Regulation of Sex Hormone Binding Globulin and Insulin-like Growth Factor Binding Protein-1

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by

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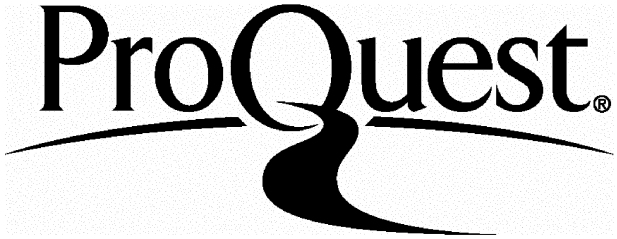

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Abbreviations

BMI	Body Mass Index
CBG	Corticosteroid Binding Globulin
cDNA	copy Deoxyribose nucleic acid
CV%	Coefficient of variation (percentage)
DHT	Dihydrotestosterone
Δ-4-OH-A	Delta-4-hydroxy androstenedione
E_2/E	Oestradiol
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
FCS	Fetal Calf Serum
FSH	Follicle stimulating hormone
GH	Growth Hormone
Hep G2	Hepatocarcinoma G2 cells
^{125}-I	Radiolabelled ^{125}-Iodine
I	Insulin
IGF-1	Insulin-like growth factor-1
IGFBP(1-6)	Insulin-like growth factor binding protein (1-6)
IRMA	Immunoradiometric assay
LH	Luteinising hormone
MCR	Metabolic Clearance Rate
mRNA	messenger-Ribose Nucleic Acid
NSB	Non-specific binding
OGTT	Oral glucose tolerance test
P	Progesterone
PBS	Phosphate buffered saline
PCOS	Polycystic Ovary Syndrome
PHLS	Public Health Laboratory Services
QCL/H	Quality control Low/High
RIA	Radioimmunoassay
SD	Standard Deviation
SE	Standard Error
SHBG	Sex Hormone Binding Globulin
T	Testosterone
T_4	Thyroxine
TBG	Thyroid binding globulin

Publications associated with this thesis

Diet induced changes in sex hormone binding globulin and free testosterone in women with normal or polycystic ovaries: Correlation with serum insulin and insulin-like growth factor1. D.S. Kiddy, D.Hamilton-Fairley, M.Seppälä, R.Koistinen, V.H.T.James, M.J.Reed, S.Franks Clinical Endocrinology 1989;31:757-763.

Effect of insulin-like growth factor-type 1 (IGF-1) and insulin on the secretion of sex hormone binding globulin and IGF Binding Protein (IBP-1) by human hepatoma cells. A.Singh, D.Hamilton-Fairley, R.Koistinen, M.Seppälä, V.H.T.James,S.Franks,M.J.Reed, Journal of Endocrinology 1990; 124:R1-R3

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Response of sex hormone binding globulin (SHBG) and insulin-like growth factor binding protein (IGFBP-1) to an oral glucose tolerance test (OGTT) in obese women with polycystic ovary syndrome before and after a low calorie diet. D.Hamilton-Fairley, D. Kiddy, V.Anyaouku, R.Koistinen, M.Seppälä., S.Franks. Clinical Endocrinology 1993;39:363-367.

Abstracts

Abnormal 24 hour insulin secretion and the relationship with sex hormone binding globulin (SHBG) and insulin-like growth factor binding protein-1 (IGFBP1) in women with polycystic ovary syndrome D.Hamilton-Fairley, D.White, M.Griffiths, V.Anyaoku, R.Koistinen, M.Seppälä, S.Franks Journal of Endocrinology 1992 ;132 (Suppl):43 Oral presentation.. Submitted to Clinical Endocrinology March 1995.

Steroid Hormone regulation of sex hormone binding globulin (SHBG) and insulin-like growth factor binding protein-1 (IGFBP1) in hepatocarcinoma G2 cells. D.Hamilton-Fairley, R.Koistinen, M.Seppälä., S.Franks. Journal of Endocrinology 1991;131 (suppl):49

Declaration

In this thesis I undertook all the SHBG measurements in both the clinical and in-vitro studies. I undertook all the cell culture experiments presented in this thesis with the exception of the first in-vitro experiment using insulin which was performed by Anita Singh. The clinical studies were done in collaboration with Deborah Kiddy and Dr Davinia White. All data was collated and analysed by myself.

ABSTRACT

Women with polycystic ovary syndrome (PCOS) present most commonly with hirsutism and/or anovulation. The prevalence of hirsutism and/or menstrual irregularity is increased in obese women with PCOS compared with lean women with PCOS. It is,therefore, possible that factors associated with obesity exacerbate the symptoms and the underlying hormonal abnormality of this condition. Sex hormone binding globulin (SHBG) and insulin-like growth factor binding protein-1 (IGFBP1) concentrations are reduced in PCOS and decline during puberty in boys and girls. The regulation of sex hormone binding globulin has been thought to be primarily by the sex steroid hormones and that of IGFBP1 by insulin.

The hypothesis of this thesis is that dietary factors are more important than the sex steroids in the regulation of hepatic SHBG production and that this is of relevance in understanding the relationship between obesity and hirsutism in women with polcystic ovary syndrome. In addition insulin also regulates hepatic IGFBP1 production implying that the two binding proteins may be co-regulated. The role of sex steroids in the regulation of IGFBP1 is not known.

The regulation of hepatic production of SHBG and IGFBP1 by insulin, IGF-1 and the sex steroids was studied in-vitro by cell culture experiments on Hepatocarcinoma G2 cells. The relationship of dietary factors on androgen metabolism and circulating SHBG and IGFBP1 concentrations was investigated in three separate studies. In the first two studies women with PCOS were studied before and after calorie restriction and during an oral glucose tolerance test (OGTT). A third study conducted over a 24 hour period investigated the diurnal variation in SHBG and IGFBP1 related to that of insulin and IGF-1.

The cell culture experiments demonstrated a role of insulin and IGF-1 as inhibitors of hepatic SHBG production but only insulin inhibited the secretion of IGFBP1. The effect of the sex steroids was less clear since testosterone increased SHBG production while oestradiol had no effect. The sex steroids had no effect on IGFBP1 production.

Weight loss following a four week very low calorie diet was associated with a significant increase in SHBG and IGFBP1 concentrations mirrored by a decrease in insulin

and IGF-1. This resulted in a decrease in free testosterone although the total testosterone concentration did not change. The inverse relationship of SHBG and IGFBP1 with insulin was further confirmed in the OGTT and 24 hour study although the role of IGF-1 was less clear. The time course of the changes was significantly longer for SHBG than IGFBP1.

These studies demonstrate that insulin is a primary regulator of SHBG and IGFBP1 synthesis by the liver. The two binding proteins may be co-regulated by insulin but other factors including the sex steroids may alter their half-lives in the circulation and therefore their serum concentration under various physiological and pathological conditions.

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CHAPTER 1

INTRODUCTION

1.1. SEX HORMONE BINDING GLOBULIN (SHBG)

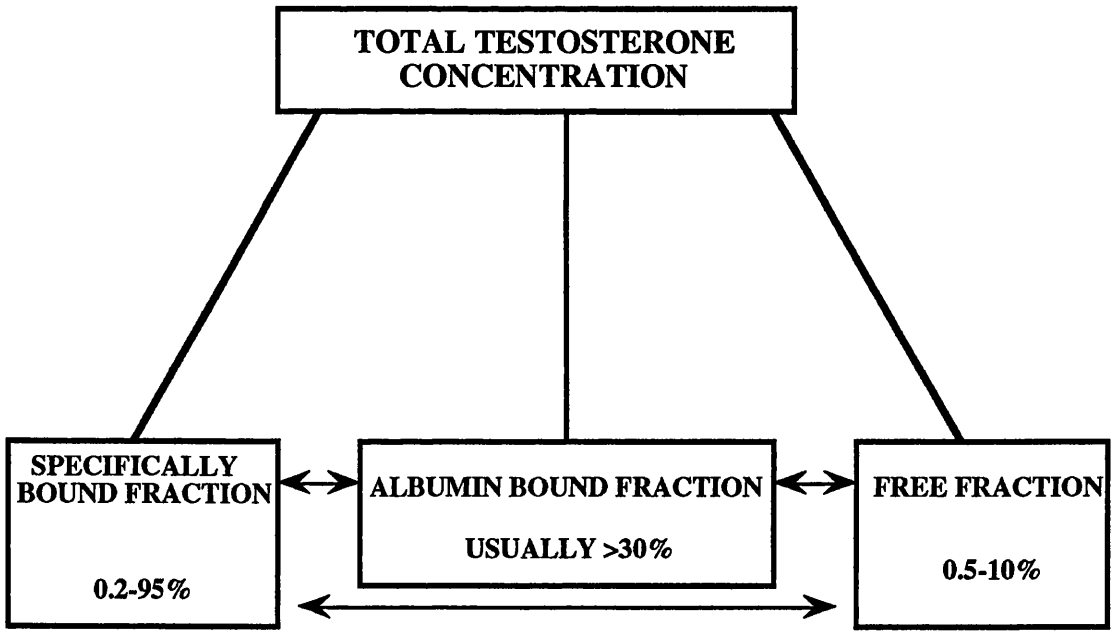
1.1.1 DISCOVERY AND STRUCTURE

The existence of a sex hormone binding protein in human serum was first discovered by Mercier Bodard et al. and Rosenbaum et al. in 1966. Since then its structure has been elucidated and its amino acid sequence established chemically and confirmed by the isolation and sequencing of a cDNA for SHBG isolated from a human liver library (Hammond et al. 1987, Que and Petra 1987, Gershagen et al. 1987). SHBG is a specific steroid binding glycoprotein and circulates in plasma as a dimer. The SHBG monomer is a single chain polypeptide containing 373 amino acids, 3 polysaccharide chains and 2 disulfide bonds. The circulating molecule is a homodimer which binds a single steroid molecule (Petra et al. 1983, Hammond et al. 1986). The steroid binding site of the molecule is thought to reside in a hydrophobic area from Leu-267 to Leu-281 which contains a region of alternating leucine residues (Petra et al. 1986) although more recent data has suggested that histidine 235 is also a steroid binding site. This residue may abut onto other areas of the SHBG molecule in its tertiary structure (Khan et al. 1990). The relative binding affinities of the steroid hormones to SHBG are: dihydrotestosterone, 100; testosterone, 45; oestradiol, 22; oestrone, 5; oestriol, 0.2. Although the affinity of SHBG for dihydrotestosterone is the greatest testosterone is the principal ligand in both males and females. The binding of the sex steroids to SHBG is reversible although its binding capacity may change under certain physiological and pathological conditions (Vermeulen et al. 1969).

1.1.2. ROLE OF SHBG IN ANDROGEN METABOLISM

Testosterone exists in serum in three states (Westphal 1971): free, albumin bound and SHBG bound. It is generally considered that SHBG bound testosterone is not available for uptake by tissues in vivo although opinion is mixed as to whether the biologically active androgen in serum is confined to the free fraction (~2%) or includes the larger proportion (~20-80%) of serum testosterone that is bound to albumin (Pardridge 1986) and that bound to SHBG. The inter-relationship between these three states is shown in Figure 1.1.

FIGURE 1.1. Transport of steroid hormones in human plasma



The most commonly held view is that the biological activity of a given hormone is affected by its unbound (free) rather than protein bound concentration in the plasma - the Free Hormone hypothesis - (Robbins and Rall 1957, Tait and Burstein 1964, Sandberg et al. 1966) and that the hormone enters the cell by diffusion with little or no active transport mechanism. In a recent review of the subject by Carl Mendel (1989) he states, in support of the hypothesis, that "for all hormones studied, in combination with all binding proteins studied, spontaneous dissociation of the protein-hormone complex has always been found

to be rapid enough to account for the observed tissue uptake of hormone entirely via the pool of free hormone". Many other factors play a part in the tissue uptake of hormones including plasma flow, influx and intracellular elimination. In accordance with this scheme an equilibrium exists, therefore, between the bound and free hormone both intra- and extracellularly and this may vary from tissue to tissue.

The human liver removes 40-50% of the testosterone presented to it in the plasma (Siiteri 1986a). This is close to the maximum predicted unidirectional uptake of testosterone calculated from the dissociation rates of testosterone from its plasma binding proteins. Intracellular concentrations of testosterone in the liver are, therefore, likely to be a function of the amount of hormone that can dissociate from the binding proteins during sinusoidal transit. In normal women the liver is the principal site of testosterone metabolism and very little is metabolised in peripheral tissues. In men and hyperandrogenic women proportionally more of the testosterone is metabolised peripherally and it is here that the concentration of free hormone is thought to be important. The metabolic clearance rate (MCR) of testosterone is inversely related to the concentration of SHBG in plasma in several physiological and pathological conditions (Vermeulen et al. 1969).

The contribution of the albumin bound fraction of testosterone has aroused much debate and several studies have shown that the free testosterone concentration plus that which is albumin bound is a better predictor of androgen metabolic status than the free testosterone concentration alone - whether the latter was calculated or measured (Rosenfield 1971, Lawrence et al. 1981, Mathur et al. 1981, Cunningham et al. 1983, Cumming and Wall 1985) - and this is supported by the findings of Siiteri et al. (1982). Since the dissociation constant of testosterone from albumin is considerably lower than that of SHBG [K_A SHBG $2x10^9$; Albumin $4x10^4$ (Heyns and De Moor 1971, Dunn et al. 1981)], the free hormone hypothesis is not disproved but other rate-limiting steps may be involved in determining the final concentration of free hormone available for uptake.

Immunocytochemical localisation of plasma steroid binding proteins within cells has been

reported (Bordin and Petra 1980) although their biological role has not been proven (Siiteri 1986a and b) and their concentrations are low. An endocytic mechanism for steroid uptake bound to SHBG and corticosteroid binding globulin (CBG) has been put forward and strengthened by the finding of "specific" binding sites for plasma steroid binding proteins on prostatic carcinoma (Hyrb et al. 1985, Rosner et al. 1986b) and endometrial cell membranes (Strel'Chyonok et al. 1984). Transport of bound steroids into target cells could provide several advantages over the free diffusion model, in particular protection from metabolism ensuring that adequate concentrations of hormone reach nuclear receptors and the possibility of up and down regulation of receptors which would allow more specific hormone delivery to target tissues (Siiteri 1986a). This theory remains speculative and the evidence is not strong enough to supercede the free hormone hypothesis.

The distribution of testosterone and oestradiol in serum shows a considerable difference between the sexes (Table 1). Testosterone is bound in similar proportions to albumin and SHBG in the male while in the female a greater proportion is bound to SHBG which, if the free hormone hypothesis applies, reflects the fact that the dissociation constant of albumin is less than that of SHBG, thereby allowing testosterone to be more readily available to peripheral tissues in men than women. The proportion of testosterone or oestradiol in each fraction changes under differing physiological (and presumably pathological) conditions

Table 1.1. Distribution of testosterone and oestradiol in human serum

		Distribution %		
Hormone	Condition	Free	Albumin-bound	SHBG-bound
Testosterone	Male	2.2	50	44
	Female	1.4	30	66
	Pregnancy	0.2	4	95
Oestradiol	Male	2.3	78	20
	Female	1.8	61	37
	Pregnancy	0.5	12	88

and this may reflect an alteration in the circulating concentration of SHBG whether by changes in the production or clearance of the protein.

The function of SHBG seems, therefore, to be to act as a reservoir for the sex steroid hormones, predominantly testosterone and oestradiol. Under appropriate conditions the MCR of these hormones may be altered and SHBG may protect the hormones from hepatic degradation and glomerular filtration, "buffer" free hormone concentrations and provide an increased (or decreased) circulating pool of hormone that can be delivered to tissues (Mendel 1989). For the purposes of this thesis I have therefore assumed that the free hormone hypothesis is valid in hyperandrogenic women and the calculation of free hormone concentrations, therefore, takes no account of albumin concentration.

1.1.3 REGULATION OF SHBG

Circulating SHBG concentrations are low in neonatal cord blood and rise rapidly in the first 10 days of life, then more slowly until the age of two months, after which the levels remain fairly constant until puberty. During puberty the levels decline rapidly in both sexes but more in boys than girls so that adult levels of SHBG are significantly higher in women compared with men (Forest et al. 1986).

The concentration of SHBG in plasma has been thought to be primarily regulated by the sex steroids and, in particular, testosterone and oestradiol (Anderson 1974, Bond and Davis 1987) since hyperandrogenic conditions are associated with low levels of SHBG and conditions in which the oestradiol is raised with an increased SHBG concentration (See Table 1. 2.).

SHBG levels are known to be high during pregnancy. The rise is seen from seven weeks gestation (Pearlman et al. 1967) reaching a peak in the third trimester (Heyns and De Moor 1971, Rosner 1972, Vermeulen et al. 1969). The evidence that this is associated with increased oestrogen levels is supported by the rapid decline immediately post partum when

SHBG concentrations return to pre-pregnancy levels within 4-5 days (Anderson 1974) although the correlation between oestrogen levels and SHBG has been found to be weak (Skjöldebrand et al. 1988).

