men here described is outstanding.

The binding/ Western ratio for testosterone, was similar in all groups, as expected, including the morbidly obese men who also had the lowest levels of immunoreactive SHBG. The hormones' binding ratios did not show the expected uniform pattern, strongly deviated in morbid obesity instead; however this deep change affected only men. Last, but not least, the relationship between the binding capacity of SHBG and the circulating hormone levels showed large differences for testosterone and estradiol (Figure 2), in addition to the marked difference due to sex, largely a correlate of the higher circulating testosterone levels of men. In any case, the percentage of hormone levels vs. binding in control women was in the range of 1.5 % whereas that of testosterone was fivefold higher. These values were not within the expected range of work for SHBG to exert its transporting function under normal conditions; i.e. when the maximal proportion of binding sites bound to estradiol represent only a small fraction of the maximal
binding capacity of the protein, leaving no room for regulation. Consequently, the data on estradiol-related transporting capacity of SHBG did not match the currently accepted model of dual transport of androgen and estrogen for SHBG.

The results obtained do not support the assumption that SHBG is a protein indistinctly binding testosterone and estradiol as part of its physiological function. The data presented for males, even if only ascribed to the controls and non-extreme obesity (i.e. excluding the morbidly obese men), could not justify the transporting and regulatory function of SHBG for estradiol because of the sustained low level of binding site occupancy. This non-complying situation is magnified when compared with the SHBG-testosterone relationships, since all and every one of the anomalies described for estradiol were fulfilled as expected. The phylogenetic closeness of SHBG to the testicular testosterone-binding protein \(^{10}\), and the data presented here suggest that SHBG acts, essentially, as a testosterone-binding globulin. The fact that SHBG also binds estradiol must be put in perspective \(^{59}\), especially given the marked differences in low circulating levels of estradiol compared with testosterone. Competence for a single binding site per monomer could not justify a functional role of the protein for estradiol, at least in males: Their much higher levels of testosterone than women compounded by the higher affinity of SHBG for the androgen, may result in negligible estrogen binding as regulatory mechanism \textit{in vivo} (at least in males and non-ovulating women). The critical role of estradiol for bone \(^{60}\), energy homeostasis \(^{61}\) and reproductive system in both females and males \(^{62}\) has been clearly established, requiring a finer regulation of estrogen in males, playing functions not fully coincident with those of females. The kinetics of binding of testosterone and estradiol did not match their expected function (which nevertheless is exerted successfully), especially when we consider that SHBG is also a carrier for hormones that bind to specific membrane sites to transfer their charge \(^{63}\). In addition, most studies on SHBG function were centered on testosterone \(^{64, 65}\). As a comparison, CBG is devoted to the control of cortisol (or corticosterone in rodents), but also binds progesterone and a number of other ligands \(^{66}\); nevertheless, no role of CBG in transport or control of progesterone has been definitively proven. There is thus a clear parallelism between both serpins.

The apparent increase of estradiol-binding "capacity" in morbidly obese men should be explained in terms of increased SHBG protein levels, but they were not. Furthermore, testosterone levels were unaffected, and the ratios for testosterone binding / Western blot were uniform for all men (and women) groups, a situation completely different to that of estradiol. A possible explanation of this inconsistency must lie on the existence of more than one type of SHBG protein, with at least one specifically binding estradiol, and the other to testosterone. Thus justifying the data observed in this study, despite testosterone alone responding as expected. This interpretation is coincident with previous reports that found different molecular species of SHBG binding either androgen or estradiol, but not both (in cirrhotic patients) \(^{54}\), as well as the importance and common occurrence of SHBG alleles \(^{22}\).

The extreme change in estradiol binding of morbidly obese men in comparison with the other groups could not be explained by the measured (Western) SHBG protein. But testosterone complies under the same conditions. Since estradiol levels were much lower, a plausible Estradiol-Binding Globulin should be present in the morbidly obese men plasma in lower levels than the "current" or "testosterone-prone" SHBG. This situation mimics that described for cirrhosis \(^{54}\). The "estradiol-prone" SHBG, structurally close to "testosterone-prone" SHBG should be formed by differential post-translational selective cleavage \(^{26}\) of the SHBG transcript \(^{22}\), thus preserving the similarity and similar (albeit probably not identical) immunoreactive properties \(^{24}\) of both SHBG molecular species. The protein measured in this
study had immunoreactive properties obviously dependent of the affinity and specificity of the antibody used, decanted towards one or other of the isoforms postulated, a situation parallel to that described by our group in rat CBG. Thus, in fact, we were not measuring SHBG (under the one molecule-for-all-hormones concept) but either a composite (with unknown proportions of both isoforms) or essentially one of these isoforms, that closer to bind regulate testosterone. Anyway, a change in proportions of both forms of SHBG or changes in molar specific immunoreactivity may uncover the existence of more than one molecular species of circulating SHBG. The speculative ascription of different molecular species to the roles of transport and binding of estradiol and testosterone may help in understanding the complex conundrum of SHBG in relation with the control of sex hormone transport, function and their relationships under conditions of pathological alterations of energy homoeostasis (30). Sex and age intensify the negative effects of MS, as observed in Koreans. Insulin is known to modulate the levels of testosterone through SHBG, but what about estradiol? Binding of estradiol or testosterone to SHBG monomers (and probably dimers) changes their shape, thus affecting the conditions of binding to the cell membrane SHBG receptor sites.

