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Sex hormone-binding globulin gene expression in the liver: drugs and the metabolic syndrome

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## Abstract

Sex hormone-binding globulin (SHBG) is the main transport binding protein for sex steroid hormones in plasma and regulates their accessibility to target cells. Plasma SHBG is secreted by the liver under the control of hormones and nutritional factors. In the human hepatoma cell line (HepG2), thyroid and estrogenic hormones, and a variety of drugs including the antioestrogen tamoxifen, the phytoestrogen, genistein and mitotane (Op'DDD) increase SHBG production and *SHBG* gene promoter activity. In contrast, monosaccharides (glucose or fructose) effectively decrease *SHBG* expression by inducing lipogenesis, which reduces hepatic HNF-4 $\alpha$  levels, a transcription factor that play a critical role in controlling the *SHBG* promoter. Interestingly, diminishing hepatic lipogenesis and free fatty acid liver biosynthesis also appear to be associated with the positive effects of thyroid hormones and PPAR $\gamma$  antagonists on *SHBG* expression. This mechanism provides a biological explanation for why SHBG is a sensitive biomarker of insulin resistance and the metabolic syndrome, and why low plasma SHBG levels are a risk factor for developing hyperglycemia and type 2 diabetes, especially in women. These important advances in our knowledge of the regulation of *SHBG* expression in the liver open new approaches for identifying and preventing metabolic disorder associated diseases early in life.

## Introduction

Plasma sex hormone-binding globulin (SHBG) binds testosterone and estradiol with high affinity, and selectively transports sex steroid hormones in human plasma (Westphal, 1986, Siiteri et al., 1982, Hammond, 1990). Circulating SHBG is a major determinant of the metabolic clearance of sex steroid hormones, and it modulates their access to target tissues. For this reason, measuring plasma SHBG levels provides an estimate of the amounts of circulating non-protein-bound, or “free,” sex steroids in the blood (Selby, 1990). Alternatively, SHBG is an integral part of a membrane based steroid signaling pathway in certain responsive tissues (Hryb et al., 1990), and interactions with plasma membrane proteins, such as megalin, may facilitate the intracellular uptake of SHBG in complex with its steroid ligands (Hammes et al., 2005).

Hepatocytes are the primary site of plasma SHBG biosynthesis (Khan et al., 1981), and changes in the blood levels of SHBG are influenced by hormonal, as well as metabolic and nutritional status (Anderson, 1974, Rosner et al., 1984, Pugeat et al., 1995). Circulating SHBG is a homodimeric plasma glycoprotein with a single steroid-binding site within each subunit. The mechanism of steroid ligand interactions with the two potential steroid-binding sites of the SHBG homodimer (Grishkovskaya et al., 2000), and the identification of the structural domains that directly or indirectly account for the steroid-binding specificity of human SHBG have been resolved at the atomic level by crystal structure analysis (Grishkovskaya et al., 2002), although some questions remain (see review by Hammond et al. this issue).

## *SHBG gene*

The gene (*SHBG*) encoding human SHBG is located in the p12-p13 region on the short arm of chromosome 17 (Berube et al., 1993). The eight exons encoding secreted SHBG span 3.2 kb of genomic DNA (Hammond et al., 1989). Transcripts containing the exon 1 sequence encoding the

signal for secretion are expressed primarily in liver, and have also been found in the kidneys of transgenic mice expressing human *SHBG* transgenes. Although an SHBG cDNA was initially cloned from a human liver cDNA library, several testis specific transcripts have also been identified (Hammond et al., 1989, Gershagen et al., 1989). These testicular cDNAs contain a unique 5' sequence corresponding to a genomic SHBG sequence found approximately 1.9 kb upstream of the translation initiation codon for the SHBG precursor polypeptide.