Oral oestrogen therapy is known to increase SHBG concentrations (refs. Table 2). Percutaneous or parenteral administration (intramuscular, vaginal) of oestrogen has minimal effects on SHBG concentrations despite similar serum concentrations of 17β-oestradiol being achieved by all routes of administration (Elkik et al.1982, Goebelsmann et al.1985, Judd 1987). The effect of oral oestrogen on SHBG concentrations may, therefore, represent a pharmacological rather than a physiological effect on the liver. Dietary oestrogens derived from foods high in fibre (phyto-oestrogens) may also contribute to an increase in SHBG concentrations by entering the enterohepatic circulation (Adlercreutz et al. 1987).

Studies conducted during the menstrual cycle have usually shown no change in SHBG concentrations (Anderson 1974, Pearlman et al. 1967) although there is some evidence of an increase in the luteal phase both in women with regular ovulatory cycles (Dowsett et al. 1985, Plymate et al. 1985) and women undergoing ovulation induction with gonadotrophins (Dowsett et al. 1985, Ben-Rafael et al. 1986,87). This has been interpreted as a response to the high serum oestradiol levels. In addition, the low SHBG levels found in women with polycystic ovary syndrome (PCOS) have been reported to return towards normal during ovulatory cycles induced by the anti-oestrogen clomiphene citrate (Eden et al. 1989) although this may be a direct oestrogenic effect of the clomiphene itself (Dray et al. 1970, Marshall et al. 1972).

The effect of androgens on SHBG concentrations is variable. Oral administration has been associated with a decrease in SHBG levels (Anderson 1974, Plymate et al. 1983) while parenteral administration, similarly to oestradiol, does not affect SHBG levels significantly (Plymate et al. 1983). In men, SHBG levels remain constant until the age of 50 after which they gradually increase until aged 80 they are double that of young men (Maruyama et al. 1984). This is not related to a decline in testicular function nor testosterone concentrations

although the diurnal variation in SHBG reported in men becomes blunted with age (Plymate et al. 1989). The variation in the concentration of SHBG following oral administration of testosterone is dose dependent and, therefore, thought to reflect the androgen/oestrogen balance (Anderson 1974). The finding that women who were hirsute and/or hyperandrogenic had lower SHBG levels than non-hirsute women further supported this concept. Since neither total testosterone nor free testosterone concentrations were found to be a good marker of clinical hirsutism or androgen status the ratio between SHBG and testosterone was investigated. Several studies have found the ratio to be a better indicator of androgen status although the principal determinant of this ratio is SHBG and not testosterone (Rosenfield 1971, Mathur et al. 1981, Cunningham et al. 1983, Wilke and Utley 1987). The evidence for a principle role of SHBG in determining the availability of testosterone to tissues is therefore strong but, as can be seen in Table 1. 2, there are a number of other factors that may alter SHBG concentrations which are independent of changes in sex steroid concentrations.

Liver disease can affect circulating concentrations of SHBG. Women with primary biliary cirrhosis and men with alcoholic liver disease both have raised SHBG levels. In men this has been ascribed to raised levels of oestradiol but in women no differences in sex steroid levels have been reported although these women are often anovulatory (Valimaki et al. 1984). In addition, orchidectomy has little effect on SHBG concentrations despite a significant drop in testosterone levels (Leionen et al. 1979, Carlström et al. 1985) and there is little change in SHBG levels after the menopause (Maruyama et al. 1984). In addition many drugs have been shown to alter the concentrations of SHBG and CBG (Pugeat et al. 1981).

The relationship between Growth Hormone (GH) and SHBG in several disease states including acromegaly (decreased SHBG) and chronic liver disease (increased SHBG) as well as in physiological states such as fasting (increased SHBG) led von Schoultz and Carlström (1989) to postulate that GH may directly regulate SHBG production by the liver. This was supported by the finding that SHBG levels declined during puberty in both sexes, a period when growth hormone levels increase significantly.

The decline in SHBG levels during puberty has aroused interest because it occurs in both sexes, although the decrease is greater in boys. The finding is a consistent one having been reported by many groups in girls (Apter et al. 1984, Bartsch et al. 1980), boys (Blank et al. 1978, Belgoroskyand Rivarola 1986) and in both (Lee et al. 1984, Holly et al. 1989). The concept that these changes are causally associated with sex steroid production is supported by the finding of raised levels of SHBG in age-matched boys with hypogonadotrophic hypogonadism (Plymate et al. 1983, Vermeulen et al. 1969) and in subjects with testicular feminisation due to androgen resistance (Rosenfield et al. 1971). This does not, however, explain the decrease seen in girls and, furthermore, a longitudinal study by Cunningham et al. (1983) showed a decrease in SHBG concentrations in both sexes that was independent of pubertal status. Insulin and insulin-like growth factor-1 both increase linearly during puberty (Smith et al. 1988) and a strong inverse correlation between insulin (Holly et al. 1989), IGF-1 (Belgorosky et al. 1986) and SHBG has been reported during puberty. These factors were therefore proposed as possible regulators of SHBG concentrations.

The association of altered SHBG levels in various pathological disorders has, therefore, provided further evidence that the regulation of SHBG is unlikely to be mediated solely by sex steroids. SHBG levels are low in hypothyroidism but return to normal with the administration of thyroxine; the levels are high in thyrotoxicosis but return to normal values within three months of treatment (Anderson 1974). Furthermore, SHBG synthesis by hepatocarcinoma G2 (Hep G2) cells in vitro is stimulated by thyroxine (Rosner et al. 1984, Plymate et al. 1988). There is, however, no correlation between thyroxine levels and SHBG levels during puberty and this casts doubts on thyroxine being a primary regulator of SHBG, at least during sexual development.

Hyperprolactinaemia has been associated with decreased SHBG levels (Vermeulen et al. 1984, Winters and Troen 1984) and SHBG synthesis by Hep G2 cells has been shown to be inhibited by prolactin (Plymate et al. 1988)

Table 1.2. Factors and conditions associated with an altered SHBG concentration

	References
Increased SHBG	
Increased thyroid hormone	Vermeulen et al.1969, Anderson 1974, Petra et al.1985, Cavaliere et al.1988.
Increased oestrogens:	
Pregnancy	Pearlman et al.1967, Vermeulen et al.1969, Rosner 1972, Uriel et al.1981.
Luteal phase of menstrual cycle	Plymate et al.1985, Dowsett et al.1985
Exogenous oestrogens	Vermeulen et al.1969, Rosner 1972, Odlind et al.1982, Bartsch et al.1977, van Kammen et al.1975,Hammond et al.1985, Thomas et al.1987.
Liver disease	Rosner 1972, Tavernetti et al.1967, Valimaki et al.1984, Terasaki et al.1988.
Phenytoin	Victor et al.1977.
Tamoxifen	Sakai et al.1978.
Prolonged stress	Aakvaag et al.1978.
Prostatic carcinoma	Bartsch et al.1977, Leionen et al.1979, Haapiaien et al.1988, Pugeat et al.1988, Carlström et al.1987.
Anorexia Nervosa	Estour et al.1986, Pugeat et al.1988.
Aging men	Bartsch et al.1977, Davidson et al.1983, Maruyama et al.1984, Tenover et al.1987, Plymate et al.1989.
Dietary content	Adlercreutz et al.1987, Anderson et al.1987,Reed et al.1987, Belanger et al.1989.
Decreased SHBG	
Obesity	Vermeulen et al.1969, Glass et al.1977, Plymate et al.1981, Kopelman et al.1981, Laatikainen et al.1983, Grenman et al.1986, Kiddy et al.1990, Weaver et al.1990.
Hyperandrogenaemia in women	Vermeulen et al.1969, 1971 Rosenfield 1971, Lawrence et al.1981, Mathur et al.1981, Biffignandi et al.1984, Carlström et al.1987,Wilke et al.1987.
Androgen replacement	Anderson 1974, Plymate et al.1983.
Hyperprolactinaemia	Lobo & Kletsky 1982, Glickman et al.1982, Winters & Troen 1984, Vermeulen et al.1984, Plymate et al.1988.
Increased Growth Hormone	Vermeulen et al.1969, De Moor et al.1972, Von Shoutz & Carlström 1989.
Menopause	Maruyama et al.1984, Lundberg et al.1985,Lapidus et al.1986
Danazol/Gestrinone	Schwarz et al.1981, Nilsson et al.1983, Gershagen et al.1984, Dowsett et al.1986.
Glucocorticoids	Vermeulen et al.1969, Stanczyk et al.1985, Blake et al. 1988

Body mass is, however, probably the most important factor influencing SHBG levels (Siiteri et al. 1982, Franks 1989). It has been shown in men (Glass et al.1977) and in women (Kopelman et al. 1980, Plymate et al. 1981) that obesity is associated with a decreased SHBG concentration. In contrast SHBG levels are found to be raised in women with anorexia nervosa despite the almost universal finding of amenorrhoea and low oestradiol Estour et al. 1987). In the obese patient, fasting is known to increase SHBG (Kopelman et al. 1981) and in anorexic patients SHBG concentrations decrease during refeeding. The topographical distribution of fat and the size of the adipocytes at various sites are also important determinants of SHBG levels with strong inverse correlations between SHBG and various markers of obesity, including waist:hip ratio and fat distribution in age and sex matched individuals. The various components of diet have been studied and the protein/ carbohydrate ratio was found to reciprocally alter SHBG levels (anderson et al. 1987). Dietary lipids have also been reported to alter SHBG concentrations in men with little or no change in weight or testosterone concentrations (Reed et al. 1987). Women with PCOS have been reported to have abnormal lipid profiles and therefore an increased cardiovascular risk (Wild et al. 1985) which is worsened by obesity (Wild et al. 1988b) implying that dietary factors may contribute to the abnormal levels of SHBG found in women with PCOS.

Obesity is associated with hyperinsulinaemia (Olefsky 1976). An association between hyperandrogenaemia and hyperinsulinaemia has been reported (Burghen et al. 1980, Shoupe et al. 1983, Chang et al. 1983, Dunaif et al. 1987,Pasquali et al. 1989) and this has been shown to be independent of obesity in women with PCOS (Jialal et al. 1987, Dunaif 1989b). The interrelationship between hyperandrogenaemia, obesity, hyperinsulinaemia and decreased concentrations of SHBG may be an important one and this is supported by the preliminary finding that insulin inhibits SHBG secretion by Hepatocarcinoma G2 (Hep G2) cells (Plymate et al. 1988).

Following the discovery that Hep G2 cells secrete most of the plasma binding proteins including SHBG (Knowles et al. 1980, Khan et al. 1981,1984) several groups have explored the possible regulation of SHBG using these cells as an in-vitro model for its hepatic

synthesis. Oestradiol and, paradoxically, testosterone have both been shown to increase SHBG production by Hep G2 cells (Lee et al. 1987, Plymate et al. 1988) although other groups have not found any change in SHBG levels when these steroids were added to the medium (Rosner et al. 1984, Mercier Bodard and Baulieu 1986). Binding of oestradiol to cytosolic receptors could not be demonstrated in the hepatoma cell line (Rosner et al. 1984) but high affinity nuclear oestradiol binding sites have been demonstrated. The functional integrity of these receptors has been shown by the fact that physiological concentrations (20nM) of oestradiol lead to a two-fold increase in apolipoprotein secretion (Tam et al. 1985). Other steroid hormones that have been investigated include dihydrotestosterone leading to an increase in SHBG synthesis, cortisol to a reduction and pregnanediol which had no effect. The finding that cortisol inhibits SHBG production is interesting since dexamethasone has been associated, in vivo, with an increase in SHBG which is thought to be mediated by a decrease in adrenal steroid production (Blake et al. 1988). In women with PCOS who are hyperandrogenaemic, treatment with dexamethasone increased SHBG levels which was presumed to be secondary to a significant fall in testosterone and androstenedione concentrations (Cunningham et al. 1983).

The regulation of circulating SHBG concentrations is therefore likely to be multifactorial and includes effects of sex steroids, thyroxine and peptide hormones such as prolactin, growth hormone, insulin and IGF-1. The site of regulation may be at the level of the hepatocyte by a direct effect on the rate of protein synthesis but there may also be post-translational modification of the structure of SHBG thereby affecting its rate of clearance from the circulation.

1.2. INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP1)

1.2.1 STRUCTURE AND NOMENCLATURE OF IGF BINDING PROTEINS

Insulin-like growth factors (IGF) I and II are growth hormone dependent peptides that are structurally related to insulin. They have anabolic and mitogenic properties in vivo and in vitro but are also potent hypoglycaemic agents (Froesch et al. 1985). They circulate in the serum bound to specific binding proteins so that less than 3% of IGF-1 is free in plasma. Six distinct insulin-like growth factor binding proteins (IGFBP) have been fully characterised and their amino-acid sequences determined (Binoux et al.1986, Ooi and Herrington 1988, Shimasaki and Ling 1991). Their nomenclature became increasingly complex as each was defined by the species, fluid or cell line in which they were discovered. Initially each binding protein discovered was thought to be unique until they were sequenced and the great degree of homology was appreciated. They have now been classified according to their molecular size and a uniform nomenclature was designated in 1990 by an international committee (Anonymous 1990). The binding proteins are well characterised in man. The total number of amino acid residues range from 216 for IGFBP6 to 289 for IGFBP2. Except for IGFBP6 all human IGFBP's contain 18 homologous cysteines; twelve are located at the N-terminal while the remaining six are distributed at the C-terminal. The residues at the N-terminal appear to be essential for IGF-I and -II binding. The principal carrier of IGF-1 in the circulation is a growth hormone dependent IGFBP of 150-200 kilodalton (kDa) now designated IGFBP3. The precise function of the other IGFBP's has not been fully elucidated because each one is produced by different tissues and their regulation also varies. There is increasing evidence that they act as paracrine and autocrine factors in the mediation of the mitogenic effects of the IGF's (Shamasaki and Ling 1991). This thesis concentrates on IGFBP1 because of the role it may play in reproductive function (Suikkari 1990).

IGFBP1 has equivalent binding affinities for both IGF-1 and IGF-11 although the latter is its principal ligand.. IGFBP1 was originally isolated from amniotic fluid (Chochinow et al. 1977, Drop et al. 1979, Povoa et al. 1984) and later from Hep G2 cells (Povoa et al. 1985).

Other sites of synthesis include many cell types; endothelial cells (Bar et al 1987) fibroblasts (Conover 1990a), decidual and secretory endometrium (Rutanen et al. 1985, 1986), amniotic fluid (Povoa et al. 1984, Baxter et al. 1987, Busby et al. 1988) and human preovulatory follicles (Seppälä et al. 1984). The amino-acid sequence of IGFBP1, a non-glycoslated protein, has been determined (Lee et al.1988, Brewer et al. 1988, Brinkman et al. 1988a, Julkunen et al. 1988). This sequence indicates that pre-IGFBP1 consists of 259 aminoacids. The putative signal peptide is 25 residues long and the mature protein thus contains 234 amino acids and has a molecular mass of 25,293 Da. The sequence is cysteine rich at the N-terminus. While the cysteines at the N-terminus are necessary for IGF binding (Hardouin et al. 1991) the cysteine at residue 226 also seems to be essential for maintaining the intact tertiary structure of the protein since a deletion or frameshift results in the loss of IGF binding (Brinkman et al. 1991). Human IGFBP1 has an Arg-Gly-Asp (RGD) sequence near the C-terminus. These sequences allow binding of the protein to the cell surface and this may enable IGFBP1 to facilitate the transport of IGF-1 to its receptors. In addition there are regions of clustered Proline, Glutamine, Serine and Threonine residues (PEST) at position 89-114 . These regions are associated with proteins which have a short half-life.

A cDNA for IGFBP1 has been isolated from a human decidual cDNA library and a single mRNA species of 1.6kb has been detected in Hep G2 cells, human liver, secretory endometrium, early and late pregnancy decidua. The human IGFBP1 gene has been characterised. It spans 5.2 kb and contains four exons (Brinkman et al. 1988b, Cubbage et al.1989). The IGFBP1 gene is found on chromosome 7 (Brinkman et al 1988a). It has not been found to be expressed in human adrenal, kidney, proliferative endometrium or placenta (Julkunen et al. 1988). IGFBP1 contains four repeats of the nucleotide sequence ATTTA in the 3' untranslated region of its mRNA. These sequences are found in similar positions in other mRNAs with a short half-life which would allow a prediction that IGFBP1 mRNA is unstable (Shaw and Hamen 1986).

1.2.2. ROLE OF IGFBP1

The function of the insulin-like growth factor binding proteins which are expressed in varying quantities by different organs and cell types is unclear. In the circulation they bind more than 95% of the IGF present in plasma (predominantly IGFBP3) thereby prolonging the half-life of the IGFs and preventing the insulin-like hypoglycaemic effects of IGF. The precise role of IGFBP1 is unclear since it has been reported to enhance the effect of IGF-1 on thymidine incorporation and proliferation of fibroblasts and smooth muscle cells in-vitro (Elgin et al. 1987) and in other reports to inhibit the binding of IGF-1 to its receptor in endometrium and therefore inhibit IGF activity (Rutanen et al.1988). One report has suggested that IGFBP1 may prevent trophoblastic invasion of the endometrium by inhibiting the action of IGF-1 on its receptors in the placenta (Ritvos et al 1988). It has therefore been proposed that IGFBP1 plays an autocrine/paracrine role in regulating IGF-1 action at a cellular level while IGFBP3 acts as a storage protein for IGF-1 in the circulation.

