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SEX HORMONE-BINDING GLOBULIN (SHBG) IN THE OVARY

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RESUM

La proteïna transportadora de les hormones sexuals (SHBG) va ser descrita a finals dels anys seixanta amb les funcions de transportar els esteroides sexuals i de regular-ne la biodisponibilitat. Des de fa ja bastant temps, es coneix també que les cèl·lules testiculars de Sertoli expressen la proteïna que lliga els andròsgens (*androgen-binding protein* o ABP), que està codificada pel mateix gen que la SHBG, però glicosilada diferentment. Les funcions de l'ABP han estat àmpliament estudiades, i actualment se sap ja que l'ABP és un dels reguladors locals de l'espermatogènesi. Nogensmenys, la presència de la SHBG en l'ovari ha estat molt poc estudiada. L'objectiu del present article és el de revisar el que actualment es coneix sobre l'expressió de la SHBG en la gònada femenina, incloent-hi les troballes recents del nostre grup que demostren la presència de la SHBG en els fol·licles ovàrics, particularment en el citoplasma de les cèl·lules de la granulosa, en algunes cèl·lules de la teca i en els oòcits de fol·licles primordials, primaris i secundaris, i també en el cos luti. A més, l'expressió del gen de la SHBG s'ha evidenciat també en les cèl·lules granulosoluteíniques en pacients sotmeses a fertilització *in vitro*. Això suggereix que aquestes cèl·lules constitueixen una font local de SHBG en l'ovari humà. Aquestes noves dades suggereixen la implicació de la SHBG en la fisiologia ovàrica.

Paraules clau: proteïna transportadora de les hormones sexuals (SHBG), ovari, cos luti, fluid fol·licular.

SUMMARY

Sex hormone-binding globulin (SHBG) was described in the late sixties as a hepatic carrier protein for sex steroids and was thought to regulate their bio-availability. It has also been known for many years that testicular Sertoli cells express androgen-binding protein (ABP), which is encoded by the same gene as SHBG, but which is differentially glycosylated. The possible roles

of ABP have been extensively studied, and this protein may be one of the local regulators of spermatogenesis. In contrast, very few authors have investigated the presence of SHBG in the ovary. The aim of the present paper is to provide a survey of what is currently known about the expression of SHBG in the female gonad, including our own recent findings that SHBG was present in ovarian follicles. This was true, particularly in the cytoplasm of granulosa cells, some theca cells, and oocytes in primordial, primary, and early secondary follicles, as well as in the corpus luteum. Furthermore, the expression of the SHBG gene has been demonstrated in granulosa-lutein cells from *in vitro* fertilization patients, indicating that these cells are one of the local sources of SHBG in the human ovary. These new data suggest an involvement of SHBG in ovarian physiology.

Keywords: sex hormone-binding globulin (SHBG), ovary, corpus luteum, follicular fluid.

INTRODUCTION

Sex hormone-binding globulin (SHBG) was classically thought to originate in the liver and was believed to carry steroid hormones in blood serum, especially dihydrotestosterone, testosterone, and estradiol (Joseph, 1994). Thus, the main function of SHBG was considered to be the regulation of their bioavailability, according to the free hormone theory, which postulated that only the free and unbound fraction of steroids would be able to cross the cell membrane and interact with their intracellular receptors (Siiteri *et al.*, 1982; Mendel, 1989). A homologous but differentially glycosylated protein, called androgen-binding protein (ABP), was known to be produced in the testis by Sertoli cells and to be secreted into the seminiferous tubules (French and Ritzen, 1973; Hagenas *et al.*, 1975), where it was believed to regulate the local concentration of androgens.

