J. Steroid Biochem. Molec. Biol. Vol. 37, No. 5, pp. 733–739, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

ESTROGEN AND ANDROGEN REGULATION OF SEX HORMONE BINDING GLOBULIN SECRETION BY A HUMAN LIVER CELL LINE

SIMON E. J. EDMUNDS, ANDREW P. STUBBS, ANTONIO A. SANTOS and MARK L. WILKINSON*
Gastroenterology Unit, United Medical and Dental School of Guy's and St Thomas', Hospitals, 18th Floor, Guy's Tower, St Thomas', Street, London SE1 9RT, England

(Received 22 May 1990)

Summary—Both estrogens and androgens have been shown to stimulate sex hormone binding globulin (SHBG) secretion *in vitro* in the hepatocellular carcinoma cell line, Hep G2, in contrast to the expected inhibition by androgens from *in vivo* studies. However, such *in vitro* stimulation was only demonstrated at high steroid doses, generally in serum-containing medium, with added Phenol Red. In the present study, Hep G2 cells were grown in serum-free medium, without Phenol Red, under the influence of testosterone (T) (0, 0.5–500 nM) and ethinyl estradiol (EE_2) (0, 50 pM–500 nM). Levels of secreted SHBG and albumin were correlated with androgen receptors in cytosolic (ARc) and nuclear (ARn) fractions and with DNA levels. In the presence of increasing T levels, SHBG levels fell to 39% of control values at 5 nM T ($P = 0.047$), rising to 97% of control at 500 nM. Conversely, incubation with EE_2 produced a rise in SHBG secretion of more than 100% at 0.5 nM ($P < 0.02$) which was sustained to 50 nM ($P < 0.005$). DNA levels did not change with the addition of testosterone or EE_2, with the exception of a 15% reduction at 5 nM EE_2 ($P < 0.05$). Albumin levels in the medium were not significantly altered by either steroid. However, in response to T, androgen receptor (AR) levels were reduced in cytosolic (42% of control) and nuclear (22%) fractions at 5 nM, and these changes in ARc and ARn correlated with SHBG levels over the range of T concentrations ($P = 0.04$ and $P = 0.017$, respectively). Nuclear estrogen receptor (ER) increased over 10-fold at 5 and 50 pM EE_2 ($P < 0.001$) and maintained a 5-fold increase to 50 nM ($P < 0.001$). Cytosolic ER was reduced at 0.5 and 5 nM but recovered at 50 nM, correlating with SHBG levels ($P < 0.001$). These findings are consistent with the hypothesis that estrogens and androgens regulate SHBG synthesis in man by direct, specific, probably receptor-mediated effects on hepatocytes. Hep G2 cells grown in serum-free medium are a suitable experimental system for further study of this phenomenon.

INTRODUCTION

Sex hormone binding globulin (SHBG), the major specific circulating binder of potent sex-steroids in man and several mammalian species, is produced by hepatocytes *in vitro* [1]. *In vivo*, circulating levels of SHBG are increased by estradiol at physiological or pharmacological levels [2–5], while androgens appear to reduce levels of the protein [4, 6]. Whether these effects are directly mediated by the steroids via hepatocyte steroid receptors is unclear. The liver is known to be a target organ for both estrogens and androgens, and receptors for both classes of steroid are present in benign [7, 8] and malignant [9] liver tissue. The human hepatocellular carcinoma cell line Hep G2 possesses estrogen receptors (ER) [10] and is known to secrete SHBG [1] as well as many other proteins secreted by normal liver tissue [11]. Production by Hep G2 of certain proteins is known to be sensitive to sex-steroid stimulation [10, 12]. However, there is a lack of agreement on the effects of steroid hormones on production of SHBG by Hep G2 cells.