The varying role of IGFBP1 between tissues may be dependent on the concentration of the binding protein itself, its affinity for the ligand or the concentration of ligand within the tissue. The protein is synthesised in many tissues and has a short half-life. The circulating concentration may therefore represent solely the hepatic production of IGFBP1, a combination of tissue and hepatic production and/or the rate of clearance of the protein from the circulation.

1.2.3. REGULATION OF IGFBP1

IGFBP1 was originally thought to be growth hormone dependent since IGFBP1 levels were reported to be low in acromegaly and raised in panhypopituitarism (Drop et al. 1984). The clear diurnal variation of IGFBP1, with the highest levels found between 2200 and 0800 hours which related to the nocturnal rise in growth hormone initially supported this view but the finding that IGFBP1 concentrations dropped very rapidly after the ingestion of a meal (Baxter and Cowell 1987) suggested that nutritional variables might be more important. The correlation of IGFBP1 levels to nutritional intake was found to be more

significant than growth hormone although subjects that received GH injections showed a 51% reduction in their fasting levels of IGFBP1 (Busby et al.1988b).

Circulating IGFBP1 does not appear to be regulated directly by IGF-1 since there is little diurnal variation in the plasma concentration of IGF-1. Furthermore, no correlation between serum concentrations of IGF-1 and IGFBP1 has been reported under normal physiological conditions (Zapf et al.1980, Suikkari et al. 1988). A negative correlation of IGFBP1 with IGF-1 has been reported following prolonged exercise in both normal and diabetic men (Suikkari et al.1989d). IGF-1 levels are reduced in malnutrition and after several days fasting (Philips 1981, Clemmons et al. 1981,Isley et al.1983) while IGFBP1 levels increase. Under these conditions insulin concentrations also declined such that the correlation of IGFBP1 to IGF-1 was similar to that of insulin. The diurnal changes in IGFBP1 correlate very closely to the changes in insulin with the highest IGFBP1 concentrations being found between 2200 and 0800 when insulin levels are lowest (Cotterill et al. 1988). There is now substantial evidence that insulin is negatively correlated with IGFBP1 in vivo whether in patients with abnormalities of insulin secretion, such as insulinomas or hyperinsulinaemia (Suikkari et al. 1988, 1989a) or in children going through puberty when insulin secretion increases and IGFBP1 levels decline (Holly et al. 1988). These data suggest that insulin is an important inhibitor of IGFBP1 secretion. In vitro studies have shown that insulin directly inhibits IGFBP1 secretion by endometrial cells (Rutanen 1985,1986)and Hep G2 cells (Conover and Lee 1990b). After insulin induced hypoglycaemia, however, plasma IGFBP1 levels show a rise instead of the expected fall (Cotterill et al. 1988, Yeoh and Baxter 1988). This suggests that glucose may also play a role in IGFBP1 regulation in certain conditions such as Type 1 diabetes (Holly et al. 1989) and this has been confirmed in vitro in fetal liver cells (Lewitt and Baxter 1989) and Hep G2 cells (Cotterill et al. 1989).

IGFBP1 levels are raised in early pregnancy and high concentrations are found in amniotic fluid (Rutanen et al. 1982) suggesting that the decidualised endometrium represents the source of this IGFBP1. The contribution of the uterus to circulating concentrations of

IGFBP1 in physiological conditions has been studied by Suikkari et al. (1987). The circulating levels of IGFBP1 were similar in pre and post menopausal women although the IGFBP1 levels fell immediately following hysterectomy in premenopausal women but returned to preoperative levels within five weeks of the operation. It would therefore seem that the uterus does contribute to the circulating plasma concentrations but that secretion by other tissues can compensate when it ceases to function. IGFBP1 production has only been found in secretory or decidual endometrium and it was therefore thought that a cyclical variation in IGFBP1 concentrations would be detected. This has not proved to be the case although a 31% fluctuation in daily concentrations was found. In vitro experiments have demonstrated an elevated synthesis and secretion of IGFBP1 by secretory endometrial explants in the presence of progesterone (Wahlström and Seppälä 1984, Bell et al. 1986, Rutanen et al. 1986). Progesterone has also been found to increase IGFBP1 secretion in bovine fibroblasts (Conover et al. 1990c).

The complexity of the regulation of IGFBP1 suggests that secretion from each tissue type may be separately regulated by multiple factors of which growth hormone, insulin and steroid hormones have already been identified as candidates. Since SHBG and IGFBP1 concentrations are both found to decline during puberty, are decreased in acromegaly, have a strong negative correlation with insulin and are found to be increased during pregnancy, the possibility that they may be co-regulated must be considered.

1.3. THE HYPOTHESIS IN CLINICAL PERSPECTIVE

1.3.1. POLYCYSTIC OVARY SYNDROME (PCOS)

Traditionally, the classical description of bilateral sclerocystic ovaries in women presenting with anovulation or hirsutism, first described in 1935 by Stein and Leventhal, has been the principal diagnostic criteria for polycystic ovary syndrome (PCOS). The associated endocrine abnormalities of a raised luteinising hormone (LH) and testosterone (T) level have become additional indices (MacArthur et al.1958, Yen 1970, Gambrell et al.1973, Rebar et al. 1976, Yen 1980) and in some studies are considered more important than the morphological appearance of the ovaries. Results from subsequent reports using clinical, endocrine and morphological features (both ovarian biopsy and pelvic ultrasound) as diagnostic criteria has brought the realisation that PCOS is a heterogeneous condition (Goldzeiher and Green 1962, Jeffcoate 1963, Givens 1977,1984, Yen 1980) with a spectrum of clinical and biochemical presentations. The clinical presentation of PCOS includes women who are anovulatory but not hirsute as well as those who have ovulatory cycles and are hirsute. The majority of women with polycystic ovaries (PCO) on ultrasound have a raised LH and/or testosterone level (Adams et al. 1985,86, Franks et al.1989, Conway et al. 1989). There is evidence that PCOS is a familial condition (Hague et al. 1988) but the clinical and biochemical presentation of the syndrome may be determined by environmental as well as genetic factors.

Hirsutism is a distressing condition for women. The factors that most effect hirsutism are circulating androgen levels and the genetically determined response to hyperandrogenism of the individuals' hair follicles. The serum levels of total testosterone and androstenedione are raised in the majority of women with PCOS. Testosterone circulates bound to a binding protein (sex hormone binding globulin- SHBG) and albumin such that in women less than 2% of the total testosterone circulates free or unbound (see section 1.1.). The concentrations of SHBG may, therefore, profoundly affect the biological availability of testosterone to peripheral tissues. SHBG levels have been shown to be reduced in women with PCOS (Yen 1980, Plymate et al.1981) and other hyperandrogenic conditions (Vermeulen 1969

and 1971, Rosenfield 1971). Factors that control the production and clearance of this protein may therefore play a significant role in the development and severity of hirsutism in women with PCOS as previously discussed in Section 1.1.3.

The mechanism of anovulation in PCOS is poorly understood. There is increasing evidence that growth factors including insulin-like growth factor-1 (IGF-1) and insulin are involved in folliculogenesis (Adashi et al. 1985, Barbieri et al. 1986, Hernandez et al. 1988). IGFBP1 may modulate the role of IGF-1 in reproduction in a paracrine/autocrine fashion at the cellular level (Rutanen et al. 1988). Circulating levels of IGFBP1 have been reported to be low in women with PCOS (Suikkari et al.1989b) and since this may alter the biological availability of IGF-1 an understanding of the regulation of hepatic IGFBP1 production may be of relevance in understanding the pathophysiology of anovulation in PCOS.

Women with PCOS, therefore, provide a clinically important model for studying the effects of nutrition and hormones on the regulation of SHBG and IGFBP1 concentrations. In order to establish whether any changes seen in SHBG or IGFBP1 concentrations in vivo are secondary to changes in hepatic production and/or to alterations in the clearance of the binding proteins, in vitro experiments were carried out using Hepatocarcinoma G2 (Hep G2) cells as a model for hepatic SHBG and IGFBP1 synthesis. These cells have been shown to produce corticosteroid binding globulin, thyroid binding globulin and apolipoproteins in addition to SHBG and IGFBP1 and have therefore been thought to provide a good in vitro model for the investigation of the regulation of these proteins.

1.4. AIMS AND OUTLINE OF THIS THESIS

The aim of this thesis was to investigate the role of insulin, IGF-1 and the sex steroid hormones in the regulation of hepatic SHBG production in order to establish what part diet/ calorie intake may play in the pathophysiology of hirsutism in women with PCOS. In addition the relationship of IGFBP1 to insulin, IGF-1 and the sex steroids was further investigated to see if the production of the two binding proteins was co-regulated. Thus the overall aim of the studies and experiments undertaken for this thesis was to determine how alterations in calorie intake could influence the concentrations of SHBG and IGFBP1 and the mechanism by which this might occur.

In vitro experiments using hepatocarcinoma G2 (Hep G2) cells as a model for SHBG and IGFBP1 synthesis were carried out to establish the role of insulin, IGF-1, growth hormone (GH) and the sex steroids - testosterone, oestradiol and progesterone on the hepatic production of SHBG and IGFBP1.

Women with PCOS provide a clinically important model for studying the effects of nutrition and hormones on the regulation of SHBG and IGFBP1 concentrations. Initial studies were carried out looking at changes in the concentrations of insulin, IGF-1, testosterone and the binding proteins before and after calorie restriction. The interrelationship between insulin, IGF-1 and SHBG/IGFBP1 was further investigated by looking at the diurnal variation in hormone and binding protein concentrations.

CHAPTER 2

METHODS

2.1 IMMUNORADIOMETRIC ASSAY OF SEX HORMONE BINDING GLOBULIN (SHBG)

Sex hormone binding globulin (SHBG) has been measured by various methods in the past. Initial approaches utilised the binding capacity of SHBG to dihydrotestosterone. These included an ammonium precipitation technique (Anderson et al. 1976, Heyn and DeMoor 1971, Rosner 1972, Rudd et al. 1974), equilibrium dialysis (Vermeulen et al. 1971), gel filtration (Vermeulen et al. 1969), steady state gel filtration (Iqbal and Johnson 1977), polyacrilamide gel electrophoresis (Corvol et al. 1971), steady state polyacrilamide gel electrophoresis (Ritzen et al. 1974), centrifugal ultrafiltration dialysis (Hammond et al. 1980), dextran coated charcoal (Rosenfield 1971, Dennis et al. 1977), absorption of SHBG onto diethylaminoethyl cellulose (Mickelson and Petra 1974, Lee et al. 1984), absorption of SHBG onto concavilin A (Bruning et al. 1985) and aqueous partition (Shanbhag et al. 1973). These methods all overcome most of the problems of non-specific binding of dihydrotestosterone (DHT) to corticosteroid binding protein and albumin but the normal serum values obtained may vary by up to 100% between methods (52±7 vs 21±2 nmol/l in men and 102±9 vs 37±4 in non-pregnant women) although the agreement beween laboratories using the same method is good (Rosner 1986a).

Assays for measuring SHBG that are dependent upon its immunological activity rather than its binding capacity have been developed. They include a radioimmunoassay (Mercier-Bodard et al. 1979, Khan et al. 1982, Cheng et al. 1983, Maruyama et al. 1984), enzyme linked radioimmunoassay (Bordin et al. 1982), enzyme linked immunosorbent assay (ELISA- Heubner et al.1987), electroimmunoassay (Egloff et al. 1981, Lee et al. 1984) and an immunoradiometric assay (Hammond et al. 1985). The immunologically based assays have several advantages, particularly in detecting low levels of SHBG, for example in men,

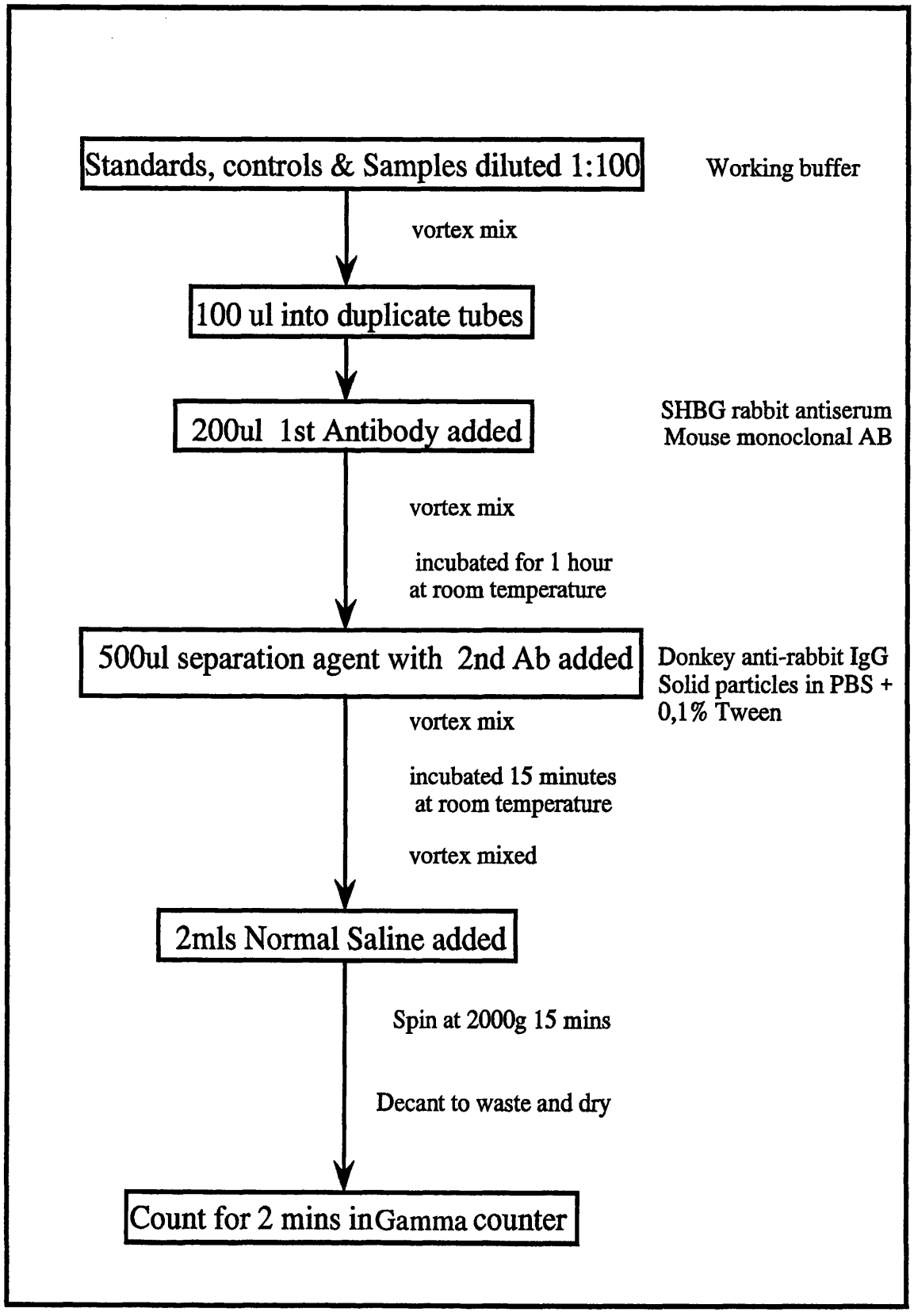
hirsute hyperandrogenic women and obese patients. Large numbers of samples can be processed and the stability of the assay means that older samples can be accurately analysed as these samples tend to lose binding capacity but not immunological activity (Rosner 1986a, Hassan et al. 1987).

The immunoradiometric assay (IRMA) was chosen for this study because the serum samples being measured came from women who were obese and hyperandrogenic and therefore, we expected the SHBG concentrations to be in the low normal range. In addition, studies in our laboratory have demonstrated a linear relationship between the SHBG results obtained by ammonium sulphate precipitation for binding capacity and those using the IRMA (Polson et al. 1987). This assay is available in kit form with standards and quality control samples. The reagents were obtained from Pharmacia, Milton Keynes, Bucks, UK. The lowest detectable concentration is 0.5 fmol per assay tube. However the acceptable assay range lies between the lowest standard (6.25nmol/l) and the 100nmol/l standard, which represents the most linear part of the curve and yields a reported intra assay precision of 1.8-4.9%. There is minimal cross reactivity with the other binding globulins including corticosteroid and thyroxine binding globulin. The procedure of this assay is based on the principles of a non-competitive "liquid-phase" immunoradiometric assay (Hammond 1985).

2.1.1. PROCEDURE

The procedure used in this assay is shown digrammatically by the flow chart Figure 2.1.1 and is detailed below. Serum samples were thawed and vortex mixed. These samples were diluted 1:100 with phosphate buffered saline (PBS-phosphate 25 mmol/l, 0.9% NaCl, 0.625% lactose, 1% bovine serum, 0.05% sodium azide - pH 7.6). SHBG standards and controls were reconstituted with 0.5 mls distilled water and diluted 1:100 with PBS. The standards were supplied at concentrations of 6.25, 12.5, 25, 50, 100 and 200 nmol SHBG per litre in human pregnancy serum diluted with bovine serum, 2.5% lactose and 0.1% sodium azide.