Over the last twenty years this basic concept of a steroid-carrying protein has been questioned because of several major discoveries: first, SHBG is expressed in several sites other than the liver and testis, especially in steroid-sensitive tissues, such as the prostate, fallopian tube, and mammary gland (reviewed in Joseph, 1994); and, second, in most of these tissues, SHBG specifically binds to the cell membrane (Strel'chyonok *et al.*, 1984; Hryb *et al.*, 1985; Fortunati *et al.*, 1991; Frairia *et al.*, 1991). This phenomenon has been considered to be receptor-mediated, but until now the molecular characteristics of the putative SHBG receptor had not been determined. Nevertheless, membrane-bound SHBG has been shown to induce an increase in intracellular cAMP (Rosner, 1990), which is even more augmented after the secondary binding of a steroid hormone to the SHBG-receptor complex (Hryb *et al.*, 1990). Another important finding concerned an alternative splicing process for the SHBG mRNA, yielding several transcript variants, in addition to the full length transcript that included eight exons with the first exon encoding the secretion signal (Gershagen *et al.*, 1989; Hammond *et al.*, 1989; Sullivan *et al.*, 1991, 1993). However, until now the function of the corresponding modified proteins had not been elucidated.

Taken together, these data strongly suggest that SHBG is more than a simple transport protein and that it may have much broader functions in cell biology, possibly as a hormone or as a locally active auto- or paracrine factor. Thus, it may play a regulatory role in the physiology of sex steroid-sensitive tissues. It has been suggested that ABP is involved in the local control of spermatogenesis, particularly in the testis (Gerard, 1995; Gerard *et al.*, 1996; Della-Maria *et al.*, 2002). Whereas the expression and function of testicular ABP have given rise to a large amount of scientific work, curiously, the presence and possible involve-

ment of SHBG in the female gonad have not been extensively investigated. The present paper proposes a systematic review of what is currently known about ovarian SH BG expression.

SHBG IN FOLLICULAR FLUID

In the ovary, the presence of SHBG was originally detected in the ovarian follicular fluid. It has been known for a long time that the total protein concentration of follicular fluid is similar to or slightly lower than that of blood serum (Perloff *et al.*, 1954; Zachariae and Jensen, 1958; Shalgi *et al.*, 1972). Thus, most of the serum proteins are also present in follicular fluid (Shalgi *et al.*, 1973). In one study, however, the author found a follicular fluid protein concentration only half that of serum (Edwards, 1974), which could be explained by the different maturation degree of the punctured follicles. The latter hypothesis is consistent with results from another group, who showed follicular fluid protein composition to become progressively more similar to that of blood serum as the follicle grew up to the pre-ovulatory stage (DiZerega *et al.*, 1983).

Initially, a protein binding to dihydrotestosterone (DHT) and estradiol was detected in the follicular fluid from three patients with polycystic ovary disease, who underwent a transvaginal puncture for ovarian cysts (Vigersky and Loriaux, 1976). This protein had high affinity and low capacity, as well as electrophoretical properties identical to those of serum SHBG. Later, SHBG and corticosteroid-binding globulin (CBG) were clearly identified in the antral fluid of pre-ovulatory follicles in healthy volunteers (Martin *et al.*, 1981).

With the development of *in vitro* fertilization (IVF) techniques, follicular fluid became an easily available material, which has been extensively studied since then. The follicular concentration of SHBG in IVF patients was found to be similar to that of blood serum, and there was a significant correlation between follicular and serum level s (Ben-Rafael *et al.*, 1986). This result was later confirmed by Andersen, who described remarkably stable SHBG concentrations within the different follicles of the same patient (Andersen, 1990). In contrast, another group detected important variations in the SHBG concentration, with some follicles having a higher concentration and others a lower one than in the blood serum (Campo *et al.*, 1989). Other authors explained the absence of any correlation between follicular fluid and serum SHBG concentrations as a possible effect of gonadotrophin releasing hormone agonists, which had not been used in the previous studies (Phocas *et al.*, 1995). Anyway, at that time the only known source of SHBG was the liver and testis, thus, follicular fluid SHBG was thought to originate exclusively in the blood, crossing the blood-follicle barrier quite easily, due to its molecular weight of about 90 kD.

