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SEX HORMONE BINDING GLOBULIN INHIBITS STRONGLY THE UPTAKE OF ESTRADIOL BY HUMAN BREAST CARCINOMA CELLS VIA A DEPRIVATIVE MECHANISM

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A controversy exists for many years about the role of sex hormone binding globulin (SHBG) in the uptake of estradiol by the cells. Using the estradiol-sensitive human breast carcinoma cell line MCF-7 and SHBG isolated from human serum by a new method, we observed a strong inhibition of estradiol uptake. The inhibition was higher when the concentration of the hormone was low. On the other hand, there seemed to be a lag period in inhibition when the concentrations of SHBG were very low, followed by an exponential increase, when the concentration exceeded a critical value. The inhibitory activity was higher when SHBG was added before or along with estradiol in the cell culture, as well as when the incubation period was elongated, while was dramatically minimized by the presence of dihydrotestosterone. Despite the inhibition of estradiol uptake caused by SHBG, the distribution of the hormone in various cell components remained practically the same. In conclusion, all indications from experimental data seem to suggest a simple deprivative mechanism being responsible for the inhibitory activity of SHBG on estradiol uptake by MCF-7 cells in culture.

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INTRODUCTION

Sex hormone binding globulin (SHBG) is a serum glycoprotein binding specifically androgens with high and estrogens with lower affinity (Pearlman and Grepy, 1966, Mickelson and Petra, 1978) compared to albumin which binds steroids with high capacity but low affinity. It is known for many years that about 98% of the plasma estradiol is bound to these proteins. The role of this bindings, however, concerning the bioavailability of this hormone remains controversial.

The discovery of sex steroid binding protein (SBP) one year after the suggestion by Tait and Burnstein (1964) that the unbound steroids in plasma incorporate into tissues, came at appropriate time. The newly discovered protein was proposed as a binder controlling the passive diffusion of free hormone into tissue. Studies on the metabolic clearance of sex steroids (Vermeulen *et al*, 1969, Bardin and Lipsett, 1967), as well as in vitro experiments with cultures of endometrial cancer cells (De Ryck *et al*, 1985) supported this hypothesis. In the past few years, however, a number of papers appeared, demonstrating the presence of membrane receptors for SHBG in the cells of various tissues like decidual endometrium (Strel'chyonok *et al*, 1984), premenopausal endometrium (Fortunati *et al*, 1991), endometrial adenocarcinoma (Fortunati *et al*, 1992) and prostate (Hryb *et al*, 1985). The existence of these receptors seems to propose a new role for SHBG. Among the various suggestions concerning the new role of SHBG, one could mention the uptake of steroid hormones by target cells via a receptor-mediated endocytosis (Strel'chyonok and Avvakunov, 1990) and the induction of a cellular second messenger c-AMP by the complex SHBG-membrane receptor (Rosner, 1990).

In spite of the experimental material collected during the last years supporting these hypotheses, the problem of the contribution of SHBG on the uptake of steroid hormones by the cells seems to be present. In a recent work (Fortunati *et al*, 1993) it was studied using the human breast carcinoma cell line MCF-7, reportedly containing intracellular immuno-reactive SHBG (Bordin and Petra, 1980) and specific membrane sites for the bindings of SHBG (Porto *et al*, 1992) and its transport via endocytosis

into the cell (Porto *et al*, 1991). The findings indicated that the addition of SBP in the cell culture did not influence the cellular uptake of estradiol.

In the present study, using the same cell line, we observed a remarkable and consistent inhibition of estradiol uptake by SHBG isolated from human serum by a new method.

MATERIALS AND METHODS

Chemicals

Steroids were products of Sigma Chemie, Deutschland, while radiolabelled steroids were products of Amersham, UK. The specific activity of estradiol (2,4,6,7,16,17-^{3}H) was 140-170 Ci/mMole and that of dihydrotestosterone (1,2,4,5,6,7-^{3}H) 100-150 Ci/mMole. The charcoal was a product of Merck, Germany and the dextran-32 of SERVA, Germany. Diphenyloxazole (PPO) and POPOP were products of Sigma, as well as DEAE-Cellulose and Sephadex G-200. Triton X-100 was a product of SERVA, Germany. The scintillation fluid for the measurement of radioactivity contained 4 gr of PPO and 0.4 gr of POPOP per liter of a Toluene-Triton X-100 mixture (2:1). All disposable material for cell culture was product of Nunk, Denmark.