Figure 2.1.1. Flow chart of SHBG IRMA assay



5ml test tubes were labelled in duplicate for total counts, non-specific binding (NSB), standards, controls and samples. 100µl of the diluted standards, controls and samples were added to the appropriate test tubes. 100µl of PBS alone was added to the NSB tubes. Equal volumes of SHBG rabbit antiserum and ^{125}Iodine-labelled mouse monoclonal antibody in PBS buffer were mixed (maximum activity 3µCi) and 200µl added to the standards, controls, samples and the total tubes. The total tubes were set aside for counting later. The rest of the tubes were vortex mixed and incubated for one hour at room temperature. A clean magnetic bar was placed in the separation agent containing the second antibody (donkey anti-rabbit rabbit IgG) covalently bound to solid particles in PBS buffer containing 0.1% Tween. The mixture was stirred on a magnetic plate to keep the solid phase homogenously in suspension. 500µl was added to all the tubes except the totals. The tube contents were vortex mixed and incubated for 15 minutes at room temperature. The samples were vortex mixed again and 2mls of normal saline added immediately. The tubes were spun at 2000g for 15 minutes at room temperature.

The supernatant was decanted to waste and the pellets left to dry for 20 minutes on absorbent paper. The residual activity was counted for 2 minutes per tube on a gamma counter.

The assay was performed in an identical manner on the medium from the Hepatocarcinoma G2 (Hep G2) cells except that 750µl of undiluted sample was used. The final concentration of SHBG was calculated by multiplying the obtained value by a factor that would express it relative to the total volume and then divided by the number of cells. (see section 2.3.3.)

2.1.2 QUALITY CONTROL.

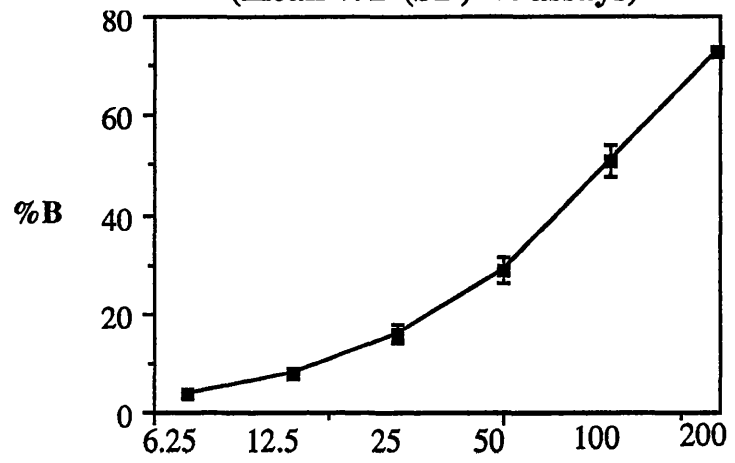
Quality control of pipetting techniques of 10 µl aliquots and serial dilution of label are shown in Appendix B. The Farmos SHBG kit was provided with two control sera samples from the lower and the upper portion of the normal range. The recommended values for these fell in a range; the lower (QCL) 9.0-12.2 nmol/l and the upper (QCH) 81.0-106 nmol per litre. Using these control sera the intra-assay coefficient of variation (CV) of the assays carried out in this thesis (CV%-mean (SD)) for both media and sera was 1.8 (1.4)% for the

QCL and 1.6 (0.5)% for the QCH value (12 assays). A pooled serum sample from 10 individuals (QC) was used as a quality control between assays and batches of antibody. The value for this QC was between 45 and48 nmol/L which is in the mid-range of the normal value for women. The inter-assay coeffiecient of variation was calculated in eight assays and the mean (SD) was 4.7 (1.7)%.

2.1.3. CALCULATION OF THE STANDARD CURVE.

The NSB tubes were used to calculate the specific binding of each standard. The value for each standard was calculated automatically by subtracting the mean counts per minute for the NSB tubes from each of the counts for the standards. These values were expressed as a percentage difference from the NSB counts (B_0) although the standard curve did not include a zero value but began at 6.25nmol/L. The doses in each standard were plotted along the 'x' axis after log transformation. The percentage difference from B_0 was plotted on the 'y' axis. The curve was drawn by spline function curve fitting. No smoothing factor was required. The mean percentage difference from the non specific binding was calculated from the standard curves of 14 assays and is shown with the standard deviation for each point in figure 2.1.2.

Fig 2.1.2 SHBG-IRMA standard curve (mean %B (SD)-14 assays)



Since both the samples and the standards were diluted 1:100 the results could be read straight from the curve without further calculation. The $\%B_0$ of the lowest standard (6.25nmol/l) was significantly different ($p<0.001$- from 14 assays) from the highest (200nmol/l). Similarly the counts for the lowest quality control and the highest were significantly different ($p<0.005$). Values below 6.25nmol/l were read simply as low since the slope of the curve at this concentration was rather flat, making accurate readings more difficult. In this thesis only two serum samples fell into this category and their values were excluded from analysis. Similarly values over 200nmol/L could not be accurately read. None of the serum samples fell into this category. One experiment involving thyroxine stimulation of hepatocarcinoma cells had all the values outside this range. The assay was therefore repeated using only 500µl of medium which brought the values into the more linear part of the standard curve. Adjustments in the final concentration per million cells were made accordingly. The precision profile for the standard curve calculated from 14

The precision profile can be seen to be consistent across the range of concentrations of SHBG despite the alterations in the slope of the standard curve with very little variation from one assay to another.

Table 2.1. Precision data for the standard curve of the SHBG-IRMA assay.

Concentration of SHBG (P) nmol/L	Slope of curve $\%B_0$/log [P] n=14	Precision SD/slope n=14
6.25	4.9	0.3
12.5	7.25	0.31
25	11.4	0.3
50	16.9	0.32
100	25.35	0.26
200	31.5	0.06

2.2. IMMUNOFLUOROMETRIC ASSAY OF SERUM IGFBP1 CONCENTRATION.

These assays were undertaken in Finland by Dr Koistinen (Koistinen et al. 1987b) for measuring serum concentrations of IGFBP1. IGFBP1 purified from human amniotic fluid (Koistinen et al.1987a) was used to raise polyclonal antibodies in rabbits. Affinity-purified first antibodies were bound to polystyrene microtitre wells. After solid phase separation of bound and free fractions the second antibodies were labelled with europium (III) chelate. In the assay, 25µl of standard or sample in duplicate was incubated with 200µl of assay buffer overnight at $4^{o}C$. After another wash 200µl of Eu-labelled antibody (100ng) at a dilution of 1:560 was added and incubated at room temperature for 2 hours. After washing, 200µl of enhancement solution (LKB Wallac, Turku, Finland) was added and, after 5 minutes, the fluorescence of the microtitre wells was measured. The sensitivity of the assay was 0.5µg/l, the intra-assay variation was 2-7% and inter-assay variation was 7-11%. The method has high specificity.

2.3. RADIOIMMUNOASSAY OF IGFBP1 IN MEDIUM FROM HEP G2 CELLS.

The assay was undertaken by Dr Eeva-Maja Rutanen in Finland (Rutanen et al. 1982) for the measurement of IGFBP1 concentrations in the medium from Hep G2 cells collected at the end of the experimental period. Purified IGFBP1 was obtained from Behringwerke AG, Marburg, West Germany. IGFBP1 was iodinated using the chloramine-T method. Anti-IGFBP1 antiserum was diluted 1:4000 to bind 50% of radiolabelled IGFBP1. 1-1000ng/ml IGFBP1 was used to prepare a standard curve. 100µl IGFBP1 standard or undiluted serum sample was incubated overnight with 100µl antiserum containg normal rabbit serum. Antibody bound and free radioactivity was separated by adding 200µl of 1:20 diluted sheep antirabbit gamma globulin serum and 0.5ml of 16% polyethylene glycol. After mixing, the samples were centrifuged and precipitates counted in a gamma counter.

The smallest detectable concentration of IGFBP1 was 4.6ng/ml. The intra-assay coefficients of variation were 8.9% at the IGFBP1 level of 25ng/ml and 5.9% at the level of 60-70ng/ml. The interassay coefficients of variation were 6.8% at 30 ng/ml and 4.9% at 175 ng/ml (six assays each).

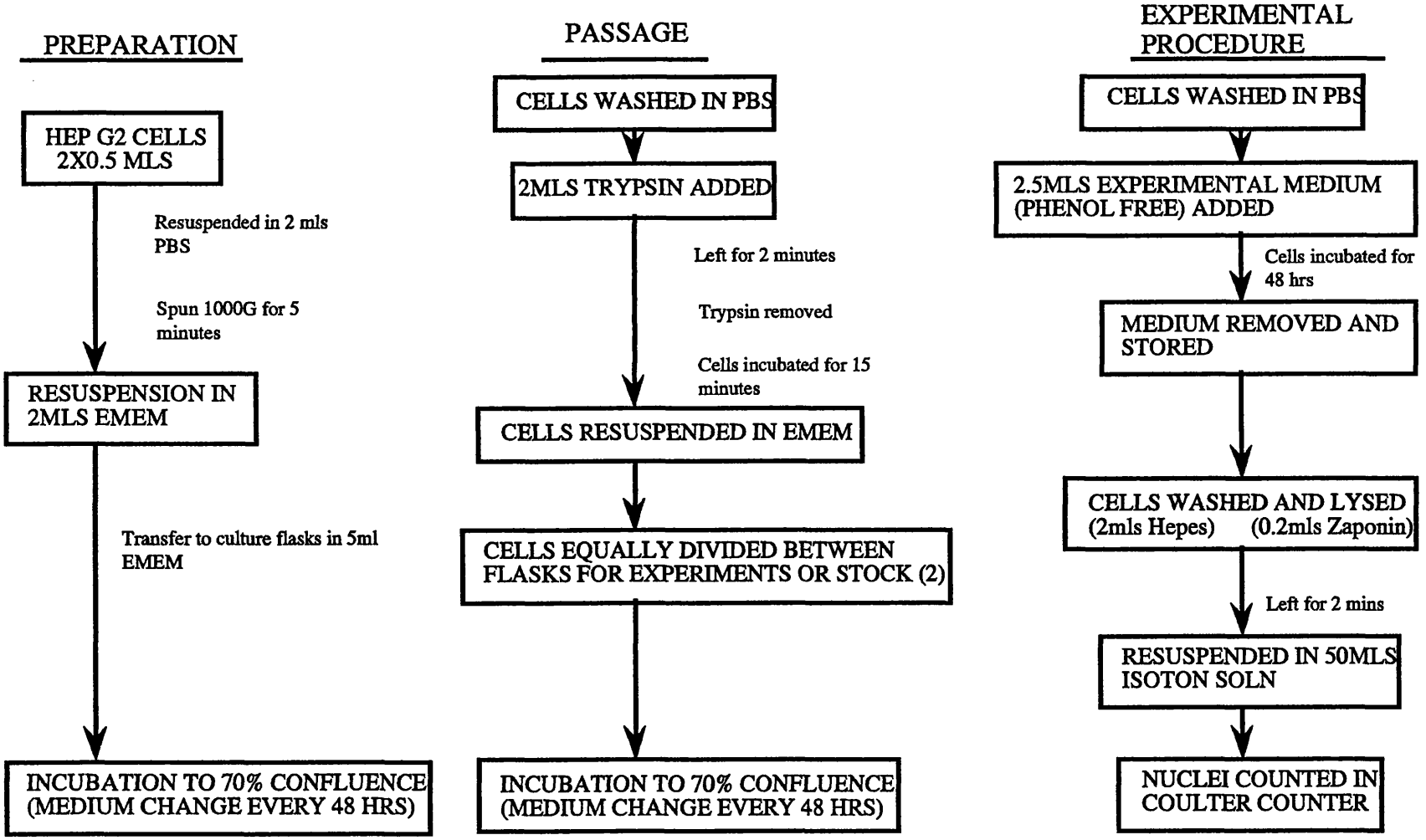
2.4. CULTURE OF HEPATOCARCINOMA G2 CELLS

The method of preparing and culturing the hepatocarcinoma G2 cells (Hep G2) is shown diagrammatically by the flow chart (Figure 2.4.1) which includes the initial preparation of the cells, the passage of the cells prior to experiments and the procedure used to harvest the media and count the number of cells in each flask. The procedures are detailed below and were done under aseptic conditions in a cell cabinet. All solutions were autoclaved prior to being used.

The hepatocarcinoma G2 cells (Hep G2) were obtained, frozen in a suspension of medium, from PHLS Centre Porton Down, Wiltshire, UK. They were defrosted and separated into two aliquots of 0.5 mls and resuspended in 2mls of 0.1M phosphate buffered saline (PBS) and spun at 1000g for 5 minutes at room temperature. The culture method used followed that of Lee et al. (1987). The supernatant was carefully removed and the cells resuspended in 2mls Eagles minimum essential medium (EMEM) modified with Earle's salts and 20mmol/l Hepes buffer. All media were supplemented with 2mmol L-glutamine/l (both-Flow Laboratories, Irvine, Strathclyde, U.K.), 10mmol sodium hydrogen carbonate/l, 1ml/ 100ml non-essential amino acid concentrate and 10% fetal calf serum (FCS) (both from Gibco, Paisley, Strathclyde, U.K.). The cells were transferred into two $25cm^3$ sterile flasks and the volume of medium in each flask was increased to 5 mls. The cells were incubated at 37°C with 95/5% air/carbon dioxide until they were 70% confluent with regular changes of medium approximately every 48 hours.

Passage of the cells was performed by removing the medium and washing the cells in 3mls PBS. Trypsin, buffered with ethinyl diamino tetra acetic acid (EDTA) (Flow Laboratories) was added and the cells left for two minutes. The trypsin was removed and the cells incubated for 10-15 minutes until they were seen to be moving down the wall of the flask. They were resuspended in an appropriate volume of supplemented EMEM and incubated until they reached 70% confluence.

Fig.2.4.1 PREPARATION AND CULTURE OF HEPATOCARCINOMA G2 (HEP G2) CELLS

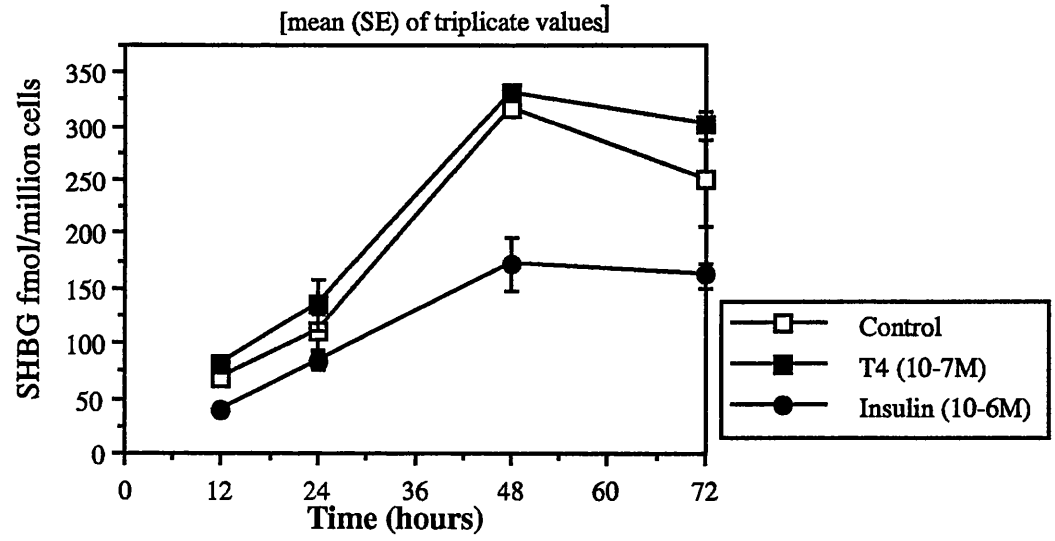


EMEM: Eagles' minimum essential medium PBS: phosphate buffered saline

2.4.1. EXPERIMENTAL PROCEDURE

The growth medium was removed and replaced with serum free, phenol free medium supplemented as above (Autopon-Flow laboratories). Phenol-free medium was used because phenol has been shown to interact with oestrogen receptors in some cell lines. The medium was removed and stored at -20°C until analysed for SHBG and IGFBP1. The cells were washed once in phosphate buffered saline (PBS) solution and the nuclei released. This was done by adding two mls of 0.01M Hepes buffer to the flasks and allowing them to stand for three minutes. 0.2 mls Zaponin (from Coulter Electronics Luton, UK.) was added before suspension in Isoton solution and counting in a Coulter counter. The time course for the secretion of SHBG by Hep G2 cells is shown in Figure 2.4.2. This shows the production over 72 hours by Hep G2 cells in EMEM alone, EMEM with thyroxine (T4) 10^{-7}mol/L or EMEM with insulin 10^{-6}mol/L. This demonstrates that maximum production of SHBG by Hep G2 cells cultured in experimental media is at 48 hours. Medium was therefore removed after 48 hours in all experiments.

Figure 2.4.2. Time course of SHBG production



CHAPTER 3
IN-VITRO STUDIES

3.1. EFFECT OF INSULIN, INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) AND GROWTH HORMONE (GH) ON THE SECRETION OF SEX HORMONE BINDING GLOBULIN (SHBG) AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP1) BY HEPATOCARCINOMA G2 CELLS.