An additional clue to the importance of the antagonistic gender-related function of cortisol and testosterone can be directly derived from the analysis of their concentration ratios, markedly decreased with obesity in women and less affected in men. These differences are difficult to explain with the assumption of a unique SHBG regulating both testosterone and estradiol, since the cortisol / estradiol concentration ratios were affected by obesity but not by sex. A similar picture can be drawn from the ratios of both sex hormones versus insulin: no effects were observed for estradiol, but men (albeit not women) showed marked effects of obesity, depending on its severity. These data hint at a much more significant (and gender-related) implication of testosterone in obesity. This is in agreement with the sex-related incidence of the development of MS with age, but also with the known relationship of SHBG (probably the isoform controlling testosterone availability) and the incidence of MS and the development of gender-related increased cardiovascular risk. These effects may be largely independent of the protective anti-inflammatory effect of estrogen. The lack of clear discrimination of SHBGs and their separate control of estrogen and androgen, has obscured the implication of testosterone (or the lack of it) in contrast with the surge of glucocorticoids in the development of cardiovascular risk in women and old men in parallel to increased frailty. Since the fall in testosterone levels, at least in men, tend to be related to age, and this occurs in parallel to a fall in SHBG levels, we can assume that the results obtained in the present study should be extended to a larger population, with a wide age range. The important amount of data available on the dynamics of testosterone action and protein deposition in conjunction with the main anabolic hormone, insulin, countered by corticosteroids show that testosterone is a critical factor in the aging process. The discriminating role of the postulated dual SHBGs may help understand better the gender-related process of aging by establishing a clear mechanism of regulation of the plasma kinetics and transport of testosterone, but will also allow a deeper study of estrogens without interference.

In conclusion, our data point to SHBG as a globulin mainly centered in the control of testosterone levels, with estradiol regulation taking a markedly second place; or, simply, estradiol being controlled by a different SHBG variant. These data confirm the existence of different SHBG molecular species to discriminatingly bind estradiol and testosterone, previously reported in cirrhotic subjects. The sex-related differences in the relationships of binding versus levels of hormones and SHBG could not be explained by
assuming that a single dimeric protein may experience the dramatic changes in affinity for testosterone and estradiol (differently for both, in fact) observed in women and men, and the marked differential effects of obesity on the ability of the same protein to bind testosterone and estradiol (Box 1).

Thus, we postulate that SHBG protein must be present in at least two different molecular species, or isoforms, to explain its differential function for both hormones, specially affected by sex and obesity. These conclusions suggest a much needed establishment of analytical procedures to identify these physiologically distinct forms of SHBG, since the sole use of antibody-based measurements could not discriminate between them.

DECLARATION OF INTERESTS

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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AUTHOR CONTRIBUTIONS

JMFR, JMMN and FJO selected the volunteers, obtained the samples and established the basal metabolic conditions of the subjects, including insulin. RA, NC, ME, JAFL, MMG, JG and LM did the SHBG-related laboratory work. ME, JAFL, MMG, and XR supervised the laboratory work, organized the results and did the final statistical analyses. MA conceived the study and wrote the paper. All senior Authors participated in the discussion of the results and in shaping the final text. At the time this investigation took place, RA, NC and LM were undergraduate students.

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FIGURE LEGENDS

Figure 1 Estradiol and testosterone binding, as well as their ratio, for women and men of normal weight, obese and morbidly obese.

The data are the mean ± sem for each group. White: women, grey: men; WC = women of normal weight; WO = women obese; WMO = women morbidly obese; MC = men of normal weight; MO = men obese; MMO = men morbidly obese. Statistical analysis of the differences between groups: Two-way ANOVA, (data within the Figure) was carried out for the parameters “sex” (S) and “obesity” (O). NS represents P values higher than 0.050. One-way ANOVA for obesity in each sex group (data on top of the Figure) O-W factor “obesity” in women; O-M factor “obesity” in men. Post-hoc Duncan test for one-way ANOVAs (obesity): different letters over the columns indicate a significant (P<0.05) difference between them; the absence of letters reflects the absence of significant differences between the groups.