Androgens have been shown to have, variously, no effect on SHBG secretion by Hep G2 cells [13] or a stimulatory effect at 0.5 and 5 μM but not at lower levels [14, 15], while estrogens have no effect [13] or a stimulatory effect at 0.1–1 μM [14–17]. Paradoxically, both weak

*To whom correspondence should be addressed.
Abbreviations: SHBG = sex hormone-binding globulin; EE_2 = ethinyl estradiol; ARc = androgen receptor (cytosolic); ERn = estrogen receptor (nuclear); T = testosterone; DHT = 5α-dihydrotestosterone; DMEM = Dulbeccos's modified Eagle's medium; FCS = fetal calf serum; PhR = Phenol Red; PBS = phosphate-buffered saline; SFM = serum-free medium; DCC = dextran-coated charcoal; IRMA = immuno radiometric assay; ELISA = enzyme-linked immunosorbent assay.

stimulatory effects of tamoxifen on SHBG production [14, 16, 17] but strong inhibition by the antiandrogen cyproterone acetate [14] have been shown. All these studies employed culture media containing Phenol Red as indicator, which has known estrogenic properties [18–20], and all but two studies [15, 17] used fetal calf serum.

In the present study, Hep G2 cell culture was performed in serum-free medium, in the absence of Phenol Red, using a wide range of concentrations of testosterone (T) and ethinyl estradiol (EE_2) starting at low levels. Secreted SHBG and albumin levels were correlated with each other, and with DNA levels and also with levels of cytosolic and nuclear receptors for androgens (T-stimulated cells) and estrogens (EE_2-stimulated cells).

METHODS

Cell culture

(i) Hep G2 cell line: Hep G2 cells were obtained from the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852-1776, U.S.A.). Growth media were obtained from Gibco U.K. and Imperial Laboratories U.K. and all additives and chemicals, of the purest available grade, were obtained from Sigma U.K.

(ii) All manipulations were performed under sterile conditions in a MDH (Inter Med) laminar flow cabinet. The cells were incubated at 37°C with 5% added CO_2 in a Leec Mk II proportional temperature incubator. Each experiment was repeated a minimum of four times. Regular checks for mycoplasma infection were made (Mycotect, Gibco U.K.) and were consistently negative.

(iii) The cells were initially plated onto 75 cm^2 plastic flasks (approx. 10^7 cells per plate) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and Phenol Red (PhR) as a pH indicator.

(iv) The cells were then collected using 0.25% Trypsin–EDTA solution in phosphate-buffered saline (PBS) before centrifugation (110 *g*-bench top centrifuge IEC Centra 4X; 1000 rpm). The cellular pellet was then resuspended in DMEM with FCS and PhR.

(v) An aliquot was counted in a standard hemocytometer and 5×10^6 cells plated per 75 cm^2 flask with 6 ml of medium per flask.

(vi) After 48 h culture, the medium was discarded and the cells were washed three times with PBS; DMEM without FCS or PhR was added (supplemented with essential vitamins, amino acids, insulin and glucagon—see Table 1).

(vii) The cells were cultured for 48 h in this serum-free medium (SFM) to diminish any sex steroid-like effects of PhR and growth-stimulating effects of FCS.

Steroid incubation

(i) Tritium labeled 5-α-dihydro [1,2,4,5,6,7-^{3}H] (3.96 TBq/mmol) androstan-17β-ol-3-one ([^{3}H]DHT) and [1,6,7-^{3}H]17β-estradiol (5.55 TBq/mmol) were obtained from Amersham U.K.

(ii) Sterile testosterone (T) solutions were prepared in duplicate by evaporating acetone solubilized steroid, and then adding SFM (giving concentrations of 0, 0.5, 5, 12.5, 50 and 500 nM T). In a parallel series of experiments, similar incubations were performed with ethinyl estradiol (EE_2) at concentrations of 0, 0.005, 0.05, 0.5, 5, 50, 500 nM in place of testosterone. Steroid concentrations in the resultant medium were checked by scintillation counting.

(iii) Each flask was then incubated with 6 ml of SFM containing T at the appropriate concentrations. Final yield: approx. 10^7 cells, 100 mg wet weight.

(iv) After 48 h of steroid incubation, the medium was aspirated and stored at −20°C for albumin and SHBG assays.