3.1.1. AIMS

Hepatic SHBG production has been thought to be regulated by the sex hormones, principally testosterone and oestradiol. The foundation of this hypothesis lies in the finding that hyperandrogenic conditions and men have lower SHBG concentrations than normal women and that conditions with high oestradiol levels, such as pregnancy, are associated with increased SHBG concentrations (Anderson 1974). Evidence that insulin and IGF-1 may regulate SHBG concentrations is provided by studies on obese men and women (Plymate et al. 1981, Peiris 1989a, Nestler 1991), and pubertal boys and girls (Holly et al 1989). An inverse relationship between SHBG and insulin has been demonstrated which was independent of sex hormone levels. A similar inverse relationship between IGFBP1 and insulin was found in puberty, diabetes, obesity and PCOS (Holly et al. 1988, 1989, Suikkari et al. 1988, 1989a). During fasting and exercise IGF-1 levels decrease and both SHBG and IGFBP1 concentrations have been shown to change inversely. Factors that alter IGF-1 concentrations, eg. growth hormone (GH), may also act directly to regulate these binding proteins since obesity is associated with low levels of GH. There is evidence that GH can act directly on tissues, for example in the ovary where GH increases oestradiol production by granulosa cells in vitro (Mason et al. 1990). The aim of this study was, therefore, to investigate whether insulin, IGF-1 and/or GH directly regulate the production of SHBG and IGFBP1 by human hepatocarcinoma G2 (Hep G2) cells.

3.1.2. METHODS

The Hep G2 cells were passaged and prepared as described in chapter 2.4. All the experiments were carried out in phenol-free serum free medium . Insulin (0.1-100 μmol/ l; Sigma, Poole, Dorset, U.K.), IGF-1 (1-100 nmol/l; Bachem Inc., Saffron Walden, Essex, U.K.) or growth hormone (0.1-100ng/ml-recombinant growth hormone, kindly supplied by Novo-Nordisk, Denmark) were added and the cells incubated for 48 hours at 37^{o}C. The experiments were performed in triplicate for each point and repeated at least twice.

In all the subsequent experiments the concentration of SHBG was measured with an immunoradiometric assay (Chapter 2.1.). The medium was undiluted and 750μl was used. The concentration was calculated per millilitre of medium and then expressed as fmol per million cells (calculated from the mean of three readings from the coulter counter and multiplied by a dilution factor -total volume/volume measured (0.05ml)). IGFBP1 concentrations were measured by radioimmunoassay (Chapter 2.3.) and expressed as ng/ ml which was then calculated as nanogram per million cells as above. In the growth hormone experiments IGF-1 was also measured in the medium since an increase was expected in response to GH. It was measured in undiluted medium after acid extraction using a radioimmunoassay (Appendix A) and expressed as nanograms per million cells.

The data obtained from these experiments was not normally distributed. A Wilcoxon ranked pairs test was performed to compare the control concentrations with each experimental value. In order to establish whether or not there was a dose response a two-way analysis of variance was performed on the summative data (Larson 1991) using dose and experiment as the factors. The dose response histograms presented here represent the pooled data from five experiments with insulin, and 3 experiments for IGF-1 and growth hormone.

3.1.3. RESULTS

There was no significant difference in the response of SHBG and IGFBP1 to insulin or IGF-1 from one experiment to another and it is therefore unlikely that any variation in the distribution of the Hep G2 cells affected the results. The effects of insulin on SHBG and

Figure 3.1.1. Effect of insulin on SHBG production by Hep G2 cells

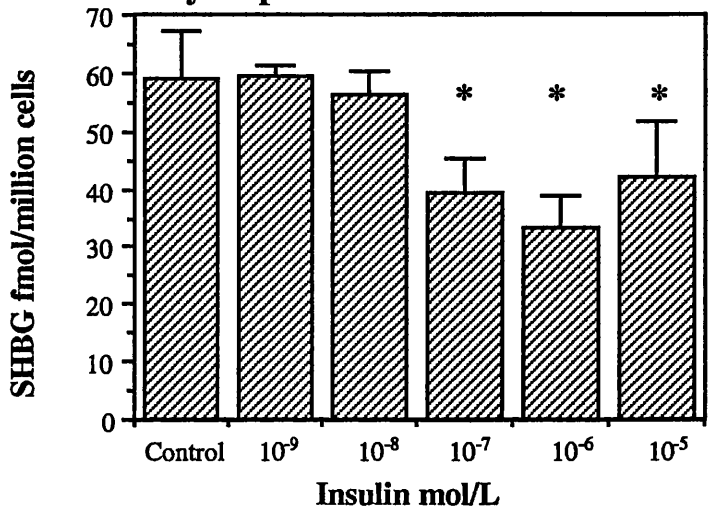
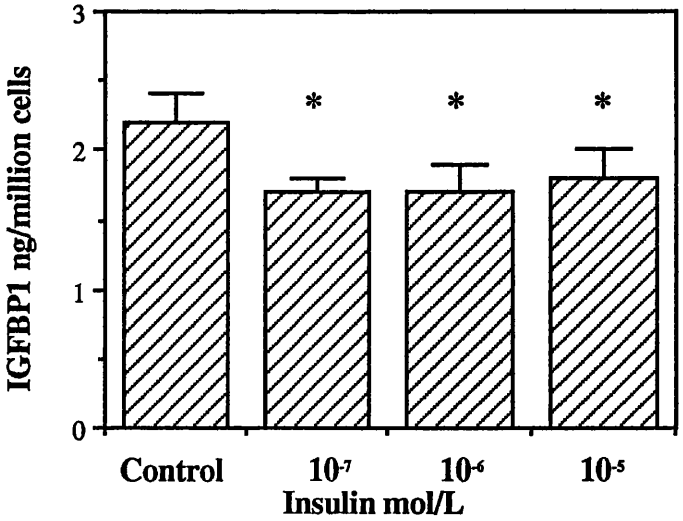


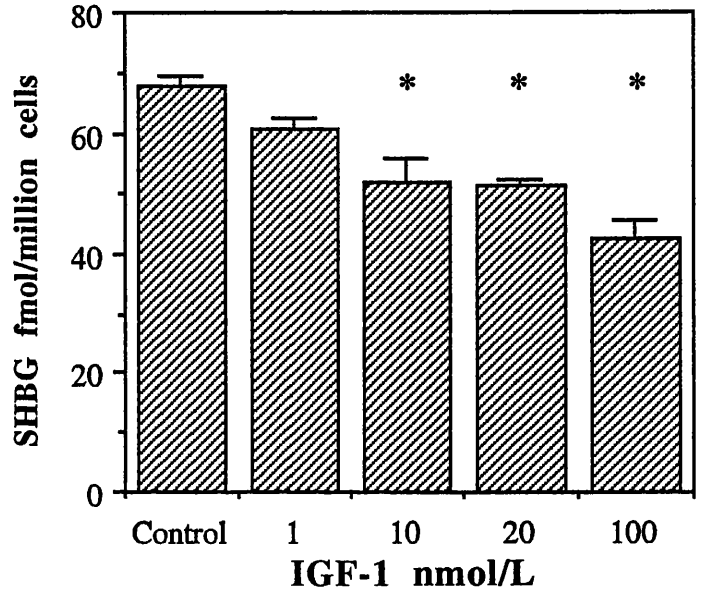
Figure 3.1.2. Effect of insulin on IGFBP1 production by Hep G2 cells



* $p<0.001^1$

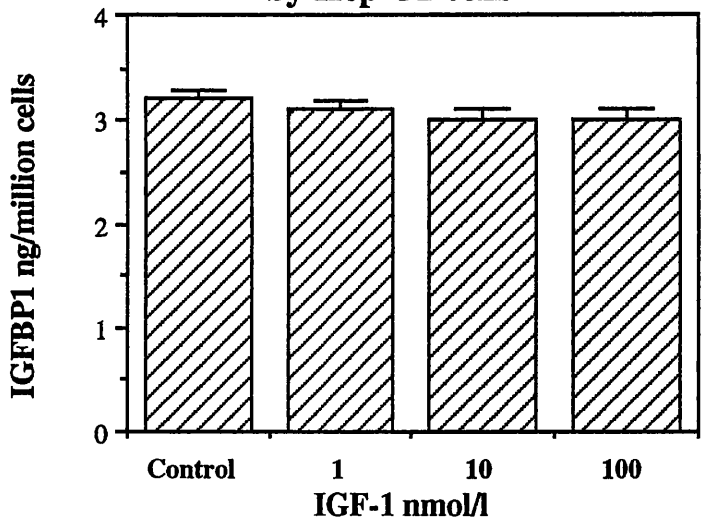
1. One way analysis of variance, factor - concentration.

Figure 3.1.3. Effect of IGF-1 on SHBG production by Hep G2 cells



* p<0.001[1]

Figure 3.1.4. Effect of IGF-1 on IGFBP1 production by Hep G2 cells



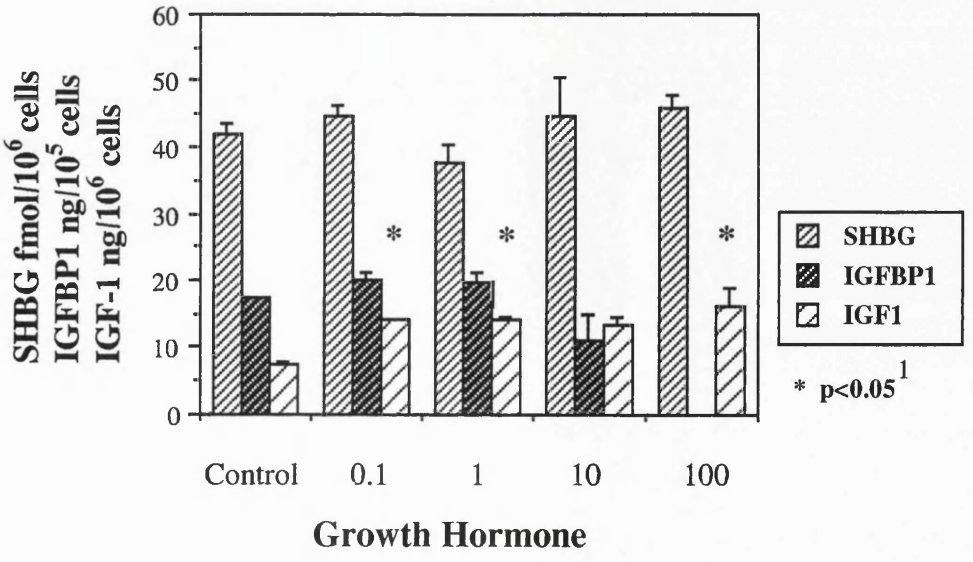
1. One way analysis of variance, factor - concentration.

IGFBP1 production are shown in Figure 3.1.1. and 3.1.2. Insulin concentrations of 0.01-100 µmol/l, which overlap with the physiological range, consistently inhibited SHBG and IGFBP1 production although this was not dose dependent. Lower concentrations of insulin did not inhibit SHBG secretion.

The inhibition of SHBG production by IGF-1 was also not dose dependent. The concentration of IGF-1 required to significantly affect the production of SHBG was a thousandfold smaller (1-20 nmol/l) than that of insulin. There was no effect of IGF-1 on IGFBP1 production even at higher concentrations (Figure 3.1.3. and 3.1.4.) In these and all subsequent figures each bar represents the mean (standard error) of triplicate values pooled from all the experiments.

Growth hormone at physiological and supraphysiological concentrations (0.1-100ng/ml) did not effect the production of either SHBG or IGFBP1. There was a significant increase in IGF-1 concentrations as expected but this was not dose dependent. (Figure 3.1.5.)

Figure 3.1.5. Effect of Growth Hormone on the production of SHBG, IGFBP1 and IGF-1 by Hep G2 cells



1. Wilcoxon ranked pairs test comparing control with each concentration.

3.1.4. DISCUSSION

The direct inhibition of SHBG and IGFBP1 production by insulin in vitro supports the hypothesis that the decrease in SHBG and IGFBP1 levels in puberty and obesity are probably mediated by insulin. The inhibition of the secretion of SHBG by insulin in Hep G2 cells has been reported previously by Plymate et al. (1988). The lack of a dose response to changes in insulin concentrationsmay have several explanations. Firstly,the interpretation of these experiments must be cautious since Hep G2 cells are transformed cells and their response cannot, therefore, be directly compared to that of adult hepatoma cells in vivo. It is, however, possible that a threshold concentration of insulin has to be reached before inhibition of SHBG is seen and that this is an all or nothing response. This is supported by the finding that IGFBP-1 is also inhibited by insulin in a non-dose dependent fashion and that these two findings are reproducible within this study, by other groups and in other cell lines (Conover and Lee 1990a&b). These findings of a threshold concentration for inhibition have been confirmed by messenger RNA studies performed by Plymate et al. (1990). The mitogenic effects of both insulin and IGF-1 have been taken into account since all the results are corrected for cell number. Statistical analysis of vriance did not show a significant difference in the dose response from one experiment to another. Other experimental factors may have influenced the result since the medium was mixed and the experiments were carried out in plastic $25cm^3$ flasks. Insulin has been shown to bind to this plastic to varying degrees and this may explain the lack of inhibition at lower concentrations.

The inhibition of SHBG production in the presence of IGF-1 at concentrations considerably smaller than those for insulin must be interpreted cautiously since IGF-1 receptors are not found on adult hepatocytes. In addition insulin is known to cross react with IGF-1 receptors. It is therefore possible that the insulin mediated inhibition of SHBG production by these cells is potentiated by cross reactivity with IGF-1 receptors. The negative correlation between SHBG and IGF-1 during fasting supports the possibility that IGF-1 inhibits SHBG production since there was a significant decrease in IGF-1 concentrations after dieting, accompanied by a significant increase in SHBG (Chapter 4.1.). The

difference was not sustained, however, and with more prolonged dieting the correlation ceased to be significant (Kiddy et al.1992). The reason is not clear but this may represent a rapid response in vivo of IGF-1 to fasting conditions which then re-equilibrate towards original concentrations as calorie restriction continues. Similar changes are seen with IGFBP1 and the IGF binding proteins may play an important role in regulating the circulating concentrations of IGF-1.

IGF-1 does not appear to directly affect IGFBP1 production by Hep G2 cells by 48 hours. This is in contrast to the results for SHBG and suggests that crossreactivity of insulin with IGF-1 receptors does not account for the inhibition of IGFBP1 production by insulin. The response of IGFBP1 to insulin and glucose has been shown to be very rapid with peak changes occuring within 6 hours (Unterman et al. 1991, Orlowski et al. 1991, Cotterill et al.1989, Lewitt et al. 1989) and it is therefore possible that a response to IGF-1 was missed by measuring the concentration at the single time point of 48 hours. Owing to financial and time constraints I have not been able to repeat the experiments to confirm or refute this possibility. The role of IGFBP1 in glucose homeostasis is becoming clearer since a negative correlation between IGFBP1, insulin and IGF-1 is found during fasting (Lewitt and Baxter 1990). A rapid response of IGFBP1 production to alterations in insulin concentrations would inversely alter available IGF-1 concentrations for tissue uptake of glucose (Suikkari et al. 1989d).

Growth hormone does not affect SHBG production despite the increase in IGF-1 levels in the medium. This is interesting since exogenous IGF-1 inhibits SHBG concentrations. The endogenous production of IGF-1 was lower in these experiments than the exogenous amounts added. In addition these cells produce large quantities of IGFBP1 and 3 which bind IGF-1, preventing binding to its receptors. The finding that IGFBP1 production is not altered by GH confirms the finding that hepatic production of IGFBP1 is independent of GH.

The results from this series of experiments support the hypothesis that SHBG and IGFBP1 may be co-regulated by insulin. The time course of such regulation probably differs between the two proteins although it is still not clear from these experiments whether this is because

of a difference in rates of synthesis or clearance. Other nutritional variables including IGF-1 may also be involved in the regulation of SHBG though this is unlikely in the case of IGFBP1.

3.2. THE EFFECT OF STEROID HORMONES AND THYROXINE ON THE SECRETION OF SHBG AND IGFBP1 BY HEP G2 CELLS

3.2.1. AIMS

The aim of this study was to investigate the effects of testosterone, oestradiol and progesterone on SHBG and IGFBP1 production. Testosterone and oestradiol have both been linked to the regulation of SHBG concentrations in serum; testosterone as an inhibitor and oestradiol as a stimulator (Anderson 1974). It is, however, not clear whether these effects are directly on hepatic synthesis or via post-translational alterations in the tertiary structure of the protein leading to an alteration in the clearance of the protein from the circulation. In vitro studies may help to clarify this problem since they look directly at hepatic production of the proteins under fixed environmental conditions.

IGFBP1 production by endometrial decidual cells has been shown to alter in the presence of progesterone although there is no data on the effects of other steroid hormones. There is also no evidence to show whether hepatic IGFBP1 production is affected by steroid hormones.

Thyroxine is known to increase the serum concentrations of SHBG and this is well described both in vivo and in vitro (De Nayer et al. 1985, Lee et al. 1987). The effect of thyroxine on hepatic IGFBP1 production is not known.