Sex hormone binding globulin from serum of pregnant women was purified by a new method as described elsewhere (Zeginiadou *et al*, submitted). Briefly, 100 ml of cold (4°C) serum was diluted with 150 ml of Tris - saline buffer (0.05M Tris - HCl pH 8.0-0.9% sodium chloride) and 212.5 ml (0.85 volumes) of aceton, precooled at −26° C, were added dropwise under vigorous stirring. Following centrifugation, the precipitate was discarded and 0.35 more volumes of aceton were added in a similar way to the supernatant. The new pellet was dissolved with 62 ml of Tris - saline buffer and ammonium sulfate fractionation followed. The fraction between 38 and 53% saturation in ammonium sulfate was collected, dissolved in Tris-HCl buffer and chromatographed on a DEAE-Cellulose column, eluted with a 0-0.25 M sodium chloride gradient in Tris buffer. The tubes containing the active protein were collected, 1.2 volumes of aceton were added to the solution and the pellet (dissolved in water) was used in the experiments. This product gave practically one fraction of protein when chromatographed on a Sephadex G-200 column coinciding

with the binding activity and also one band in polyacrylamide gel electrophoresis.

Cell Culture

MCF-7 cells (from pleuritic fluid of breast cancer) used throughout the experiments were a gift of Dr. Joyce Taylor of Imperial Cancer Research Fund, London, and are preserved for some years in the Department of Cell Culture of the Hospital. Cells were grown (in monolayers) in Dulbeco's MEM to confluency, in an incubator (Forma Scientific) under controlled conditions of 37° C and 5% CO_2, in 25 ml flasks (Costar, The Netherlands). These were diluted approximately every 48 hours, using trypsin solution that contained 100,000 units of penicillin and 0, 1 gr of streptomycin per 500 ml. Small volumes with cells were then transfered to new sterilized flasks with fresh medium.

The amount of steroid needed for each experiment was first evaporated under vacuum, in order to remove the toluene and the steroid was then dissolved in 500 ml of culture medium, which was then added to the culture dish. The day of the experiment, cells were transfered to petri dish (5 ml) at a concentration of about 1×10^6 cells per dish. After addition of the steroid and protein and incubation for the eppropriate time, the medium was changed, in order to remove any unbound steroid and protein. The cells were then harvested mechanically using a ruber scraper and centrifuged for 5 min at 4,500 $\times$ G. The pellet was then washed three times (by dispension and centrifugation) and the final precipitate was dissolved in 0,4 ml of 25% Triton X-100 solution, added into 4 ml of scintillation fluid and measured for radioactivity in a liquid scintillation beta counter (LKB).

Fractionation of Cellular Components

Cells were homogenized with 0.05 M Tris-HCl buffer pH 8.0 in a Potter-Elvehjem homogenizer. Nuclear, mitochondrial plus lysosomal, microsomal and cytosolic fractions were obtained by centrifugation over sucrose solution as described by Hogeboom (1955). Each fraction (except cytosol) thus obtained, was suspended into 0.4 ml of Triton X-100 solution (25%) and after 30 min was added in 4 ml of scintillation fluid and measured in the beta counter. Cytosol (0,4 ml), without pretreatment, was also measured in the same way.