SHBG assay

Levels of SHBG were estimated by the two-tier column assay [21]. Samples were obtained from the used culture medium after 48 h incubation with steroid. Excess added steroid was adsorbed with dextran-coated charcoal (DCC) to prevent competition with ligand in the subsequent assay. Samples (1 ml) were vortex mixed with 50 μl of 0.1% dextran and 1% activated

Table 1. Composition of serum-free medium

Supplemented Dulbecco's modified Eagle's medium Supplements	Concentrations
L-Glutamine	2 nM
Non-essential amino acids/MEM vitamins (Gibco)	5 ml/500 ml
Linoleic acid/albumin conjugate (Sigma)	2.5 μg/ml
Transferrin (Collaborative Research)	10 μg/ml
Bovine fibronectin	2.5 μg/ml
Bovine insulin	10 μg/ml
Glucagon	3 μg/ml
Triiodothyronine	10 pM
Sodium selenite	10 nM
$ZnSO_4$	5 nM
$CuSO_4$	5 nM

charcoal in 10 nM Tris–5 nM $CaCl_2$ buffer and then centrifuged at 900 *g* for 15 min. The supernatant was then diluted 1 : 1 with Tris–$CaCl_2$ buffer prior to standard assay procedures. Representative samples at each concentration of steroid were estimated for SHBG using an immunoradiometric assay (IRMA) (Farmos, distributed by Pharmacia U.K. or an enzyme-linked immunosorbent assay (ELISA) (Dako Patts).

Steroid receptor assay

In those samples incubated with testosterone, androgen receptor levels were measured using the two-tier, affinity immobilization micro assay [22] modified for use in tissue culture as described elsewhere [23].

Estrogen receptor assay

In the cells incubated with EE_2, estrogen receptors (ER) were measured by the same technique but with the substitution of 17β-estradiol as steroid ligand. The nuclear pellet was kept for DNA assay.

DNA assay

DNA measurement was performed using an ethidium bromide fluorimetric technique [24]. DNA was used as a measure of cell number because variable "clumping" of Hep G2 cells limited the accuracy of automated cell counter estimations.

Statistics

Statistical analysis was performed using the two tailed *t*-test and regression analysis with the Minitab (IBM) and Statistical Analysis for Clinical Sciences package [25].

RESULTS

Sex hormone binding globulin was detected in all batches of used culture medium, concentrations ranging from 59 to 289 pmol/flask, K_d: 10^{-9}–10^{-8}. These results, which were obtained using the ligand binding assay, were confirmed by IRMA or ELISA. The response of Hep G2 cells to testosterone in terms of SHBG production was markedly biphasic [Fig. 1(a)]. The production of SHBG was maximal in the absence of added steroid and fell at low levels of added steroid, reaching a nadir of 39% of control values in the presence of an initial testosterone concentration of 5 nM. At concentrations above this level, SHBG production increased, returning to 97% of control values at 500 nM testosterone.