3.2.2. METHODS

The experiments were performed in triplicate and repeated at least once. The experimental medium was phenol free and serum free. Serum was excluded so that no extra growth factors including IGF-1 and insulin were present to influence the results.

Hep G2 cells are known to exhibit aromatase activity (Singh- unpublished data) and it was therefore thought that the effects of testosterone might be mediated by conversion to oestradiol (although this was not quantifiable by a commercially available radioimmunoassay). In order to inhibit this aromatase activity testosterone was added in the presence of delta-4-hydroxy androstenedione, a competitive inhibitor of aromatase.

Testosterone (T),17β- oestradiol (E), progesterone (P), and delta-4-hydroxy androstenedione (Δ-4-OH A) (Sigma Laboratories, Poole, Dorset, U.K.) were kindly supplied by Dr M. Reed from the Department of Chemical Pathology, St. Mary's Hospital Medical School, London. Thyroxine was also obtained from SigmaLaboratories and the lyophilised powder dissolved in unsupplemented sterile EMEM..

Two way analysis of variance was performed to establish whether there was a dose response to the addition of varying concentrations of steroid and a wilcoxon ranked pairs test was used to determine whether there was a significant difference at each concentration from the control values. The graphs presented here represent a single experiment with testosterone, pooled data from three experiments for T with Δ-4-OHA , two experiments with progesterone, oestradiol and thyroxine (mean (SE) of triplicate values from pooled experiments).

3.2.3. RESULTS

3.2.3.A. TESTOSTERONE

Testosterone was found to *stimulate* SHBG production by Hep G2 cells in a dose dependent fashion (Figure 3.2.1.). The addition of the aromatase inhibitor Δ-4-OH A (Fig 3.2.2.) did not significantly affect the response of SHBG to testosterone. Δ-4-OH A alone did not significantly alter SHBG production.

Testosterone had no effect on the secretion of IGFBP1 (Fig 3.2.1.)

Figure 3.2.1. Effect of testosterone on SHBG and IGFBP1 production by Hep G2 cells.

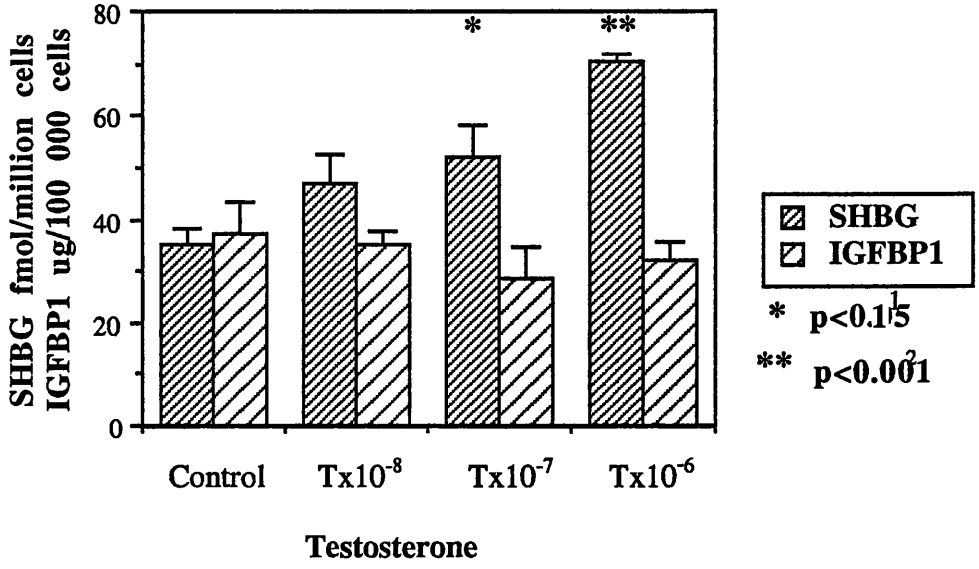
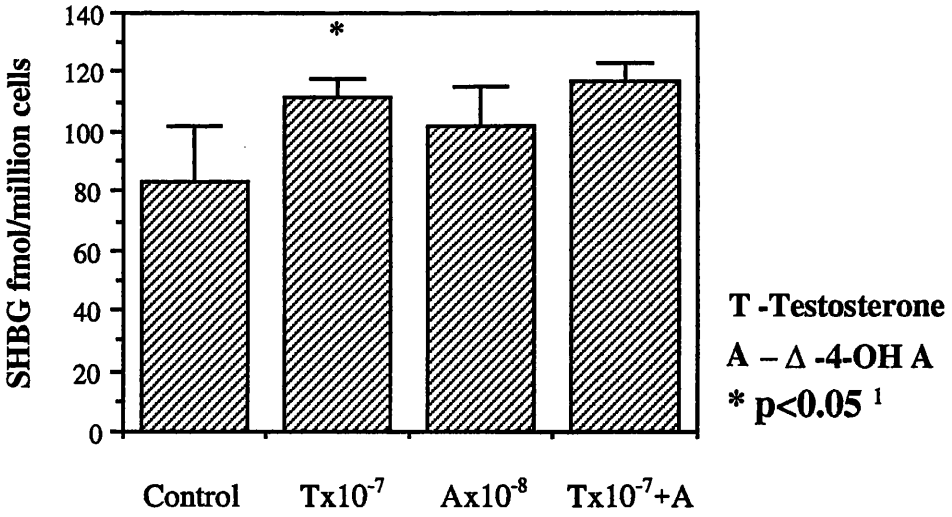


Figure 3.2.2. Effect of Testosterone on SHBG production by Hep G2 cells in the presence of an aromatase inhibitor



1. Wilcoxon ranked pairs test comparing control with each concentration.
2. One way analysis of variance, factor - concentration.

3.2.3.B. OESTRADIOL

Oestradiol, at physiological and supraphysiological concentrations, had no effect on SHBG production by Hep G2 cells under serum free conditions (Figure 3.2.3.). None of the experiments showed any change in IGFBP1 production.

3.2.3.C. PROGESTERONE

Progesterone had no consistent effect on the production of either SHBG or IGFBP1 by Hep G2 cells.

Figure 3.2.3. Effect of oestradiol on the production of SHBG and IGFBP1 by Hep G2 cells

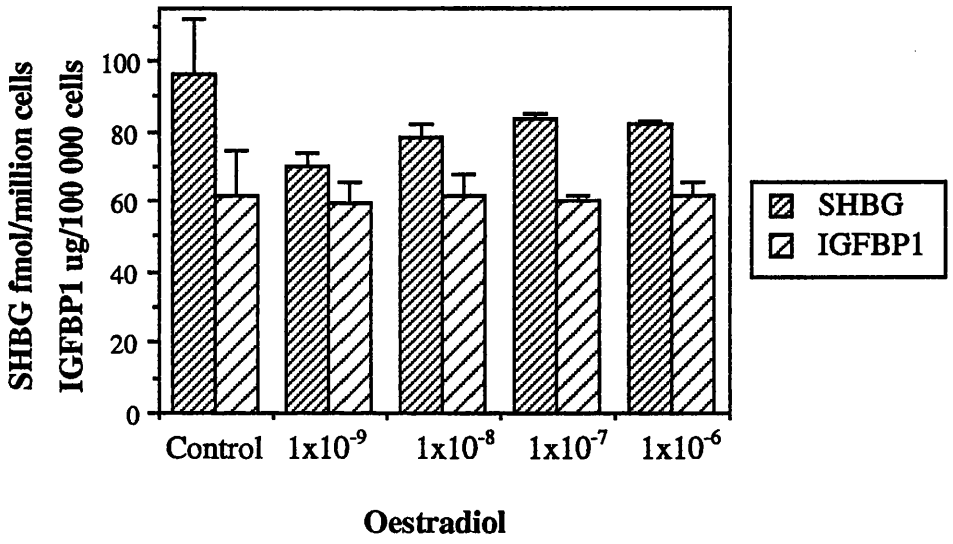
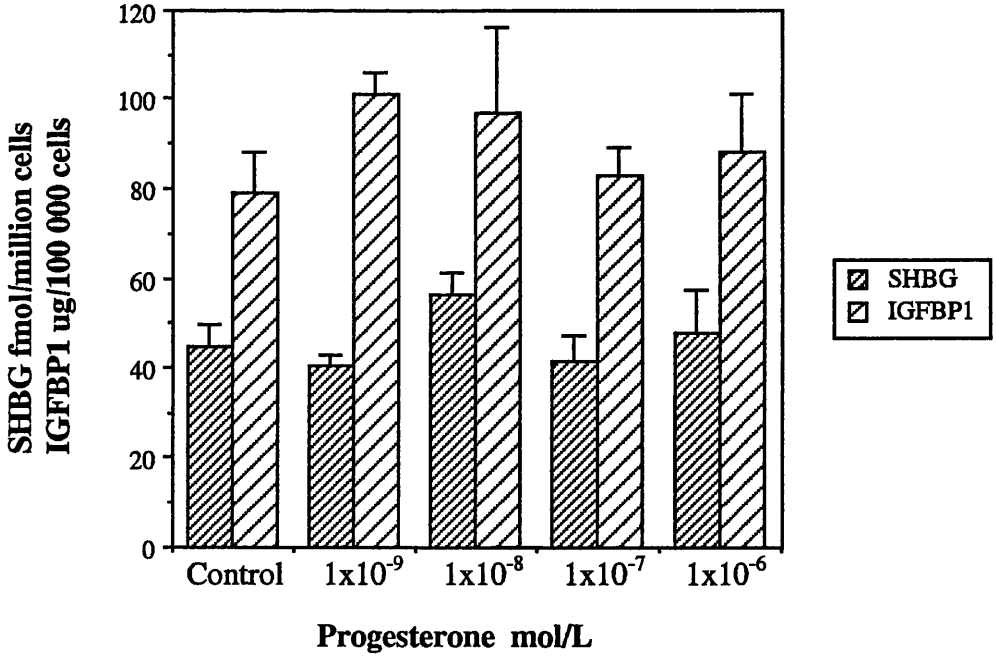


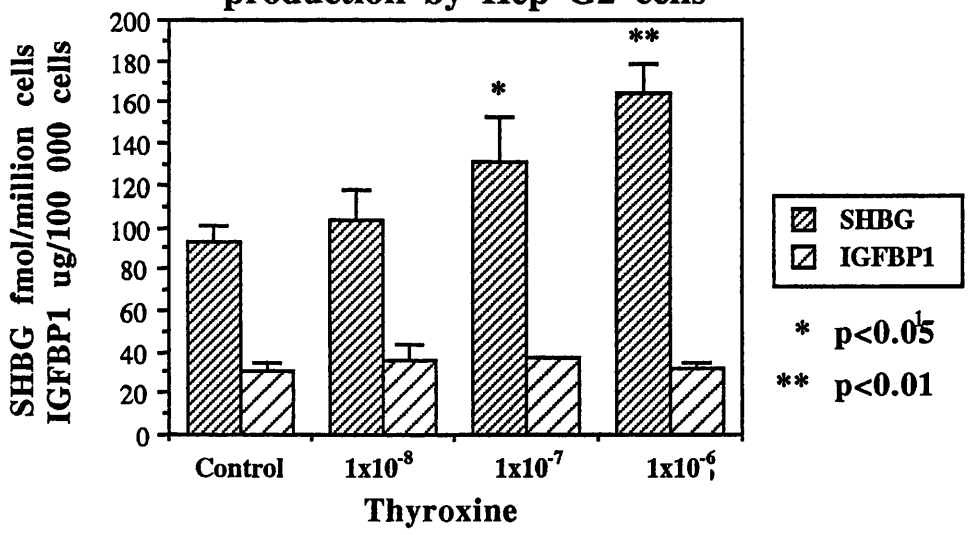
Figure 3.2.4. Effect of progesterone on SHBG and IGFBP1 production by Hep G2 cells



3.2.3.D. THYROXINE

The production of SHBG by Hep G2 cells was significantly increased by thyroxine. This was dose dependent $p<0.001$ - one way analysis of variance, factor-concentration) (Figure 3.2.5) and significant at physiological concentrations.

Figure 3.2.5. Effect of thyroxine on SHBG and IGFBP1 production by Hep G2 cells



1. Wilcoxon ranked pairs test comparing control with each concentration.

3.2.4. DISCUSSION

The theory that testosterone and oestradiol are primary regulators of hepatic production of SHBG is not supported by the results from these experiments. The possibility that the stimulation of production by testosterone might be through conversion to oestradiol is not confirmed since Δ-4-OH A did not interfere with the effect of testosterone. Although the number of samples available for statistical analysis is small in this series the stimulation by testosterone has been found by other groups (Lee et al. 1987). Recent data from Plymate et al. (1991) provides evidence to confirm that this is a primary effect of testosterone on hepatocytes by showing an increase in SHBG mRNA in cultured Hep G2 cells under similar conditions.

Oestradiol, in contrast to the findings of Lee et al. (1987), does not stimulate SHBG production under serum free conditions. The result obtained here is, however, similar to the findings of Rosner (1984) and Mercier-Bodard (1986) who found that the presence or absence of serum in the growth medium altered the results of the experiment. In view of the results from these experiments the physiological changes which have been observed in response to both oestrogen (increase) and testosterone (decrease) (Chapter 1) may not represent an alteration in the hepatic production of SHBG. These changes may, therefore, be secondary to changes in the clearance of SHBG, possibly by alteration in the glycosylation of the tertiary structure of the protein (Tardivel-Lacombe and Degrelle 1991).

The sex steroids do not appear to affect the production of IGFBP1 by these cells in this small series of experiments. Progesterone has been found to increase the production of IGFBP1 by decidual cells (Rutanen 1988). The regulation of IGFBP1, therefore, appears to vary from one cell type to another. The difference in the response of the two binding proteins to testosterone and oestradiol suggests that their hepatic synthesis may not be co-regulated by the sex steroids.

Thyroxine increases SHBG production by Hep G2 cells in a dose dependent manner

confirming the findings of other groups (Rosner et al. 1984, Plymate et al. 1990). A recent report has shown that thyroxine acts at a transcriptional level, increasing significantly the amount of mRNA for SHBG (Plymate et al. 1991). Thyroxine is generally believed to act in a typical steroidal fashion in regulating transcription of specific genes in those cells that have a thyroxine receptor (Evans 1988, Carson-Jurica et al. 1990). The physiological reasons for thyroxine inducing the SHBG gene is unclear. The increase in SHBG during pregnancy may only partly be due to a direct action of thyroxine on hepatic synthesis since oestrogen has been reported to alter the sialylation of thyroxine binding globulin (TBG) so prolonging its half-life (Ain et al. 1987) and a similar mechanism may exist for SHBG (Tardivel-Lacombe and Degrelle 1991) supporting the concept that the sex steroids modulate SHBG concentrations post-translationally but do not affect hepatic production.

3.3. EFFECT OF INSULIN ON SHBG PRODUCTION BY HEP G2 CELLS IN THE PRESENCE OF THE STEROID HORMONES AND THYROXINE.

3.3.1. AIMS

Hepatic production of SHBG and IGFBP1 appears, from the data presented here, to be directly inhibited by insulin. Clinical observation suggests that the sex steroids are important regulators of SHBG concentrations. The previous section (supported by results from other groups) suggests that testosterone and oestradiol do not act directly on the liver to alter SHBG concentrations but may act post-translationally. The aim of these experiments was to investigate whether the presence of insulin would alter the response of SHBG to the sex steroids and thyroxine.

3.3.2. METHODS

All the experiments were performed in triplicate and repeated at least twice. Insulin at a concentration of $1x10^{-5}$ was used for all the experiments. The medium used contained no phenol and no serum was added in any of the experiments. In each experiment three controls were set up; for the steroid being tested, for insulin and the medium alone.