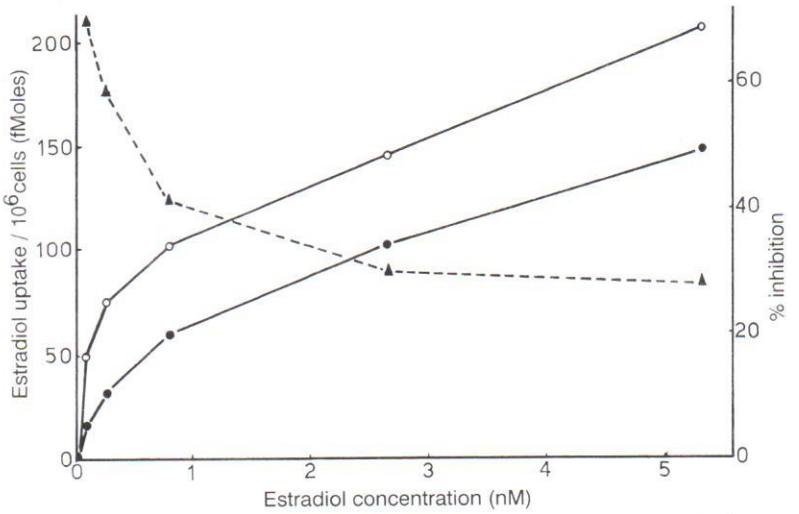

FIGURE 1 Effect of SHBG on estradiol uptake by MCF-7 cells, in response to the hormone concentration. Dishes containing 10^6 cells were used and SHBG (20 pMoles) was added at the beginning of the incubation, 10 min before the estradiol addition. The cells were incubated for two hours. ○——○ Estradiol uptake, ●——● Estradiol uptake in the presence of SHBG, ▲----------▲ Inhibition of estradiol uptake in the presence of SHBG (%).

Measurement of SHBG

A dextran-coated charcoal method was used for the measurement of the globulin binding capacity. In brief, the incubation mixture contained in a final volume of 0.25 ml the following: 0.025 M Tris-HCl buffer pH 8.0, increased concentrations of estradiol (0.1, 0.2 and 0.4 μCi, corresponding to 0,7, 1.4 and 2.8 pMoles) and 10-15 μl of SHBG preparation. After 10 min at 0° C (in an ice-water bath), one ml of dextran-coated charcoal suspension (1 gr of charcoal and 0.1 gr of dextran-32 per 100 ml of Tris-HCl buffer) was added and shaked. After exactly 5 min, the tubes were centrifuged for 5 min at 4,500 × G and 0,4 ml from the supernatant were added in vials containing scintillation fluid (3 ml) and measured in the β-counter. A Scatchard plot was made with the three values for the estimation of binding capacity.

RESULTS

The influence of SHBG on the uptake of estradiol by the cells is shown in Figure 1. It is evident that SHBG exhibits a strong inhibitory activity on the

uptake, especially when the concentrations of the hormone are very low. In this case, the inhibition is nearly complete. The inhibitory activity minimizes quickly with the increase of estradiol concentration and ceases when it surpass 4-5 nM. An inhibition around 27%, however, remains as a result of the SHBG presence.

The level of SHBG in the cell culture is essential concerning the extent of estradiol uptake. As can be seen in Figure 2, very low concentrations of SHBG have not any inhibitory activity. In fact, it seems that a lag period preceeds as the concentration of the globulin is increasing. It is followed by rapid increase of inhibition, when the concentration exceeds a critical point. After that, the uptake inhibition proceeds very slowly, leading to a platau of uptaken hormone.

The inhibitory activity of SHBG is higher when it coexists with estradiol from the beginning of its addition, as can be seen in Table I. More profound difference in the inhibitory activity was observed when the duration of estradiol presence in the cell culture was changing. As can be seen in Figure 3, the estradiol uptake increases with the time until about 40 min.