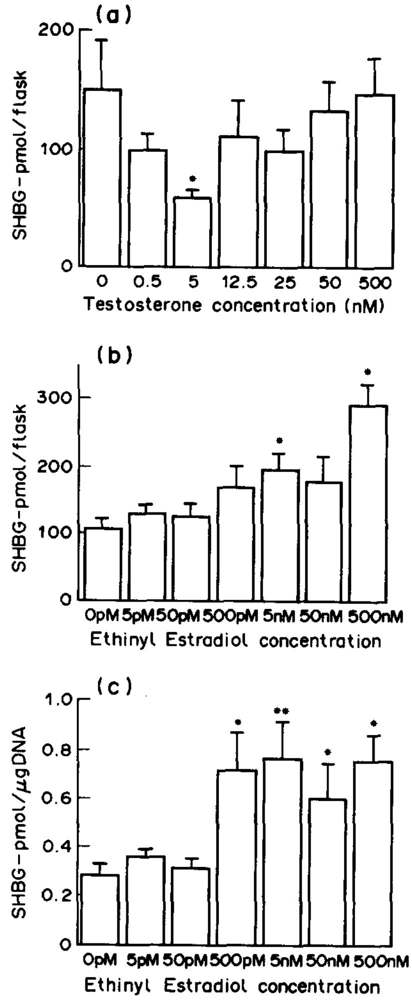

Fig. 1. The effect of testosterone and ethinyl estradiol on SHBG secretion by Hep G2 cells. (a) SHBG secretion expressed as pmol/flask (mean ± SEM), after 48 h culture in serum-free, Phenol Red-free medium (SFM), supplemented with increasing concentrations of testosterone (0–500 nM, after evaporation of the ethanol vehicle). As DNA production was unaltered, correction for DNA did not alter the pattern of results (*$P < 0.05$, $n = 8$). (b) SHBG secretion expressed as pmol/flask after 48 h culture in SFM supplemented with increasing concentrations of ethinyl estradiol (0–500 nM), in the same manner as for (a) (*$P < 0.05$, $n = 6$). (c) SHBG secretion data from (b), re-expressed as pmol SHBG per μg DNA, showing the increase in SHBG levels at all concentrations above 50 pM (*$P < 0.05$, **$P < 0.01$, $n = 6$).

Production of SHBG was markedly increased from 0.5 to 500 nM EE_2 [Fig. 1(b)]. There was little effect of the steroids on Hep G2 cell growth. No significant change in DNA concentration was detected at any level of testosterone or EE_2 studied except for a 15% reduction in DNA at 5 nM EE_2 ($P < 0.05$). In those samples where it was possible to express SHBG values per mg DNA, a similar pattern of falling SHBG secretion at 0.5 and 5 nM testosterone was obtained, but this amounted to only four experiments at each level of testosterone and the differences were not significant. In the case of EE_2, where six or more values were obtained at each concentration of steroid, the changes in SHBG were similar to those expressed per flask, and were significant [Fig. 1(c)]. In the testosterone-stimulated cells, the alterations in SHBG secretion correlate with the alterations in AR binding capacity, both cytosolic and nuclear. This relationship continued up to testosterone level of 500 pmol (data not shown). Levels of SHBG correlated with ARc ($R = 0.44$, $P = 0.04$) and particularly with ARn ($R = 0.515$, $P = 0.017$) (Fig. 2).

The levels of ERc ranged from 32 to 258 fmol per mg soluble protein or approx. 1.4 to 7.4×10^3 binding sites per cell and levels of ERn ranged from 20 to 293 fmol/mg protein or 2.5 to 8.4×10^3 binding sites/cell. Nuclear ER, which was just detectable in the absence of added EE_2, rose sharply at the lowest level of added steroid and remained significantly above control values up to the highest level of EE_2 tested. Responses of cytosolic ER were different and only showed a significant fall at the 0.5 and 5 nM values (Fig. 3).

There was a close correlation ($R = 0.83$, $P < 0.001$) between SHBG and ERc, when ER was expressed as fmol/μg nuclear pellet DNA (Fig. 4).

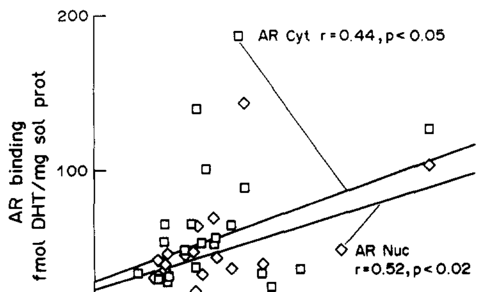

Fig. 2. Correlation between SHBG secretion and nuclear (ARn) and cytoplasmic (ARc) androgen receptor levels: Hep G2 cells were cultured for 2 days after plating in FCS and phenol red (PhR)-containing medium, before a 2 day "wash-out" period in serum-free, PhR-free medium (SFM). Next, for a further 48 h they were cultured in SFM supplemented with testosterone (0–500 nM). Medium was then aspirated and SHBG concentration determined by ligand binding assay and expressed as pmol/flask. The cells were further cultured for 18 h in SFM to wash out added testosterone and then harvested by scraping. Nuclear and cytoplasmic fractions were obtained and the AR levels determined by ligand binding microassay and expressed as fmol/mg soluble protein ($n = 4$, 2 plates per point). Results were analyzed by linear regression.