Figure 3.3.1. Effect of oestradiol on SHBG production by Hep G2 cells in the presence of insulin

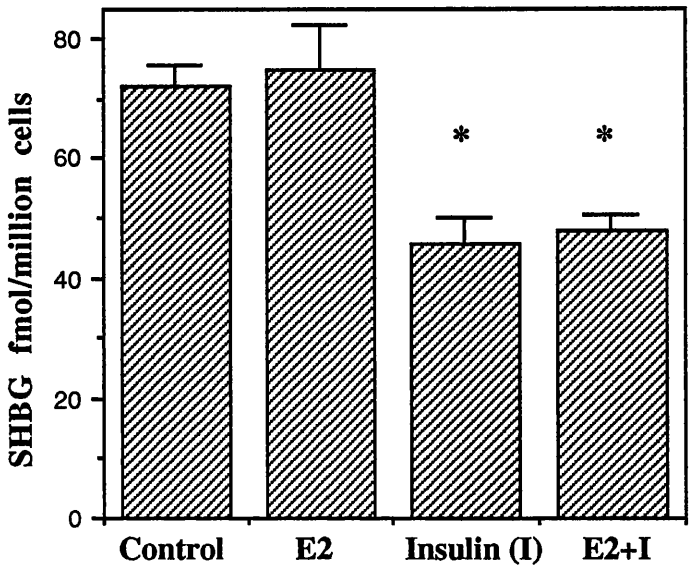
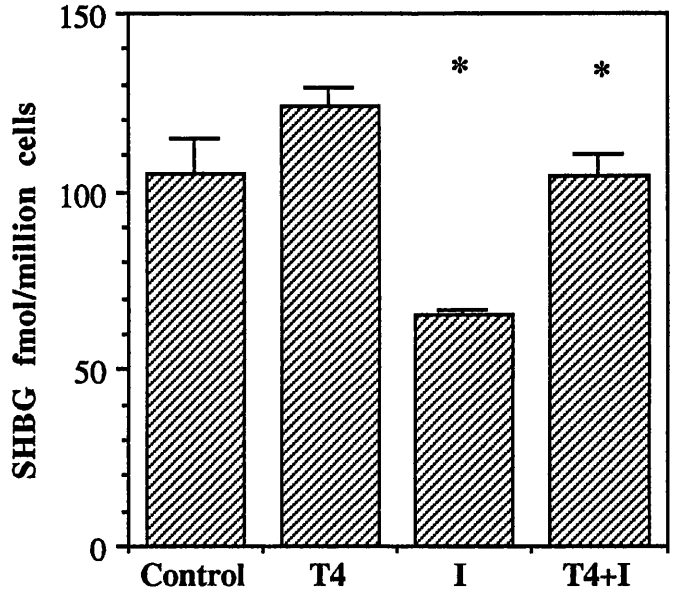


Figure 3.3.2. Effect of thyroxine on the production of SHBG by Hep G2 cells in the presence of insulin



*** p<0.001[1]**

1. Wilcoxon ranked pairs test comparing each concentration with controls

3.3.3. RESULTS

In the experiment s with insulin and testosterone; testosterone was found to stimulate SHBG production as before. The addition of insulin negated this increase but levels were similar to the control value and significantly greater than the concentrations found with insulin alone. [Control (mean (SE)): 76.9 (3.0), T:85.6 (3.9), Insulin: 65.3 (3.9) $p<0.01$, T+Insulin: 81.6 (0.6)].

In an experiment in which oestradiol and insulin were added to the medium together the concentrations of both SHBG and IGFBP1 in the medium were decreased below control values and were similar to those found with insulin alone (Figure 3.3.1.)

In the presence of thyroxine, insulin overcame the stimulant effect of thyroxine on SHBG and SHBG levels were similar to control values (Figure 3.3.2.). In all the experiments insulin alone significantly inhibited SHBG and IGFBP1 concentrations.

3.3.4. DISCUSSION

These experiments provide further evidence that insulin is likely to be an important inhibitor of SHBG. This inhibitory action overcomes the stimulatory effects of both testosterone and thyroxine although the levels remain similar to control values. In the presence of oestradiol, insulin appears to inhibit SHBG production as effectively as it does when acting alone. This data supports the hypothesis that insulin is a more important regulator of hepatic SHBG production than both the sex steroids and other hormones. Although SHBG and IGFBP1 may both be regulated by insulin it is unlikely, from the evidence provided here and from other groups (Mercier-Bodard 1986, Rosner et al.1984), that the proteins are co-regulated by other factors including thyroxine, growth hormone and the sex steroid hormones. The observation that people with conditions associated with hyperinsulinaemia - non insulin dependent diabetes mellitus, obesity, PCOS, acromegaly- have lower SHBG concentrations than controls may be due to primary inhibition of hepatic production but could be due to increased clearance. No evidence is available, at present, for the latter possibility so primary hepatic inhibition remains the most likely hypothesis.

CHAPTER 4
CLINICAL STUDIES

4.1. CHANGES IN SEX HORMONE BINDING GLOBULIN AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 IN WOMEN WITH NORMAL OR POLYCYSTIC OVARIES DURING A LOW CALORIE DIET.

4.1.1. AIMS

Women with polycystic ovary syndrome (PCOS) who are anovulatory have lower SHBG and IGFBP1 concentrations than weight-matched women with normal ovaries. These women are known to be insulin resistant (Dunaif and Graf 1989a,b; Robinson et al.1992). Obese women with or without PCOS are also insulin resistant and have been found to have lower SHBG concentrations than their lean counterparts. In the previous chapter insulin was shown to be an important inhibitor of hepatic SHBG and IGFBP1 production in-vitro. These results from in-vitro studies should be interpreted with caution when applied to human physiology since the cells are in isolation and have been transformed into immortal cell lines. Weight loss is associated with a reduction in insulin concentrations and we were therefore interested in investigating the role of weight loss following calorie restriction on insulin, insulin-like growth factor-1 (IGF-1), SHBG and IGFBP1 concentrations in obese women with normal ovaries and those with PCOS.

4.1.2. PATIENTS AND METHODS

Subjects were recruited from the endocrinology, gynaecological endocrinology and infertility clinics at St. Mary's Hospital, London and the Samaritan Hospital for Women. The control group was recruited from volunteers who were either patients with non-endocrine causes of infertility or members of staff working in the Department of Metabolic Medicine

at St. Mary's Hospital Medical School. Each subject was weighed and measured to determine their body mass index (BMI-weight (kg)/height(m)^2). The normal range is 19-25 kg/m^2 and therefore a BMI ≥25.1-30 kg/m^2 was considered "overweight" and obesity as a BMI ≥30.1 kg/m^2. All of the subjects had a haemoglobin of >12.0 g/dl and were euthyroid. None of the women was on any long term medication. The women had not taken the oral contraceptive pill or other hormonal preparation in the three months preceding the study.

Five obese women with polycystic ovary syndrome were investigated. Their mean (SD) BMI was 35.7 (3.2) kg/m^2. All had a BMI of >30.1 kg/m^2. They were hirsute (Ferriman-Galwey score >7), had polycystic ovaries on ultrasound, and had either a raised serum testosterone, raised luteinising hormone (LH), or both (Adams et al. 1985, 1986; Franks 1989). The upper limit of normal for testosterone (2.6nmol/L) and LH (11.1IU/L) was taken as greater than two standard deviations from the mean of 60 women with normal ovaries and regular menstrual cycles (Adams et al. 1986). The control group comprised six women with regular menses who had normal ovaries on ultrasound scan and whose body weight was at the upper limit of, or slightly greater than, normal (mean 25.5 (2.2)). All subjects were given the diet for two weeks and, in the PCO group alone, for a total of four weeks. This study was performed with the approval of the local Ethical Committee (Parkside Health Authority). Each subject signed a consent form prior to starting the diet.

The diet was a very low calorie diet of 350 kcal/day provided by Cambridge Nutrition Ltd., Norwich, Norfolk, UK (The Cambridge diet) and comprised 33g protein, 42.7g carbohydrate and 2.9g fat per 100g. The subjects were allowed a free fluid intake of water or low calorie drinks.

Blood samples were taken after an overnight fast on the day before starting the diet (day 0) and again after two weeks and four weeks (PCOS group only) of the diet (weeks 2 and 4). The samples (30mls) were taken from an antecubital vein without a tourniquet and allowed to stand for two hours before being spun at 2000g, 4^{o}C for 10 minutes. The serum was

separated and the samples stored at -20^{o}C until analysed for testosterone, free testosterone, insulin, IGF-1, SHBG and IGFBP1. Statistical analysis of the differences in the results before and after dieting and between the PCO and control group was by Wilcoxon ranked pairs test. One subject with PCOS had a serum sample taken daily for the first seven days and on alternate days for the following week (Day 14). The samples were analysed for SHBG and IGFBP1 only.

Testosterone was measured by radioimmunoassay (Wheeler and Luther 1983). The percentage free testosterone was measured by an ultrafiltration method and the free testosterone was calculated (Vlahos et al. 1982). Insulin was measured by radioimmunoassay (RIA)(Hampton et al. 1986) and IGF-1 by RIA after acid-ethanol extraction of the serum (Daughaday et al. 1980). [Details of these assays are listed in Appendix A] SHBG was measured using an immunoradiometric assay (Farmos, Pharmacia, Milton Keynes U.K.) The serum concentrations of IGFBP1 were measured by an immunofluorometric assay as described by Koistinen et al. (1987b). The details of these assays are outlined in Chapter 2. (Sections 2.1. and 2.2.).

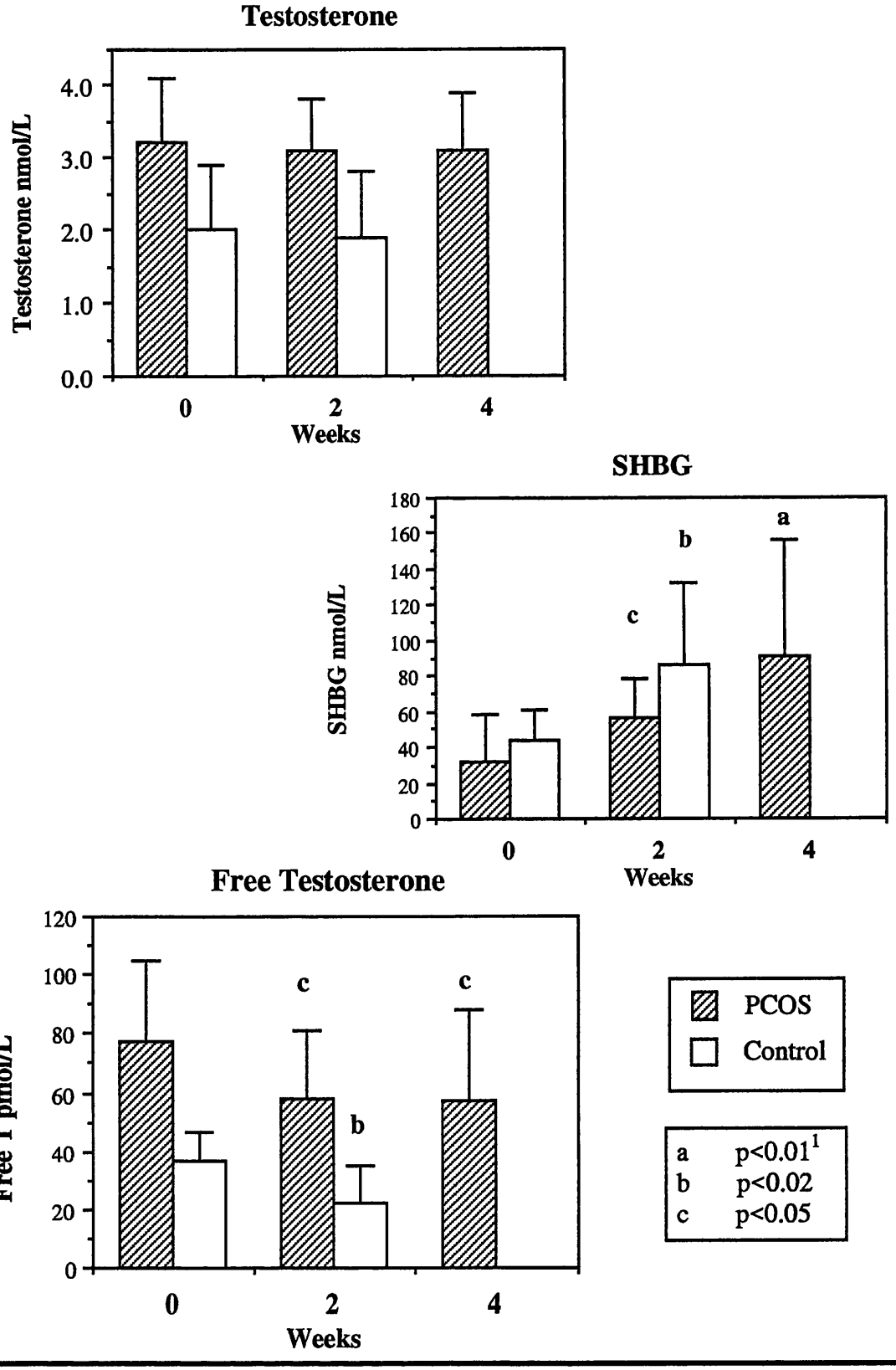
4.1.3. RESULTS

All of the subjects lost weight on the low calorie diet. At two weeks the mean (SD) weight loss was 5.2 (2.4) kg in the PCO group and 4.3 (1.0) kg in the control group. After four weeks the PCOS group had lost 7.1 (2.8) kg which was 6.8 (2.1) % of their initial weight.

The changes in total testosterone, SHBG and free testosterone are shown diagramatically in Figure 4.1.1. There was no change in levels of total testosterone in either the PCO or control group as a result of calorie restriction. SHBG concentrations, however, had doubled at two weeks in both groups and remained elevated at four weeks in the PCO group. There was a significant fall in free testosterone levels over the same period:25% in the PCO group and 40% in the controls. The results for each individual both before and after dieting are

Figure 4.1.1.

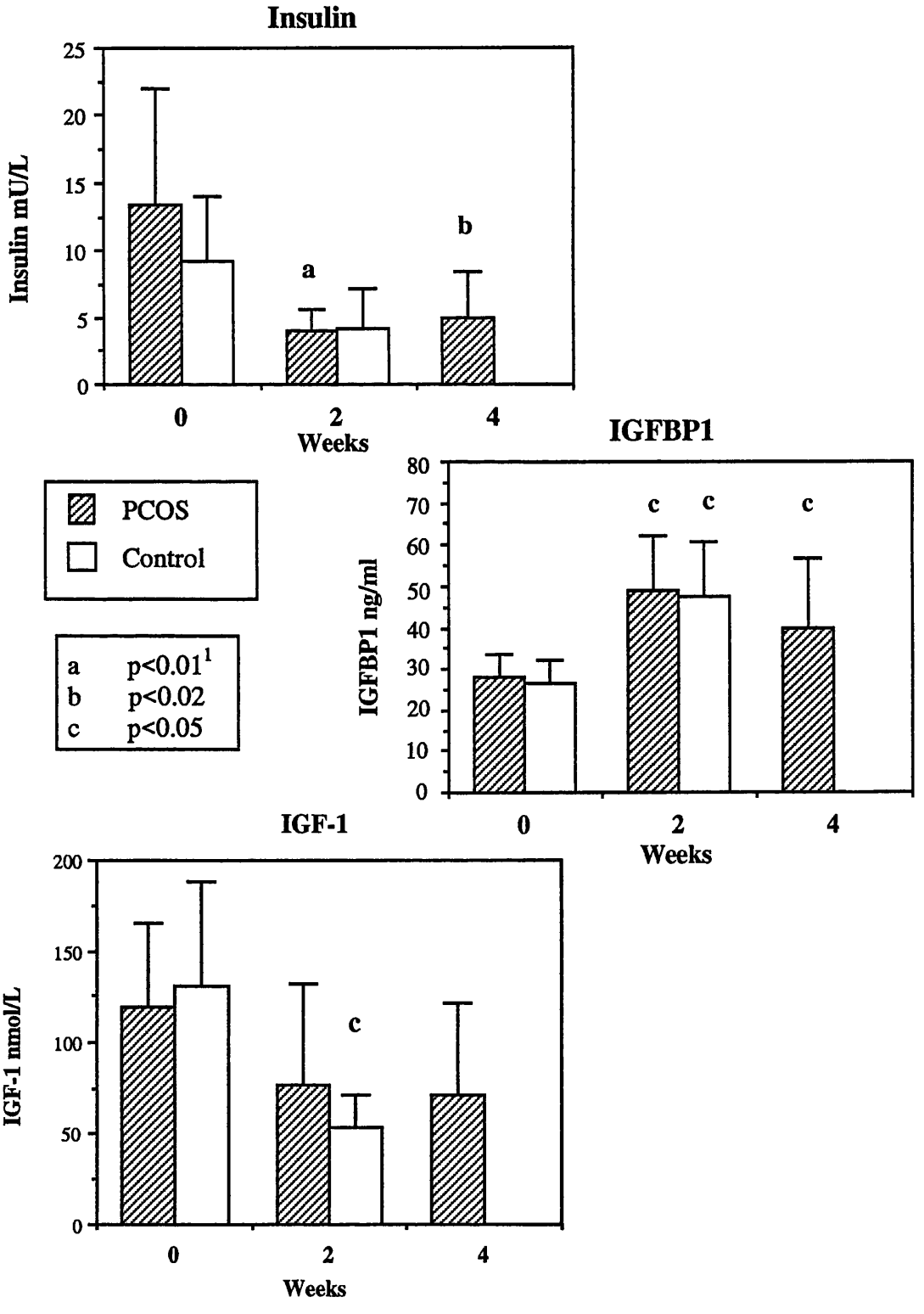
Changes in Testosterone, SHBG and free testosterone concentrations during and after calorie restriction



1. Wilcoxon ranked pairs test comparing results at each interval with week 0

Figure 4.1.2.

Changes in Insulin, IGFBP1 and IGF-1 concentrations during and after calorie restriction



1. Wilcoxon ranked pairs test comparing results at each interval with week 0

shown in Appendix D: Table A, and the overall data in Table 4.1.1.

The changes in insulin, IGF-1 and IGFBP1 during the diet are shown in Figure 4.1.2. Serum insulin levels fell in both groups of subjects. This was a significant decrease in the women with PCO but not in the controls because although five out of six showed a fall in insulin, one subject had slightly higher levels at 2 weeks than at the start of the study. IGF-1 concentrations were significantly lower after two weeks and the serum IGFBP1 concentration showed a significant rise in both groups. This rise was greatest at 2 weeks in the PCO group but was still significantly higher than the control value after 4 weeks.