Figure 2 Relationship between the levels of estradiol or testosterone in plasma with respect to the binding ability of this same plasma, expressed as percentages. Figure conventions are the same as in Figure 1.

Figure 3 SHBG Western blot of women and men of normal weight, obese and morbidly obese. Figure conventions are the same as in Figure 1; a.u. = arbitrary units (the MC mean value was attributed the arbitrary value of 100).

Figure 4 Ratios of estradiol and testosterone binding versus the SHBG Western blot data, for women and men of normal weight, obese and morbidly obese. Figure conventions are the same as in Figure 1.

Box 1 The SHBG dual hormone-binding conundrum

Summary of the available reasons and experimental data proving the unsustainability of the SHBG dual role controlling / transporting estradiol and testosterone.

T = testosterone, E2 = estradiol
Table 1  Basic characteristics of the human subject groups used in the present study

<table>
<thead>
<tr>
<th>parameters</th>
<th>units</th>
<th>women</th>
<th>men</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>normal weight</td>
<td>obese</td>
<td>morbid obese</td>
</tr>
<tr>
<td>number of individuals</td>
<td></td>
<td>14</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>age</td>
<td>years</td>
<td>36 ± 2</td>
<td>32 ± 2</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>weight</td>
<td>kg</td>
<td>61 ± 1</td>
<td>90 ± 3</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>height</td>
<td>m</td>
<td>1.66 ± 0.01</td>
<td>1.64 ± 0.02</td>
<td>1.64 ± 0.02</td>
</tr>
<tr>
<td>BMI</td>
<td>kg/m²</td>
<td>22.2 ± 0.4</td>
<td>33.4 ± 1.0</td>
<td>45.0 ± 1.3</td>
</tr>
<tr>
<td>fasting plasma glucose</td>
<td>mM</td>
<td>4.73 ± 0.13</td>
<td>4.76 ± 0.12</td>
<td>4.97 ± 0.15</td>
</tr>
<tr>
<td>HbA1C</td>
<td>%</td>
<td>4.6 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td>1.25 ± 0.18</td>
<td>2.43 ± 0.36</td>
<td>2.44 ± 0.33</td>
</tr>
<tr>
<td>total plasma cholesterol</td>
<td>mM</td>
<td>4.53 ± 0.13</td>
<td>5.00 ± 0.29</td>
<td>5.23 ± 0.27</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>mM</td>
<td>1.51 ± 0.11</td>
<td>1.30 ± 0.08</td>
<td>1.31 ± 0.11</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>mM</td>
<td>2.69 ± 0.17</td>
<td>3.09 ± 0.24</td>
<td>3.18 ± 0.29</td>
</tr>
<tr>
<td>plasma triacylglycerols</td>
<td>mM</td>
<td>0.779 ± 0.054</td>
<td>1.37 ± 0.30</td>
<td>1.66 ± 0.19</td>
</tr>
</tbody>
</table>

The values are presented as mean ± sem for each group. Statistical analysis of the differences between groups (2-way ANOVA) was carried out for the parameters "sex", "obesity" and their interaction. NS represents P values higher than 0.050.
Table 2  Hormone levels in serum of women and men with normal weight, obese or morbidly obese.