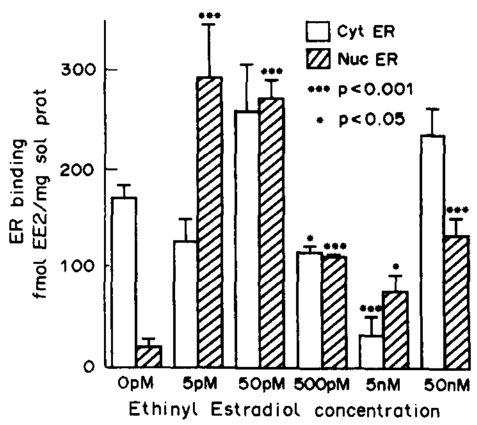

Fig. 3. The effect of ethinyl estradiol concentration on ER levels in Hep G2 cells: Hep G2 cells were cultured for 2 days after plating in FCS and PhR containing medium before a 2 day "wash-out" period in serum-free, PhR-free medium (SFM). Next, for a further 48 h they were cultured in SFM supplemented with ethinyl estradiol (0–50 nM). The cells were further cultured for 18 h in SFM to wash out added steroid and then harvested by scraping before fractionation into nuclear and cytoplasmic components. Receptor levels in nucleus (ERn) and cytoplasm (ERc) were determined by ligand binding microassay and expressed as fmol EE_2 bound per mg by soluble protein (mean $\pm$ SEM) ($n = 4$, 2 plates per point).

Alterations in the levels of albumin were not significant and did not correlate with changes in SHBG.

DISCUSSION

The generally held view that the liver was the source of SHBG was confirmed in 1981 when

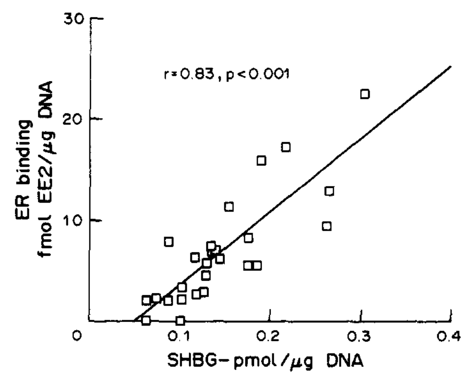

Fig. 4. Correlation between SHBG secretion and cytoplasmic estrogen receptor (ERc) levels: Hep G2 cells were cultured in the same manner and the same methodology used as for Fig. 2, except for the substitution of ethinyl estradiol (0–50 nM) as the active steroid. In this experiment, the nuclear pellet was assayed for DNA content. Estrogen receptors were therefore expressed as fmol per μg nuclear pellet DNA. Cytoplasmic receptor levels for each point ($n = 4$, 2 plates per point) were correlated with SHBG production (expressed as pmol SHBG per μg nuclear pellet DNA).

Rosner and co-workers demonstrated production of SHBG by Hep G2 cells [1]. However, how this secretion is regulated has been a much more controversial matter. To date, studies in Hep G2 cells have shown either no response in terms of SHBG production to sex steroids in the growth medium [13], response to high levels of estradiol or tamoxifen [16, 17] or stimulation by both estrogens and androgens at high levels (500 nM) [14, 15]. It has recently been shown that these changes are accompanied by an increase in SHBG mRNA [17]. Conversely, *in vivo* work has demonstrated a correlation between the estrogen/androgen balance and circulating SHBG levels [4], although not in all circumstances [28, 29]. Likewise, orally (but not parenterally) administered estrogens cause sharp rises in SHBG levels [2].