#### *SHBG proximal promoter*

Mice expressing a 4.3 kb human *SHBG* transgene direct expression to the liver and kidney (Janne et al, 1998). These experiments indicated that approximately 850 bp of promoter sequence located upstream of exon 1 contains sufficient information to direct expression to the fetal and adult liver and kidney. This region of the human *SHBG* proximal promoter has been characterized in some detail in terms of transcription factor binding sites and functional studies., and DNaseI footprinting, EMSA analysis and reporter gene assays (Janne and Hammond, 1998; Hogeveen et al., 2001) lead to the identification of key transcriptional elements in the promoter. Notably, the liver-specific *SHBG* promoter lacks a traditional TATAA box, and a TA-rich HNF-4/COUP-TF transcription factor binding site close to the transcription start site appears to replace TFIID in recruiting the transcriptional machinery, and is essential for *SHBG* expression in the liver (Janne and Hammond, 1998). Functional antagonism between HNF-4 and COUP-TF at this site appears to act as a sensor of metabolic and nutritional status, and recent studies have shown that monosaccharide-induced lipogenesis inhibits hepatic *SHBG* expression by reducing cellular HNF-4 levels, allowing a non-permissive occupation of COUP-TF at this site (Selva et al.; 2007).

Although another upstream HNF-4 binding site has little effect on basal transcription from the SHBG promoter, Selva and Hammond (2009b) have shown that this site also functions as a PPAR-response element (see below).

An Upstream Stimulatory Factor (USF-1/2) binding site present in the human *SHBG* promoter is lacking in the *SHBG* promoters of subprimate species and has recently been shown to account for phylogenetic differences in testicular *SHBG* expression between rodents and humans (Selva et al, 2005b). This site has little effect on hepatic *SHBG* expression, as transgenic mice expressing a human *SHBG* transgene lacking this USF binding site, maintain a high level of *SHBG* expression in the liver (Selva et al, 2005b). Interestingly, whereas the wild type 4.3 kb *SHBG* transgene is not expressed in the testis, the deletion of this USF1/2 binding site permitted the expression of *SHBG* in the testes of transgenic mice harboring the modified *SHBG* transgene (Selva et al., 2005b). Furthermore, Sertoli cell expression of the modified human *SHBG* transgene is responsive to FSH and retinoic acid which stimulate its expression. Thus, it appears that USF acts as a transcriptional repressor in Sertoli cells, and the presence or absence of its binding site in the *SHBG* proximal promoter therefore accounts for why *SHBG* expression in Sertoli cells differs between species.

Apart from this obvious difference in *SHBG* promoter sequences between species, the human and rodent *SHBG* promoters show a remarkable degree of sequence conservation, which only begins to diverge at the boundary of several *alu* sequences located at -732 bp from the human *SHBG* transcription start site (Janne and Hammond, 1998). A (TAAAA)<sub>n</sub> polymorphism within an *alu* sequence within the 5' region of the human *SHBG* promoter has been identified (Hogeveen et al, 2001). The number of (TAAAA)<sub>n</sub> repeats is highly variable between individuals and ranges between 6 and 11 repeats, and several studies have described a correlation between the number of repeats and the concentration of SHBG in the blood (Cousin et al., 2004).

Moreover, this polymorphism appears to have physiological significance as the number of (TAAAA)<sub>n</sub> repeats has been associated with the polycystic ovarian syndrome (Xita et al., 2003), age of menarchy (Xita et al., 2005), and bone density in elderly men (Eriksson et al., 2006). Mechanistically, Hogeveen et al (2001) have demonstrated that the transcriptional activity of the *SHBG* promoter is dependent on the number of (TAAAA)<sub>n</sub> repeats. The authors further demonstrate binding of a 46 kd nuclear factor to this sequence, and this binding is dependent on the number of (TAAAA)<sub>n</sub> repeats (Hogeveen et al., 2001).

#### *Alternative transcripts and promoters*

Human *SHBG* transcripts containing an alternative exon 1 sequence are expressed in the human testis, as well as in the testis of transgenic mice expressing an 11 kb human *SHBG* transgene. In this testis enriched alternatively spliced transcripts comprise an alternative exon 1 sequence that replaces the exon 1 sequence found in liver and kidney *SHBG* mRNA, and in humans this alternative transcript is expressed specifically in the germ cells of the testis (Selva et al, 2005a, Selva et al, 2005b). The alternative exon 1 sequence contains no apparent initiating methionine residue, and is generally believed to be non-coding. The protein product resulting from these transcripts has a different size than plasma *SHBG* and accumulates in the acrosome of spermatids and mature sperm (Selva et al., 2005a). This sperm *SHBG* isoform is capable of binding sex steroids, and its concentration in spermatozoa is correlates with sperm motility and declines with age (Selva et al., 2005a). Very little is known about the regulation of transcription from the alternative upstream promoter that controls human *SHBG* expression in male germ cells, but the testis-specific alternative human *SHBG* transcripts accumulate in a spermatogenic stage dependent manner in the testis of transgenic mice expressing a 11 kb human *SHBG* transgene (Janne et al., 1998). As reported for the rat *shbg* gene (Joseph et al 1994), many other