As to the possible role of SHBG in follicular fluid, there have been several hypotheses, all of them based on the steroid-binding properties of the protein. Thus, follicular SHBG could play a role by trapping steroids within the follicle (Edwards, 1974), regulating the bio-availability of steroids, in the follicle, especially estradiol (Baird and Fraser, 1975), and controlling the transport of estradiol into or out of the follicle (Younglai and Short, 1970; Steinglod *et al.*, 1988). However, these classical concepts need to be questioned, as it has been shown that estradiol concentration in the fluid of pre-ovulatory follicles is about 1,000 times higher than in the serum, and therefore, exceeds the binding capacity of SHBG 20 to 100 fold (McNatty, 1978; Martin *et al.*, 1981; Ben-Rafael *et al.*, 1986). Actually, intrafollicular estradiol is bound to proteins at a rate of about 95%, while the SHBG-bound fraction only accounts for 1.5 to 25% (O'Brien *et al.*, 1982; Ben-Rafael *et al.*, 1986; Andersen, 1991). Thus, other binding proteins, such

as albumin, must be involved in maintaining these very high estradiol concentrations within the ovarian follicle. Finally, the 5% of free estradiol still represents a high concentration (10^{-7} M), compared to the dissociation constant of the estradiol receptor (10^{-9} M). Therefore, it is unlikely that SHBG regulates steroid bio-availability in the follicle, as it was classically thought to do in the serum (Martin *et al.*, 1981; Ben-Rafael *et al.*, 1986).

OVARIAN SHBG IN ANIMAL MODELS

At the time when steroid-binding activities were first detected in human follicular fluid, some authors found a similar activity in bovine antral fluid (Andersen *et al.*, 1976; Cook *et al.*, 1977) and ovine follicles (Cook *et al.*, 1977; Campo *et al.*, 1985). However, no such activity could be detected in porcine follicles; this was considered to be indirect proof that intra-follicular SHBG was serum-derived, since pigs also lack serum SHBG (Cook *et al.*, 1977).

In the ewe, Campo *et al.* detected a testosterone and DHT-binding activity, not only in serum and follicular fluid, but also in the cytosolic fraction of the granulosa cells. In all of the three sites, association and dissociation constants were identical to those of testicular ABP (Campo *et al.*, 1985). Curiously, this interesting observation did not give rise to further investigations.

In his exhaustive review of the literature, Joseph reported unpublished results showing the presence of immunoreactive ABP in rat granulosa and theca cells, as well as the Northern blot detection of ABP mRNA in tissue extracts from rat ovaries (Joseph, 1994).

The expression of SHBG was further investigated using transgenic mice carrying either a rat ABP or a human SHBG transgene. In the first of these models, Northern blot analysis revealed the presence of rat ABP transcripts in whole ovarian extracts, while there was no expression in the wild-type ovaries (Joseph *et al.*, 1997). However, wild-type ovaries were shown to contain endogenous mouse ABP transcripts. Semi-quantitative analysis of these results demonstrated an increasing expression from the age of fourteen days up to the adult stage. Furthermore, ovarian extracts from transgenic mice showed a five-fold binding activity to tritiated DHT, when compared with wild-type ovarian extracts. Similar results were obtained from blood plasma and uterine extracts (Joseph *et al.*, 1997).

In contrast to the results from the Northern blot and binding assays, the ABP protein could not be localized by immunohistochemistry in either transgenic or in wild-type ovaries. To explain these discrepancies, authors suggested that the Northern blots were contaminated by mesonephrotic RNA and that the steroid-binding activity was, in fact, due to the follicular fluid. Nevertheless, the possibility of a local synthesis of ABP, in addition to its uptake from the blood could not be excluded.

Female transgenic mice presented with a lower body weight than the wild-type animals. They also showed important neurological disorders in their hind limbs and impaired fertility, with a diminished number of siblings and sometimes complete sterility. Finally, they frequently developed tumors of the pituitary, uterus, and ovary (Joseph *et al.*, 1997).