However, some authors have suggested, that the role of estrogens and androgens in the control of SHBG production is limited to a secondary modulation via the pituitary gland and that the primary mediators of hepatic production of this protein are human growth hormone and somatomedin-C (insulin-like growth factor-I) [28, 29]. It is certainly known that a number of non-sex hormones do influence SHBG production by Hep G2 cells. Thyroxine [13, 16, 17] and dexamethasone [16] have been shown to increase SHBG secretion in this experimental system, while prolactin and insulin [15] inhibited it. The present study agrees with previous work in tissue culture in that SHBG is secreted by Hep G2 cells but it differs markedly from those earlier studies in that it shows direct stimulation of SHBG secretion by estrogen and inhibition by testosterone at levels approximating to physiological values. In this it seems to support the earlier studies in intact man which suggested that SHBG secretion was controlled by the estrogen/androgen balance [4].

The reasons for the differences of the present results from other *in vitro* studies, which themselves disagreed in many areas, can only be guessed at but certain methodological differences are apparent. Firstly, no previous study in cell culture has used such low levels of steroids. Further differences include; different incubation schedules, the use of a prolonged "wash-out" period minimizing the effects of endogenous sex-steroids or those contained within the growth media prior to the experiments, the use of a sensitive ligand binding technique for SHBG, the absence of Phenol Red (a weak oestrogen [18–20]) from our media, and the avoidance of the use of ethanol to add supplementary steroids.

The effects of sex-steroids on protein production appear to be relatively specific to SHBG. There is no significant change in albumin secretion, nor in fetal steroid binding protein levels (unpublished studies), in response to EE_2. This rules out the possibility that the changes in SHBG are due to a non-specific effect on protein synthesis/secretion.

The present results show that levels of androgens and estrogens, close to those that might be found physiologically or with low dose estrogen therapy, produce effects on SHBG production in liver cells in culture which were predicted by *in vivo* studies. This suggests, in agreement with others [17], that at least some of the effects of the estrogen and androgen balance on SHBG production are mediated directly via receptors for these steroids in the liver cells. This impression is strengthened by the correlation of androgen receptor levels and SHBG production and also of ER levels and SHBG, when both are expressed in terms of the levels of nuclear pellet DNA. It had been shown previously that the reduction of SHBG levels by human chorionic gonadotrophin requires an intact 5α-reductase enzyme system [30] and that its reduction after 3 days of orally administered stanozolol requires an intact androgen receptor [31]. It is not clear however whether such androgen receptor needs to be present in the liver or in the pituitary gland/hypothalamus.

Although we have demonstrated an effect on SHBG secretion by the use of an androgen, testosterone, the rapid metabolism of this steroid by the liver means that the effect we are observing may be partly due to its metabolites. The major metabolism of testosterone in Hep G2 cells is towards androstenedione and the estrogens [32] but since the changes in SHBG are so different from those obtained with ethinyl estradiol or from the effects by using the indicator phenol red which has estrogenic properties, it seems likely that androgenic influences are also operating. The effect of DHT in this system is currently under investigation.

As it has been demonstrated recently in a number of studies that SHBG may exert at least some of its effects following binding to cell membranes (summarized in Ref. [33]) it cannot be discounted that the SHBG produced during the course of the experiment is modulating its own synthesis, and further, that any estimate of synthetic rate is likely to be an underestimate, not only because of this absorption, but also due to possible denaturation of secreted SHBG in the medium.

The present study suggests that the secretion of SHBG is controlled at least in part by the direct action of both estrogens and androgens on hepatocytes, probably mediated via steroid receptors but does not exclude additional non-steroidal influences. This system, Hep G2 cells grown in serum-free and Phenol Red-free medium, appears to be appropriate for studying the synthesis of this protein and further studies with antihormones, progestagen, enzyme inhibitors and other non-steroid hormones should help to clarify how this control is mediated. Recent evidence suggests that transcriptional events are of prime importance [17] but confirmation of these results is lacking and the mechanisms leading to the increase in mRNA remain to be examined.

Acknowledgements—S.E.J.E. was supported by the Special Trustees of Guy's Hospital. A.P.S. was supported by the Cancer Research Campaign and A.A.S. by the Junta Nacional de Investigacao Cientifica e Technologia, Lisbon, Portugal and M.L.W. by the New Moorgate Trust. Dr P. Tomlinson of the Department of Paediatrics at Guy's Hospital developed and gave invaluable help with the albumin assay.