alternatively spliced human SHBG transcripts exist and a novel alternative exon 1 sequence, located in the nearby *FXR2* gene has recently been reported (Nakhla et al., 2009). However, it remains questionable as to whether any of these transcripts encode a functional protein.

Figure 1 illustrates the human *SHBG* promoter with the known *cis*-elements, their relative positions and their actual sequences.

### *Regulation of SHBG expression in the liver by drugs*

#### *In vivo regulation*

Variations of plasma SHBG levels in pathophysiologic conditions, as well as its difference between sexes, led to the hypothesis that SHBG production can be stimulated by estrogens and repressed by androgens. The clinical arguments supporting this hypothesis are multiple :i) the induction of ovulation by hCG, leads to a strong increase in estradiol levels and is linked with high SHBG concentrations (Odlind et al., 1982); ii) SHBG levels are increased by oral contraceptives and menopause treatments involving the use of ethinylestradiol (van Kammen et al., 1975, Pugeat et al., 1988); iii) SHBG levels are increased under tamoxifen treatment (Boccardo et al., 1984) and can be attributed to the partial estrogenic properties of tamoxifen in the liver; and iv) the administration of drugs that have estrogenic properties, such as mitotane (Op'DDD), which is the only adrenal-specific drug available for treatment of adrenal carcinoma, is associated with increased plasma SHBG levels and the manifestation of gynecomastia in male patients undergoing mitotane treatment (Nader et al., 2006, van Seters and Moolenaar, 1991). In contrast, treatment of patients with Danazol, a drug that binds SHBG with high affinity (Pugeat et al. 1981) and which is thought to have a moderate androgenic activity, leads to a significant decrease in SHBG concentrations (Gershagen et al., 1984, Ruokonen et al., 1985).

Although it would therefore appear that that compounds with estrogenic activities increase plasma SHBG levels, while the androgenic ones decrease them, little is known about the exact molecular mechanisms by which they exert these effects. However, Nader et al. (2006) showed that Mitotane increased the expression/secretion of SHBG by mechanism(s) requiring the presence of the estrogen receptor ( $ER\alpha$ ) in HepG2 cells (Nader et al., 2006), which suggests that at least part of the response of SHBG levels to estrogenic compounds may be mediated by  $ER\alpha$ .

Finally, some but not all studies have found a decrease of SHBG levels under treatment with synthetic glucocorticoid, such as dexamethasone, in patients with polycystic ovary syndrome (PCOS) (Blake et al., 1988) and in children with acute lymphoblastic leukaemia (Wallace et al., 2003)

#### *In vitro* regulation of SHBG expression in the liver

Since the biosynthesis of SHBG takes place in the liver, the human hepatoma cell line HepG2 has been classically used to study the expression/secretion and hormonal regulation of this protein. Many *in vitro* studies have tested the effect of sex steroids (estradiol and testosterone) and glucocorticoids on *SHBG* expression and or the secretion of SHBG, but the results have been inconsistent. The secretion of SHBG was found to be stimulated by high levels of estradiol, dexamethasone (Mercier-Bodard and Baulieu, 1986) and cortisol (Loukovaara et al., 1995), with a slight increase of SHBG mRNA expression upon estradiol treatment, whereas DHT treatment was accompanied by a slight decrease in SHBG protein expression (Mercier-Bodard et al., 1991). Other studies showed a stimulation of SHBG secretion by estradiol and testosterone (Lee et al., 1987, Plymate et al., 1988a). In contrast some groups failed to show any effect of these drugs on *SHBG* expression/secretion (Rosner et al., 1984, Payne et al., 1997) or found a negative effect of

cortisol on SHBG secretion (Lee et al., 1987, Wang et al., 1988). High levels of estradiol and testosterone were also found to increase SHBG levels inside the cells, but not its release into the culture medium (Loukovaara et al., 1995, Kalme et al., 1999). In one of the most rigorous study of this kind (Edmunds et al., 1990) physiological concentrations of steroids were used and it was reported that estradiol stimulates the secretion of SHBG in a dose dependant manner, whereas testosterone has a biphasic effect characterized by an inhibition of SHBG secretion at low doses, and an absence of effect at more elevated ones.