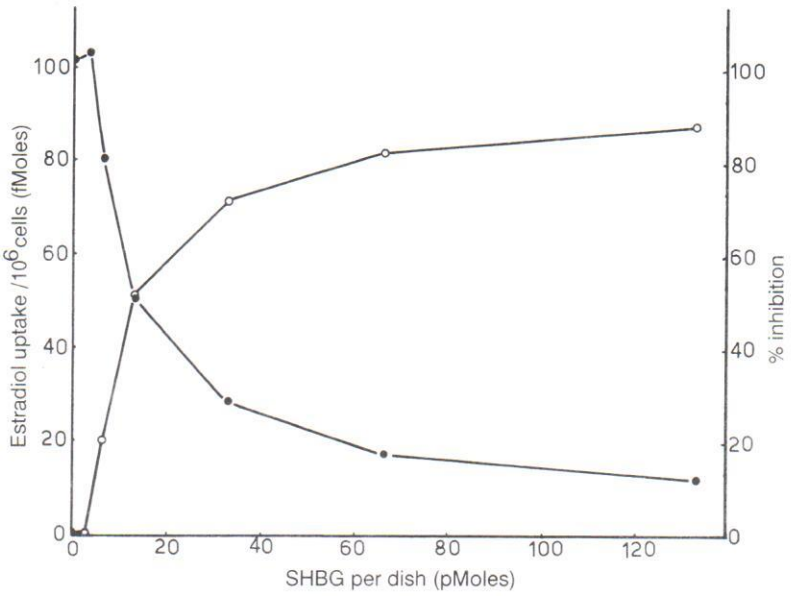

FIGURE 2 Effect of SHBG concentration on the estradiol uptake by MCF-7 cells. SHBG was added in the beginning of incubation (two hours) while the labelled estradiol (1 nM) 10 min later. ●——● Estradiol uptake, ○——○ Uptake inhibition by SHBG (%)

TABLE I Effect of the SHBG addition time on the rate of estradiol uptake by MCF-7 cells

Time of estradiol addition (min from the beginning)	Estradiol uptake (fMoles/10^6 cells)	Time of SHBG addition (min from the beginning)	Estradiol uptake (fMoles/10^6 cells)	Uptake inhibition (%)
		0	47.7	42.7
0	83.2			
		90	55.1	33.8
		30	51.7	38.9
30	84.6			
		90	60.1	20.0
		60	50.0	41.5
60	85.4			
		90	67.8	20.6

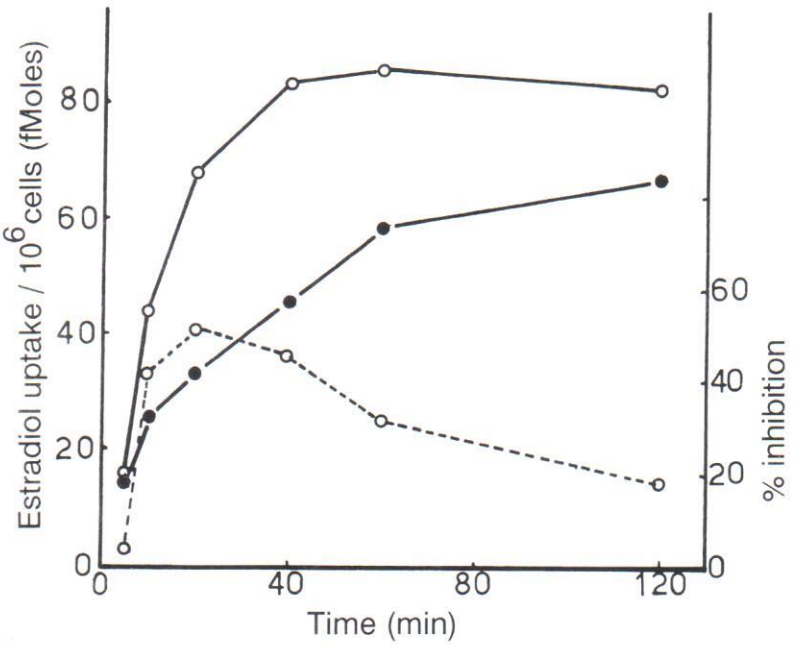

FIGURE 3 Effect of the time of estradiol presence in the culture on the inhibitory activity of SHBG. One nM of estradiol and 10 pMoles of SHBG (added 10 min before estradiol) were contained in each dish. ○——○ Estradiol uptake in the absence of SHBG, ●——● Estradiol uptake in the presence of SHBG, ○- - - - - ○ Uptake inhibition by SHBG (%).

TABLE II Distribution of estradiol in subcellular components in the absence and presence of SHBG

Subcellular component	Estradiol content (fMoles) − SHBG)	Percentage of the total	Estradiol content (fMoles) + SHBG)	Percentage of the total	Inhibition of uptake (%)
Nuclei	61.8	71.0	31.3	69.5	49.4
Mitochondria	4.0	4.6	3.6	8.0	10.0
Microsomes	3.8	4.4	1.6	3.6	57.9
Cytosol	17.4	20.0	8.5	18.9	51.1

After that time the uptake remains practically stable. The inhibition of the uptake by SHBG (added before estradiol in the culture) is higher for short times of incubation, falling steadily as the time increases.