REFERENCES

1. Khan M. S., Knowles B. B., Aden D. P. and Rosner W.: Secretion of testosterone-estradiol-binding globulin by a human hepatoma-derived cell line. *J. Clin. Endocr. Metab.* **53** (1981) 448–449.
2. Vermeulen A.: Physiology of the testosterone-estradiol binding globulin in man. In *Steroid–Protein Interactions* (Edited by R. Frairia, H. L. Bradlow and G. Gaidano). *Ann. N.Y. Acad. Sci.*, New York, Vol. 538 (1988) pp. 103–111.
3. Dowsett M., Atree S. L., Virdee S. S. and Jeffcoate S. L.: Estrogen-related changes in sex hormone binding globulin levels during normal and gonadotropin stimulated menstrual cycles. *Clin. Endocr.* **23** (1985) 303–312.
4. Anderson D. C.: Sex hormone binding globulin. *Clin. Endocr.* **3** (1974) 69–96.
5. Van Look P. F. A. and Frohlich M.: Effects of ethinyl estradiol on plasma levels of pituitary gonadotrophins, testicular steroids and sex hormone binding globulin in normal men. *Clin. Endocr.* **14** (1981) 237–243.
6. Ruokonen A., Alen M., Bolton N. and Vihko R.: Response of serum testosterone and its precursor steroids, SHBG and CBG to anabolic steroid and testosterone self-administration in man. *J. Steroid Biochem.* **23** (1985) 33–38.
7. Duffy M. J. and Duffy G. J.: Estradiol receptors in human liver. *J. Steroid Biochem.* **9** (1978) 233–235.
8. Bannister P., Sheridan P. and Losowsky M. S.: Identification and characterization of the human hepatic androgen receptor. *Clin. Endocr.* **23** (1985) 495–502.
9. Iqbal M. J., Wilkinson M. L., Johnson P. J. and Williams R.: Sex steroid receptor proteins in fetal, adult and malignant human liver tissue. *Br. J. Cancer* **48** (1983) 791–796.
10. Tam S. P., Archer T. K. and Deeley R. G.: Effects of estrogen on apolipoprotein by the human hepatomacarcinoma cell line, Hep G2. *J. Biol. Chem.* **260** (1985) 1670–1675.
11. Knowles B. B., Howe C. C. and Aden D. P.: Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209** (1980) 497–499.
12. Coezy E., Auzan C., Lonigro A., Philippe M., Menard J. and Corvol P.: Effect of mestranol on cell proliferation and angiotensinogen production in Hep G2 cells; relation with the cell cycle and action of tamoxifen. *Endocrinology* **120** (1987) 133–141.
13. Rosner W., Aden D. P. and Khan M. S.: Hormonal influences on the secretion of steroid-binding proteins by a human hepatoma-derived cell line. *J. Clin. Endocr. Metab.* **59** (1984) 806–808.
14. Lee I. R., Dawson S. A., Wetherall J. D. and Hahnel R.: Sex hormone-binding globulin secretion by human hepato-carcinoma cells is increased by both estrogens and androgens. *J. Clin. Endocr. Metab.* **64** (1987) 825–831.
15. Plymate S. R., Matej L. A., Jones R. E. and Friedl K. E.: Inhibition of sex hormone-binding globulin production in the human hepatoma (Hep G2) cell line by insulin and prolactin. *J. Clin. Endocr. Metab.* **67** (1988) 460–464.
16. Mercier-Bodard C. and Baulieu E.-E.: Hormonal control of SBP in human hepatoma cells. *J. Steroid Biochem.* **24** (1986) 443–448.
17. Mercier-Bodard C., Baville F., Bideux G., Binart N., Chambraud B. and Baulieu E.-E.: Regulation of SBP synthesis in human cancer cell lines by steroid and thyroid hormones. *J. Steroid Biochem.* **34** (1989) 199–204.
18. Berthois Y., Katzenellenbogen J. A. and Katzenellenbogen B. S.: Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 2496–2500.
19. Rajendran K. G., Lopez T. and Parikh I.: Estrogenic effect of phenol red in MCF-7 cells is achieved through activation of estrogen receptor by interacting with a site distinct from the steroid binding site. *Biochem. Biophys. Res. Commun.* **142** (1987) 724–731.
20. Ernst M., Schmit C. and Froesch E. R.: Phenol red mimics biological actions of estradiol: enhancement of osteoblast proliferation *in vitro* and of type I collagen gene expression in bone and uterus of rats *in vivo*. *J. Steroid Biochem.* **33** (1989) 907–914.
21. Iqbal M. J. and Johnson M. W.: Study of steroid–protein binding by a novel "two-tier" column employing Cibacron Blue F3G-A–Sepharose 4B. I—Sex hormone binding globulin. *J. Steroid Biochem.* **8** (1977) 977–983.
22. Iqbal M. J., Corbishley T. P., Wilkinson M. L. and Williams R.: A microassay for the determination of binding parameters of estrogen and androgen receptors employing affinity immobilisation on Cibacron Blue 3GA–Sepharose 6B. *Analyt. Biochem.* **144** (1985) 79–85.
23. Edmunds S. E. J., Santos A. A., Stubbs A. P. and Wilkinson M. L.: Androgen receptor levels, albumin production and growth of a human hepatoma cell line (Hep G2) in response to testosterone. (1990) (Submitted for publication).
24. Prasad A. S., Dumouchelle E., Koniuch D. and Oberleas D.: A simple fluorometric method for the determination of RNA and DNA in tissues. *J. Lab. Clin. Med.* **30** (1972) 598–602.
25. Chalmer T. (Ed.): *Data Analysis for Clinical Medicine*. International University Press, Oxford (1988).
26. Leake R. E., Freshney R. I. and Munir I.: Steroid response *in vivo* and *in vitro*. In *Steroid Hormones a*