In agreement with the clinical data observed during thyroid pathologies, most *in vitro* studies have shown that triiodothyronine (T3) stimulates the expression/secretion of SHBG in HepG2 cells (Mercier-Bodard et al., 1987, Raggatt et al., 1992, Loukovaara et al., 1995) with a dose dependant effect of T3 on SHBG mRNA expression. An additive effect of T3 and estrogenic compounds on SHBG secretion was also noted (Mercier-Bodard and Baulieu, 1986, Mercier-Bodard et al., 1987). Interestingly, the *SHBG* promoter does not contain a thyroid hormone response element, and thyroid hormones appear to control human *SHBG* gene expression via an indirect mechanism, as discussed below.

Several other hormones such as insulin, insulin-like growth factor I (IGF-I), epidermal growth factor and prolactin (Mercier-Bodard et al., 1991, Plymate et al., 1988a, Singh et al., 1990, Crave et al., 1995, Loukovaara et al., 1995, Plymate et al., 1988b) all seem to repress the production of SHBG by HepG2 cells by as yet undefined mechanisms.

#### *Drug interactions with SHBG steroid-binding sites*

We have studied the ability of some drugs to bind both SHBG and corticosteroid-binding globulin (CBG). Using computer simulation we predicted that therapeutic levels of danazol,

methyltestosterone, fluoxymesterone, and norgestrel could significantly displace testosterone from its SHBG binding site (Pugeat et al., 1981). These results indicate that binding to steroid transport proteins should be considered among the *in vivo* effects of drugs on endogenous steroid hormone levels.

An interesting application of studies of SHBG-drug interactions has been shown by Miguel-Queralt and Hammond (2008) who reported that SHBG controls the flux of natural or synthetic sex steroids across zebrafish gills, and that this highly specialized functions of SHBG can be disrupted by xenobiotic ligands of SHBG such as ethinyl-estradiol and perhaps others like bisphenol A (Déchaud et al. 1998), and this has prompted the development of *in silico* screening methods for the identification of synthetic chemicals ligands that may act as SHBG ligands (Thorsteinson et al., 2009). It is therefore possible that SHBG could influence the uptake and accumulation of xenobiotic ligands that are present and abundant in water systems, and which could act as potential endocrine disruptors through binding protein interactions in other species, including humans (Déchaud et al. 1998).

#### *Effects of insulin and monosaccharides*

Body mass index is a major determinant of SHBG concentrations in the blood of in men and women (De Moor et al., 1970; Kopelman et al. 1980). Low SHBG levels are considered to be a biomarker for the metabolic syndrome in obese patients (Laaksonen et al. 2004; Kalme et al., 2005; Tchernof et al. 1999), and are predictive of type 2 diabetes (Lindstedt, et al. 1991; Haffner, et al. 1993; Bonnet, et al. 2009) and cardiovascular disease risk (Lapidus et al. 1986; Haffner et al. 1989; Sutton-Tyrrell et al. 2005). Conversely, children with Kwashiorkor under-nutrition (Pascal et al. 2002) and women with anorexia nervosa have significantly increased SHBG levels

(Estour et al, 1986), which represent an index of nutritional status in eating disorders (Barbe et al. 1993).