The presence of SHBG in the cell culture does not influence the distribution of estradiol in the subcellular components, as can be seen in Table II. The vast majority of estradiol is localized in the nuclei, as does in the absence of the globulin (69.5% versus 71.5%), while the ratio of estradiol in the absence and presence of SHBG is practically the same in nuclei, microsomal fraction and cytosol (around 2). Only the incorporation of estradiol into mitochondria remains the same but the fraction of estradiol in these subcellular particles is very low (about 4.3% of the total).

Dihydrotestosterone (DHT), a steroid with higher affinity to SHBG, eliminates at concentrations higher than 1 μM the inhibition of estradiol uptake caused by the globulin. Incorporation of DHT itself into MCF-7 cells was not oberved at all in our experiments (results not shown).

DISCUSSION

The role of SHBG in the uptake of sex hormones by the cells remains controversial, in spite of the vast work done on the subject during last years. The controversy could be the result of the variety of the cell lines and SHBG preparations used in the experiments. SHBG itself is a matter of controversy during the last decade, concerning its size (Hammond *et al*, 1986, Danzo *et al*, 1989, Petra *et al*, 1986), structure (Petra *et al*, 1986, Namkung *et al*, 1990, Petra *et al*, 1988) and steroid binding mechanism (Orstan *et al*, 1986). It is due evidently both, to the method of globulin isolation and the method of estimation of its various characteristics.

In this work, we used a new, simple and quick method for the purification of SHBG, based mainly on acetone fractionation of the proteins. The purification procedure does not influence the binding activity of the product, as can be judjed from the high yield of the method (over 58%).

The SHBG preparation was used in culture of MCF-7 cells, which represent a typical estrogen-dependent line. The results showed emphatically a strong inhibition of estradiol concentration, being very high with hormone levels corresponding to that observed during the follicular phase of human menstrual cycle or in postmenopausal women. It is rapidly decreased when the hormone concentration is rising, having a tendency to stop when the concentration exceeds 1.3 nM. This concentration corresponds to the higher levels observed in the women during the mid-cycle phase. Even at very high concentrations of estradiol, however, a significant inhibition of hormone uptake is observed, at least when the concentrations of the globulin are moderate. In fact, when the concentrations of SHBG exceed 130 pMoles per culture dish, the inhibition of the uptake tends to reach 100%. At very low concentrations of SHBG, however, there is not any inhibition at all. It is then increasing exponentially when the concentrations of the globulin exceed 5 pMoles per dish, approaching the maximal inhibition at concentrations over 30 pMoles per dish, which correspond to the concentrations of SHBG in the serum of man.

The time duration of estradiol presence in the cell culture affects its uptake by the cells. This is valid, however, only for time periods not exceeding 30 min. After that period, the uptake is not practically changed. The inhibition caused by the presence of SHBG on the hormone uptake is strong only when estradiol is present in the culture for short time periods. After 40 min, the inhibition is gradually falling, reaching values lower than 20%. This variety of the uptake inhibition could be a consequence of estradiol excange between the extracellular fluid and the cells. In this case, the stable intracellular estradiol levels, oberved after 40 min, correspond, evidently, to the quantity of the hormone bound to its receptors in the cells and are dependent on the level of latter. An interesting observation was that when the estradiol was present for very short periods in the culture (lower of 5 min), there was not any inhibition of its uptake by SHBG. A resonable explanation of this phenomenon is that all of the measured estradiol uptake in this case was due to binding on the cell membranes and did not represent real uptake into the cells.

High dihydrotestosterone concentrations in the culture eliminate the inhibitory action of SHBG on estradiol uptake. It is evidently caused via the saturation of the globulin binding sites by DHT molecules, which posses a much higher affinity for that, compared to estradiol.

The presence of SHBG in the culture does not influence the intracellular distribution of estradiol. A deviation from the rule in the case of mitochondria seems to represent an experimental artifact, in the view of very low concentrations of estradiol observed in those subcellular particles.

The whole picture emerging from experimental data, concerning the effect of SHBG on estradiol uptake by the cells, is that of a deprivative mechanism; SHBG binds estradiol, lowering this way its pool supplying with hormone the cells. There were no indications of a mediatory role of SHBG in the transport of estradiol into the cells, at least with the particular SHBG preparation and cell line used.

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