Practical Approach (Edited by B. Green and R. E. Leake). IRC Press, Oxford (1987) pp. 205–218.
27. Adams R. L. P.: *Cell culture for Biochemists*. Elsevier/North Holland, Oxford (1980) pp. 191–195.
28. Von Schoultz B. and Carlstrom K.: On the regulation of sex-hormone-binding globulin—a challenge of an old dogma and outlines of an alternative mechanism. *J. Steroid Biochem.* **32** (1989) 327–334.
29. Holly J. M. P., Smith C. P., Dunger D. P., Howell R. J. S., Chard T., Perry L. A., Savage M. O., Cianfarani S., Rees L. H. and Wass J. A. H.: Relationship between the pubertal fall in sex hormone binding globulin and insulin-like growth factor binding protein—I. A synchronized approach to pubertal development? *Clin. Endocr.* **31** (1989) 277–284.
30. El-Awady M. K., Salam M. A., Gad Y. Z. and El-Saban J.: Dihydrotestosterone regulates plasma sex-hormone-binding globulin in prepubertal males. *Clin. Endocr.* **30** (1989) 279–284.
31. Sinnecker G. and Kohlers S.: Sex hormone-binding globulin response to the anabolic steroid Stanozolol: evidence for its suitability as a biological androgen sensitivity test. *J. Clin. Endocr. Metab.* **68** (1989) 1195.
32. Stubbs A. P., Edmunds S. E. J., Murphy G. M. and Wilkinson M. L.: Testosterone metabolism in human hepatoma (Hep-G2) cells. *Biochem. Soc. Trans.* (1990) In press.
33. Rosner W., Hryb D. J., Khan M. S., Singer C. J. and Nakhla A. M.: Are corticosteroid-binding globulin and sex hormone-binding globulin hormones? In *Steroid–Protein Interactions* (Edited by R. Frairia, H. L. Bradlow and G. Gaidano). *Ann. N.Y. Acad. Sci.*, New York, Vol. 538 (1988) pp. 137–145.