The reasons as to why plasma SHBG levels are invariably low in obese individuals of all ages and sex has been the subject of considerable debate. Although glycosylation influences the plasma clearance of SHBG (Cousin et al. 1999), there is no evidence that variations in SHBG glycosylation may contribute to low SHBG in obese humans. Interestingly, however, current evidence suggests a mechanism involving reduced SHBG production by hepatocytes in response to metabolic disorders. Early data showed that plasma SHBG levels are negatively correlated with glucose, as well as insulin (Haffner et al. 1988; Pugeat et al. 1991) and these correlations were repeatedly confirmed in literature. Although an early study reported that insulin reduces SHBG production by human HepG2 hepatoblastoma cells (Plymate et al. 1988a), a subsequent study suggested that this effect is not specific to SHBG, and may be attributed to a global reduction in protein secretion (Crave et al, 1995). The possibility that expression of the human *SHBG* gene in the liver responds to an increased exposure to monosaccharides, rather than to insulin has been recently explored. Using transgenic mice expressing human *SHBG* transgenes and HepG2 human hepatoblastoma cells, Selva et al (2007) reported that glucose and fructose reduce human SHBG production by hepatocytes, *via* a down-regulation of hepatic HNF-4 levels in concert with parallel increases in cellular palmitate levels. Moreover, inhibition of lipogenesis prevented monosaccharide-induced down-regulation of HNF-4 and reduced the concomitant down-regulation of *SHBG* expression in HepG2 cells. These unexpected findings provide insight to how plasma SHBG levels are linked to metabolic disturbances associated with excess sugar consumption (Figure2).

*Effect of thyroid hormones*

Thyroid hormones influence plasma SHBG levels under both normal and pathological conditions (Anderson 1974). The early increase in SHBG levels within the first 2-3 weeks after birth has been attributed to the postnatal maturation of hepatic sensitivity to thyroid hormones (Leger et al. 1990). In addition, plasma SHBG levels are commonly elevated beyond normal reference ranges in patients with hyperthyroidism (Coddaccionni et al. 1988), but are not increased in individuals suffering from familial thyroid hormone resistance, despite their very high blood levels of thyroid hormones (Sarne et al. 1988). Since the latter is likely due to defects in thyroid hormone action in the liver, it has been proposed that plasma SHBG measurements can be used clinically to assess end-organ sensitivity to thyroid hormones (Coddaccionni et al. 1988).

Studies *In vitro* have shown that SHBG production is enhanced by thyroid hormone treatments in concert with an increase in SHBG mRNA levels (Rosner et al. 1984; Raggatt et al. 1992; Mercier-Bodard et al. 1991). However, as mentioned above, the human *SHBG* promoter lacks a classical thyroid hormone response element thyroid. Recently studies in HepG2 cells (Selva and Hammond, 2009a) have revealed that thyroid hormone regulates *SHBG* expression indirectly through *via* thyroid hormone-mediated changes in hepatic lipogenesis and HNF-4 levels. Furthermore, in transgenic mice expressing human *SHBG* transgenes, 5 days of T3 treatment serum increased SHBG levels with a concurrent increase in hepatic HNF-4 and a decrease in cellular palmitate levels.

#### *SHBG regulation by PPAR $\gamma$*

It has been well documented that treatment with the PPAR $\gamma$  agonist, rosiglitazone, increases plasma SHBG levels, notably in patients with the polycystic ovary syndrome (Azziz et al. 2001). The human *SHBG* promoter contains a PPAR-response element (PPAR-RE). Surprisingly,

treatment of HepG2 cells with rosiglitazone reduces SHBG production and *SHBG* promoter activity by 20–25%, whereas the PPAR $\gamma$  antagonist, GW9662, increased both by 2- 3 fold (Selva and Hammond, 2009b). Moreover, these studies demonstrated that a common genetic variant of PPAR $\gamma$ -2 (Pro12 variant), which is transcriptionally more active than the PPAR $\gamma$ -2 Ala12 variant, is much more effective in repressing *SHBG* expression in HepG2 cells. This not only confirmed that PPAR $\gamma$  represses SHBG expression in liver cells, but also explains why the PPAR $\gamma$ -2 Ala12 variant influence SHBG levels in normal men ( Mousavinasab F et al, 2006).