<table>
<thead>
<tr>
<th>Plasma hormones and hormone ratios</th>
<th>Units</th>
<th>Sex</th>
<th>Normal weight</th>
<th>Obese</th>
<th>Morbid obese</th>
<th>1-way ANOVA P-obesity</th>
<th>P-sex</th>
<th>2-way ANOVA P-obesity</th>
<th>P-interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (pM)</td>
<td></td>
<td>Women</td>
<td>162 ± 29</td>
<td>173 ± 25</td>
<td>256 ± 48</td>
<td>NS</td>
<td>NS</td>
<td>0.0043</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>134 ± 16</td>
<td>124 ± 13</td>
<td>215 ± 22</td>
<td>0.0044</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (nM)</td>
<td></td>
<td>Women</td>
<td>1.19 ± 0.18</td>
<td>1.59 ± 0.23</td>
<td>2.31 ± 0.36</td>
<td>&lt;0.0001</td>
<td>0.0040</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>19.6 ± 1.4</td>
<td>17.4 ± 1.8</td>
<td>11.1 ± 1.1</td>
<td>0.0004</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol (nM)</td>
<td></td>
<td>Women</td>
<td>407 ± 38</td>
<td>374 ± 64</td>
<td>277 ± 64</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>354 ± 27</td>
<td>382 ± 34</td>
<td>333 ± 74</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td></td>
<td>Women</td>
<td>42.2 ± 5.4</td>
<td>82.5 ± 12.1</td>
<td>77.4 ± 10.9</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>41.4 ± 6.2</td>
<td>93.1 ± 17.7</td>
<td>103 ± 10</td>
<td>0.0004</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone/estradiol ratio</td>
<td></td>
<td>Women</td>
<td>6.73 ± 1.10</td>
<td>8.19 ± 0.80</td>
<td>10.3 ± 1.4</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>0.0078</td>
<td>0.0046</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>176 ± 27</td>
<td>155 ± 19</td>
<td>57.9 ± 11.5</td>
<td>0.0084</td>
<td>&lt;0.0001</td>
<td>0.0063</td>
<td>0.0045</td>
</tr>
<tr>
<td>Cortisol/testosterone ratio</td>
<td></td>
<td>Women</td>
<td>441 ± 65</td>
<td>277 ± 59</td>
<td>136 ± 37</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>19.1 ± 2.1</td>
<td>23.0 ± 2.3</td>
<td>24.2 ± 5.4</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol/estradiol ratio x10^-3</td>
<td></td>
<td>Women</td>
<td>3.23 ± 0.67</td>
<td>2.27 ± 0.53</td>
<td>1.49 ± 0.61</td>
<td>NS</td>
<td>NS</td>
<td>0.0087</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>3.16 ± 0.47</td>
<td>3.25 ± 0.40</td>
<td>1.47 ± 0.33</td>
<td>0.0280</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol/insulin ratio</td>
<td></td>
<td>Women</td>
<td>3.66 ± 0.68</td>
<td>4.43 ± 1.50</td>
<td>3.82 ± 0.88</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>4.49 ± 0.98</td>
<td>1.54 ± 0.20</td>
<td>2.26 ± 0.39</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone/insulin ratio</td>
<td></td>
<td>Women</td>
<td>31.5 ± 7.2</td>
<td>33.6 ± 12.6</td>
<td>33.6 ± 6.2</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>0.0006</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>681 ± 142</td>
<td>232 ± 32</td>
<td>112 ± 13</td>
<td>0.0020</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values are presented as mean ± sem for each group. Statistical analysis of the differences between groups (1- and 2-way ANOVA) was carried out for the parameters "sex", "obesity" and their interaction. NS represents P values higher than 0.050. Post-hoc Duncan test for 1-way anovas: different superscript letters represent significant differences between the corresponding groups.

eje@bioscientifica.com
Figure 1

Figure 1

62x15mm (300 x 300 DPI)
Figure 2 Relationship between the levels of estradiol or testosterone in plasma with respect to the binding ability of this same plasma, expressed as percentages.

Figure 2

72x26mm (300 x 300 DPI)
Figure 3 SHBG Western blot of women and men of normal weight, obese and morbidly obese

66x44mm (300 x 300 DPI)
Figure 4 Ratios of estradiol and testosterone binding versus the SHBG Western blot data, for women and men of normal weight, obese and morbidly obese

Figure 4

62x22mm (300 x 300 DPI)
The SHBG dual hormone binding conundrum

Current accepted knowledge:
A unique SHBG binds, transports and regulates the levels and availability of T and E2

Already known data that so not support the "single SHBG" current theory
- M and W show different (and varying) levels of T and E2 with similar SHBG protein concentrations
- Widely different T and E2 levels in plasma apparently do not compete or interfere on SHBG function despite using a single binding site for which they compete as ligands
- Different SHBG forms have been found in humans with structural and binding affinity differences for T and E2
- Many animals do not have SHBG, but most possess non-circulating T-binding proteins; i.e. T may be regulated independently of E2. In any case T and E2 levels are regulated in plasma despite the eventual absence of SHBG

Additional proof, presented here, against the "single SHBG" current theory
- Circulating E2 (lower than T) at most may cover 3% of the potential binding sites of concurrent plasma SHBG, which is not compatible with T occupying 65-90% of the sites. These data are not compatible with a regulation role of SHBG for E2
- Obesity affects T (but not E2) binding. This is incompatible with both hormones binding (competing) for the same SHBG binding site if the protein remains unchanged
- The ratio of hormone binding vs. protein level (Western blot) should be constant unless the protein affinity is changed (by changing the protein itself). The ratio for T is unaltered by sex and obesity, but that for E2 is affected by both

The conclusions are far reaching:
- Cannot be explained by the single type of SHBG, which brings us to assume that, at least there are two SHBGs, one regulating T and the other for E2
- Most of the methods used for the measurement of SHBG rely on their immunoreactivity, but they may detect/ measure a T- or E2-prone SHBG molecular species of a mixture of both in different proportions
- Consequently, there are no reliable methods for SHBG measurement available, in the sense that SHBG levels could not be related to a specific protein carrying out a specific function. This, largely invalidates many studies on SHBG function
- Specific discriminative methods for the SHBGs may help clarify the intuited role of estrogens and androgens on metabolic syndrome-related pathologic states.