As far as the second model is concerned, that of transgenic mice carrying a human SHBG transgene, there is no published data on females, and regarding the males, normal fertility was observed despite very high serum SHBG and testosterone levels (Janne *et al.*, 1998; Janne *et al.*, 1999).

SHBG TRANSCRIPTS IN THE HUMAN OVARY

Twenty years ago, Ito and co-workers de-

monstrated the presence of a steroid-binding protein, similar to ABP, in the cytosolic fraction of human ovarian cortex extracts. The concentration of this protein was lower in ovaries from women with polycystic ovary syndrome than in normal ovaries (Ito *et al.*, 1985).

Using RT-PCR, Misao's team demonstrated the presence of SHBG extracts in whole ovarian extracts from fifteen patients between the ages of 25 and 68 years, who underwent an ovariectomy for various medical reasons (Misao *et al.*, 1995). Their semi-quantitative approach, using an internal standard, demonstrated higher expression levels in premenopausal ovaries than in post-menopausal ovaries, suggesting the possible regulatory effect of steroid hormones on the expression of SHBG. Alternatively, it could have been hypothesized that SHBG was predominantly expressed in functional ovarian follicles, which disappear after menopause. Later, the same group demonstrated the presence of SHBG transcripts in human corpus luteum extracts from fifteen patients. Extracts from the early, mid-, and late luteal phases did not show any significant differences in the expression level (Misao *et al.*, 1997). However this level seemed to be positively correlated with the serum estradiol and the serum estradiol/progesterone ratio (Misao *et al.*, 1999*b*).

These authors also investigated SHBG expression in 34 ovarian tumors, comprising 22 malignant and 12 benign tumors. In all cases, SHBG transcripts were detected by RT-PCR, and the expression level did not show any correlation to the type of tumor or the stage of the disease. However, SHBG was over-expressed in six of the malignant tumors; three of them were endometrioid adenocarcinoma (Misao *et al.*, 1995). Actually, the presence of an estradiol-binding protein in ovarian cancer extracts had already been described ten years earlier using a cytochemical technique (Wand *et al.*, 1985). In metastatic ovarian cancers, Misao and co-workers found SHBG transcripts in only four out of ten samples (Misao *et al.*, 1999*a*). In normal, as well as in pathological ovarian tissues, alternative transcripts lacking exon 7 were also detected, but their proportion was higher in ovarian cancers than in normal ovaries (Misao *et al.*, 1998).

In a recent paper, we confirmed the results from Misao, showing the presence of full-length SHBG transcripts, as well as splicing variants lacking exon 7 in whole ovarian extracts (Forges *et al.*, 2005). We further determined granulosa-lutein cells to be one of the possible cellular origins of this ovarian SHBG expression (Forges *et al.*, 2004). In that study, we collected granulosa-lutein cells from patients, who underwent follicular aspiration for *in vitro* fertilization. Total RNA was extracted from freshly isolated cells, as well as after a 4 day culture period. In both cases, RT-PCR analysis showed the presence of full-length mRNA, whereas in our study, alternative transcripts could not be detected. We thus concluded that at least the complete, secreted SHBG protein arose from this cell type in the ovary. Recently, we demonstrated the secretion of SHBG by granulosa-lutein cells *in vitro*, using the Western blot analysis of the supernatant culture media (unpublished results).

IMMUNOHISTOCHEMICAL LOCALIZATION OF SHBG IN THE HUMAN OVARY

Except for the above-mentioned transgenic model, until very recently the presence of SHBG has not been immunohistochemically investigated in ovarian tissues. In our previous study, we showed a positive SHBG immunostaining in the cytoplasm of granulosa-lutein cells from IVF patients, which was consistent with the results of the RT-PCR analysis, demonstrating that these cells actually produced SHBG in the ovary (Forges *et al.*, 2004). However, the possibility of a simul-

taneous uptake of extra-cellular (i.e., follicular fluid) SHBG, as it has been described in rat Sertoli cells (Gerard *et al.*, 1994), could not be completely ruled out.