*SHBG: association with the metabolic syndrome and a risk factor for developing diabetes*

Numerous cross-sectional studies have suggested that SHBG concentrations are inversely associated with insulin-resistance, and in turn, with the risk of type 2 diabetes (Pugeat et al. 1991). In women with polycystic ovary syndrome, low SHBG levels are negatively correlated with body mass index and waist to hip ratio, and are associated with insulin resistance, as shown by glucose clamp studies in patients with the polycystic ovary syndrome (Ducluzeau et al. 2003). Because SHBG concentrations differ between men and women, the association between SHBG and incidence of diabetes may also differ. The relationship between low SHBG and the risk of type 2 diabetes has been reported to be stronger in women than in men (Lindstedt et al. 1991; Haffner et al. 1993). Only a few studies have reported risk estimates separately for men and women. In following a cohort of subjects who were normoglycemic at baseline but hyperglycemic at 3 years (glycemia  $\geq$  6.1 mmol/l or type 2 diabetes) and matched for sex, age, and BMI with control subjects, who remain normoglycemic at 3 years, we found that serum SHBG levels were significantly associated with the risk of developing hyperglycemia among women but not in men. The predictive value of a low SHBG concentration in women also

appears to be independent of physical activity, BMI, obesity status or insulin and indexes of insulin resistance. Moreover, SHBG levels were significantly associated with the risk of diabetes after 9 years in women (Bonnet et al, 2009). This important observation has been confirmed by Ding et al. (2009) who reported by a nested case–control study of postmenopausal women in the Women’s Health Study and in an independent cohort of men from the Physicians’ Health Study II that low circulating levels of SHBG are a strong predictor of the risk of type 2 diabetes in women and men..

#### Concluding remarks

From these recent advances in our knowledge of SHBG regulation, it is expected that alteration in circulating level of SHBG could be an attractive tool for identifying metabolic disorder-associated diseases early in life, and contribute to the development of new treatment strategies. In this regard, it is well documented that metabolic deregulation in daughters of women with polycystic ovary syndrome, occurs early during pubertal development in concert with low serum SHBG levels (Sir-Petermann et al., 2007). It remains to be determined whether a low SHBG in these children is predictive for the risk of developing metabolic disorders.

The unanticipated down regulation of hepatic *SHBG* expression by excessive monosaccharide exposures *in vitro*, should be confirmed *in vivo* in humans. The recent study by Stanhope et al. (2009) showing that consumption of fructose-sweetened but not glucose-sweetened beverages for 10 weeks increases *de novo* lipid synthesis and impairs insulin sensitivity in overweight or obese adults, implicates fructose as a key nutrient that may contribute to the epidemic of metabolic disorders. The molecular links between SHBG with the risk for

developing metabolic disorder associated diseases including hip fracture (Lee et al. 2008), breast cancer (Davidson et al. 1981; Key et al. 1982), and coronary heart disease (Reinecke et al. 2002) needs to be investigated further.

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## Legends of figures

Figure 1. The human *SHBG* promoter contains several *cis*-elements, and their relative positions within the 803 bp promoter sequence are shown as boxes, with their actual sequences listed below together with the identities of their known binding proteins (Jänne & Hammond 1998; Selva *et al.* 2005; Selva & Hammond 2009). Note that : i) the (TAAAA) repeat has a silencing activity that varies with the number of repeats, and this activity is dependent on downstream promoter elements including SP1 (or SP1 related factor) (Hogeveen *et al.* 2001) ; ii) the USF binding site represses *SHBG* transcription in Sertoli cells but has little influence on *SHBG* transcription in the liver (Selva *et al.* 2005); iii) the peroxisome-proliferator receptor-gamma binds to the DR-1 element and represses its transcriptional activity (Selva & Hammond 2009) ; iv) the DR-1 element can also bind HNF-4 $\alpha$  and COUP-TF, but this appears to have a modest influence on transcriptional activity (Selva & Hammond 2009); v) the HNF4/COUP TF binding site is the major on/off switch of the human *SHBG* promoter, with HNF4 enhancing transcription and COUP TF repressing transcription.

Figure 2. Monosaccharide-induced lipogenesis reduces hepatic HNF-4 $\alpha$  levels, which in turn attenuates *SHBG* expression. This provides a biological explanation for why *SHBG* decrease is associated with the metabolic syndrome and with an increased risk for diabetes development in humans due to excessive consumption of dietary fructose.



Monosaccharides (glucose and fructose)