In another study, we performed immunohistochemistry on paraffin-embedded tissue sections from human adult and fetal ovaries (Forges, *et al.*, 2005). SHBG immunostaining was differentially localized throughout folliculogenesis. In all stages, from primordial follicles up to the pre-ovulatory follicle, the cytoplasm in the granulosa cells contained immunoreactive SHBG. This was consistent with our previous results in granulosa-lutein cells from stimulated ovaries. In contrast to the granulosa layer, where nearly all of the cells contained immunoreactive SHBG, the theca interna of the preantral and antral stages presented with only a few isolated, positively stained cells.

Moreover, SHBG was present in the oocyte cytoplasm of primordial, primary, and some early secondary follicles, in both adult and fetal ovaries. In contrast, from the late secondary, preantral follicle up to the graafian follicle, the oocyte was always devoid of any SHBG immunostaining. Thus, the presence of SHBG in the oocyte ceased at the time that the follicle became sensitive to FSH; but, whether there was a causal relationship between the two phenomena or if this was a simple coincidence still needs to be determined.

To analyze the presence of SHBG in mature human oocytes, oocyte-cumulus complexes (OCCs) were obtained by follicular aspiration from patients, who underwent ovulation induction for intrauterine insemination and who presented with a multi-follicular ovarian response. In these patients, aspiration of the excessive follicles was performed before insemination, in order to avoid multiple pregnancies. In the OCCs, only the cumulus cells showed immunostaining, whereas the oocyte cytoplasm and the first polar body of the mature oocytes were negative. Interestingly, there was strong staining at the oocyte surface and in the perivitelline space, though not in the zona pellucida. This observation led to the hypothesis that there existed a local accumulation of SHBG, secreted by the granulosa cells, and a subsequent interaction with SHBG receptors on the oocyte membrane. SHBG-binding on the oocyte surface could either be followed by an internalization of the SHBG-receptor complex, as had been shown by our team in male germ cells (Gerard, 1995), or by the production of cAMP as a second messenger, which has been described in prostate (Nakhla *et al.*, 1990; Nakhla *et al.*, 1994) and breast cancer cells (Fissore *et al.*, 1994; Fortunati *et al.*, 1999). Cyclic nucleotides actually play an important role in the regulation of oocyte meiotic maturation (Conti *et al.*, 2002). Thus, SHBG may be secreted locally by cumulus cells and be involved in the control of meiotic arrest. However, the presence of SHBG-specific binding sites on the oolemma has not yet been investigated.

In ovarian tissue sections containing a corpus luteum, immunoreactive SHBG was found in the large luteal cells, whereas the small luteal cells were only weakly and inconsistently stained. This luteal SHBG expression profile was in accordance with the abovementioned results from the antral follicles, in that large luteal cells were classically thought to originate in the granulosa cells, while small luteal cells differentiated from thecal cells (Sanders and Stouffer, 1997).

In summary, SHBG has now evolved from a basic circulating transport protein to a locally produced and secreted cell mediator in sex steroid-sensitive tissues. In the human follicle, SHBG is differentially expressed during folliculogenesis and, after ovulation, in the corpus luteum. Granulosa-lutein cells are one of the possible sources of SHBG in the ovary. Follicular fluid SHBG may, therefore, originate not only in the serum, but also in a local secretion by the granulosa cells surrounding the follicular antrum. The role that SHBG plays in ovarian physiology still remains elu-

sive. A major breakthrough may be expected from animal models, where the SHBG gene would have been deactivated. However, until now, no knock-out experience has been reported. Thus, further knowledge about this protein will rely mainly upon *in vitro* studies. Our group is currently investigating the possible roles of SHBG in human granulosa-lutein cells *in vitro*.

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