Table 4.1.1. Table of the mean values (SD) in women with PCOS and the control group before and after a low calorie diet.

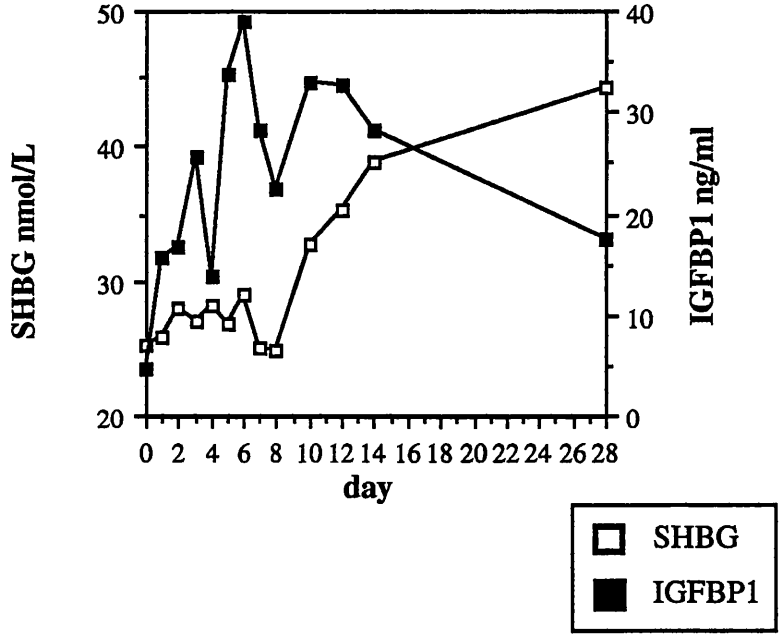
GROUP WEEKS	T nmol/L	SHBG nmol/L	Free T pmol/L	Insulin mU/L	IGF-1 nmol/L	IGFBP1 ng/ml
PCOS						
0 weeks	3.2 (0.9)	32 (26)	77 (28)	13.3 (8.7)	119 (46)	28.1 (5.5)
2 weeks	3.1 (0.7)	56 (22)	58 (23)	4 (1.5)	77 (55)	48.9 (13.2)
4 weeks	3.1 (0.8)	91 (65)	57 (31)	4.9 (3.6)	72 (49)	39.9 (16.6)
P value[1]						
2	NS	<0.01	<0.01	<0.05	NS	<0.05
4	NS	<0.05	<0.01	<0.02	NS	NS
CONTROL						
0 weeks	2.0 (0.9)	43 (18)	37 (10)	9.2 (4.8)	131 (57)	26.6 (15.7)
2 weeks	1.9 (0.9)	86 (46)	22 (13)	4.2 (2.9)	54 (18)	47.3 (24.9)
P value	NS	<0.02	<0.02	NS	<0.01	<0.05

1. Wilcoxon ranked pairs test comparing results between groups

The changes in SHBG and IGFBP1 during the first fortnight of the diet in one subject are shown in figure 4.1.3. She lost 3.1 kg during the fortnight. Her fasting insulin concentrations fell by 53.5% (18.7 to 8.7mU/L). This decrease continued over the next fortnight to a final concentration on d.28 of 7mU/L. The increase in SHBG was gradual with little change in the first week (d.0; 25.3-d.7; 25.1 nmol/L) and a more rapid rise in the second week (d.14; 38.8 nmol/L). IGFBP1 concentration rose more rapidly with a wider daily fluctuation. There was a fourfold increase in IGFBP1 concentrations at 7 and 14 days (4.8 to 28.2 ng/ml) but the increase was not sustained, with a fall in IGFBP1 levels to 17.6 ng/ml on d.28. (Data in Appendix D, Table D.1.2.)

Figure 4.1.3.

Changes in SHBG and IGFBP1 concentrations during 28 days of calorie restriction in a single subject



Significant negative correlations were found between insulin, SHBG, free testosterone and IGFBP1 in both groups (Table 4.1.2.). There was also a positive correlation of SHBG with IGFBP1 (r=0.23). These correlations were calculated using the pre - during and post - calorie restriction values in both the PCOS and control group (n=27). Since there was no significant change in total testosterone concentrations no correlation was found with either the binding proteins or insulin and IGF-1. Insulin and IGF-1 showed a significant positive correlation (r=0.40) under these fasting conditions.

Table 4.1.2. Correlations of SHBG and IGFBP1 to insulin, IGF-1, testosterone and free testosterone.

	SHBG r value	IGFBP1 r value
Insulin	-0.43*	-0.49*
IGF-1	-0.51*	-0.47*
IGF-1	0.17	0.08
Testosterone	-0.52*	0.23

r value = Spearman's coefficient. n=27.
* $p<0.02$

4.1.4 DISCUSSION

The data from this small study suggests that calorie restriction leads to marked changes in SHBG and free testosterone concentrations although total testosterone concentrations did not change. These changes were associated with inverse alterations in insulin and IGF-1 concentrations. The reciprocal changes in the concentrations of SHBG, insulin and IGF-1 support the evidence from the in-vitro data in Hep G2 cells (Chapter 3 and Plymate et al.1988) that insulin and IGF-1 may directly inhibit the synthesis of SHBG, thus effecting androgen metabolism. During calorie restriction insulin levels decline leading to an increase in SHBG production, an increased binding of testosterone and therefore a decrease in circulating levels of free testosterone even though total testosterone levels do not change.

The relationship of the changes in IGFBP1 concentrations to those in insulin, IGF-1 and SHBG imply a direct effect of insulin and possibly IGF-1 on hepatic production of IGFBP1. The evidence from the previous chapter supports the role of insulin as an inhibitor of hepatic IGFBP1 production but not that of IGF-1. Insulin has been shown to regulate IGFBP1 levels in vivo and in- vitro in many cell types (Suikkari et al 1988,1989a, Conover 1990c, Conover and Lee 1990b). The data presented suggests there may be a close relationship between the control of SHBG and IGFBP1 and is in keeping with the finding of a parallel change in these binding proteins during puberty (Holly et al 1989). The data from the single patient investigated on a daily basis suggests that the time course and the permanence of such changes in SHBG and IGFBP1 may be different, with SHBG taking longer to respond to alterations in insulin concentrations than IGFBP1. This may reflect a difference in the binding proteins' respective half-lives in the circulation.

The effect of calorie restriction on SHBG, insulin and IGFBP1 was similar in women with normal ovaries and those with PCO. Clinically these changes are likely to be more important to women with PCO because of the possible beneficial effect the alterations in androgen metabolism may have on hirsutism. There is, however, little data available discussing the effect of calorie restriction/weight loss on hirsutism (Pasquali et al. 1989) although a longer term diet has shown a significant improvement in the Ferriman-Gallwey score in women who lose >5% of their body weight (Kiddy et al. 1992). The association of obesity and oligo/amenorrhoea is well recognised in women with PCOS (Stein and Leventhal 1935, Goldzieher and Green 1962, Yen 1980) and those with normal ovaries (Hartz et al. 1979, Kopelman et al 1981). Weight loss has been reported to improve menstrual disturbance (Kopelman et al 1981, Harlass et al 1984, Kiddy et al. 1992) and this does not seem to be specific for women with PCOS.

The diet used in this study was a low fat diet. The effect that various constituents and their relative proportions within a diet have on SHBG levels has been studied. Altering the protein/carbohydrate content of diet (Anderson et al. 1987), eating a high fibre diet containing phyto-oestrogens (Adlercreutz et al. 1987) and, in men, a low fat diet have all

been shown to increase SHBG levels (Reed et al. 1987). The increase in SHBG concentrations and the increased excretion of oestrogens was thought to be mediated by alterations in the enterohepatic circulation of oestrogens (Adlercreutz et al. 1987). These studies do not report any changes in weight or insulin concentrations which, in the light of this study, may play an important role in altering SHBG concentrations. The effect on SHBG of a low fat diet in men was associated with a change in lipid profile (Reed et al. 1987) and, in women, a significant correlation between SHBG and lipoproteins has been reported (Longcope et al. 1990). The combination of changes in the lipid profile and those in SHBG may be mediated by insulin since the latter is known to alter the concentrations of the high density lipoprotein fraction. Abnormal lipid profiles have been reported in women with PCOS and this has led to conjecture that they have an increased risk of cardiovascular disease. The differences are independent of oestrogen concentrations but are reported correlate significantly with hyperandrogenism and hyperinsulinaemia (Wild et al. 1985, 1988b).

The changes in insulin concentrations after weight loss reported in this study support the view from in-vitro studies that SHBG and IGFBP1 may be co-regulated. The consequent effect on the production, transport and metabolism of androgens may influence menstrual patterns and hirsutism. These results do not, however, give us any information on the differences in the production and clearance rate of the two binding proteins and this is important in the understanding of their metabolic effects.

4.2. RESPONSE OF SEX HORMONE BINDING GLOBULIN (SHBG) AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP1) TO AN ORAL GLUCOSE TOLERANCE TEST (OGTT) BEFORE AND AFTER CALORIE RESTRICTION

4.2.1. AIMS

The evidence from the in-vitro data (Chapter3.1.) and that from the previous study (Chapter 4.1.) support the concept that alterations in insulin concentrations affect the hepatic production of SHBG and IGFBP1. The possibility that these two proteins are co-regulated, therefore, remains. The evidence presented so far, however, does not provide information on the time response of these two proteins to acute changes in insulin concentrations. An oral glucose load produces a rapid rise in insulin concentrations which return to baseline levels within a few hours.

The purpose of this study was, therefore, to determine the relationship of acute changes in insulin to those in both SHBG and IGFBP1 and secondly to examine the effect of calorie restriction on the levels of these binding proteins during an oral glucose tolerance test.

4.2.2. METHODS

Six obese subjects (mean BMI (SD) 34.2 (3.4)) with PCOS were studied before and after four weeks on the Cambridge diet (section 4.1.2.). Each subject was given a 75g oral glucose load after an overnight fast and blood samples were taken every 30 minutes for three hours. The samples were divided between a tube containing fluoride oxalate for glucose estimation and a heparinised tube for plasma preparation. The samples were spun and stored as described above. They were analysed for glucose, insulin, SHBG and IGFBP1.

Analysis of the difference in the sum of the SHBG and insulin concentrations during the OGTT before and after calorie restriction was compared using the Wilcoxon reanked pairs test. The change in the IGFBP1 levels from the starting value to each time point during the OGTT was analysed by two way analysis of variance with time and concentration as the factors..

4.2.3. RESULTS

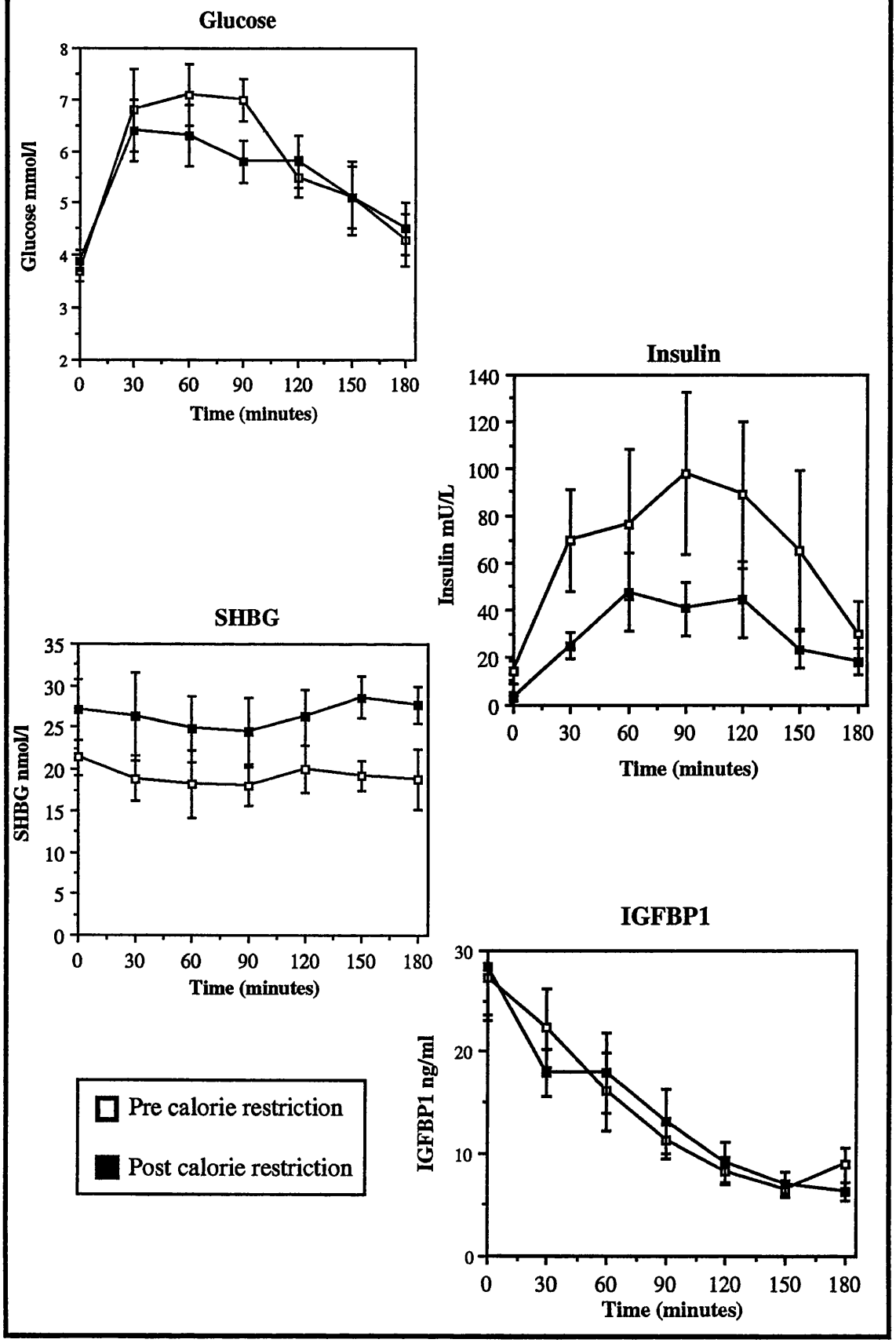
All of the subjects investigated lost weight during the four week period of the study (range1.7-9.5kg). In this study the percentage weight loss (mean 6.6%-range 2.2-9.2%) did not affect the results. The testosterone concentrations did not change during the period of calorie restriction (pre: mean (SD); 3.4 (0.3), post; 3.0 (0.5) nmol/L). The individual data is shown in tabular form in Appendix D, section D.2.1.

The changes in glucose, insulin, SHBG and IGFBP-1 concentrations during the oral glucose tolerance tests before and after calorie restriction are shown in Figure 4.2.1. Each point represents the mean (SE) of the values obtained in the six subjects.The changes in glucose and insulin levels during the three hour period are as expected following a glucose load. The fasting glucose levels and the increase following an oral glucose load did not change after calorie restriction. The sum of the insulin concentrations released during the OGTT was significantly lower after dieting than before treatment (pre: mean (SD);415.6 (349), post; 201.1 (129.4) mU/L,$p<0.03$). The fasting and the sum of the plasma SHBG levels were significantly lower before the period of calorie restriction than after (fasting; pre;21.4 (5.1), post;27.1 (8.7)nmol/L $p<0.01$, sum: pre;129.9 (40.5), post;164.3 (70.6) $p<0.03$). A negative correlation between the sum of SHBG and insulin concentrations ($r=-0.69$ $p<0.01$) was found, confirming previous reports.

During the OGTT there was no significant change in the serum SHBG levels but there was a rapid and marked decline in plasma IGFBP-1 levels over the same period. The IGFBP-1 levels continued to decline for the full three hours and analysis of variance showed a significant difference in concentration over time ($p<0.001$). The fasting concentration of IGFBP-1, however, was not significantly different from pretreatment values after four weeks of calorie restriction. IGFBP1 was significantly negatively correlated with insulin ($r=-0.39$, $p<0.03$, $n=82$) during the OGTT but not with glucose ($r=-0.15$, $p=0.7$, $n=82$).

Figure 4.2.1.

Changes in plasma glucose, insulin, SHBG and IGFBP1 concentrations during an oral glucose tolerance test before and after calorie restriction



4.2.4. DISCUSSION

The evidence from this small studysuggests that there are major differences in the time course of the response of these two binding proteins. There was a rapid decrease in IGFBP-1 concentrations as insulin levels rose whilst SHBG concentrations did not change. In contrast weight loss, accompanied by significant decreases in fasting and glucose stimulated insulin concentrations, resulted in a significant increase in baseline SHBG concentrations but no significant change in IGFBP1 levels (Kiddy et al 1992). A recent study of women with normal ovaries has shown a small but significant decrease in SHBG during an OGTT in women with normal ovaries (Holownia et al.1991). The women in this study were obese and had PCOS - both conditions associated with hyperinsulinaemia. The presence of chronic hyperinsulinaemia may, therefore, blunt the response of SHBG to acute changes in insulin levels although prolonged decreases in insulin concentrations do lead to an increase in SHBG concentrations.

The half-life of SHBG is quite long at about 7 days (Anderson et al. 1976) and since there is no apparent acute response to changes in insulin concentrations the increase in SHBG concentrations may be quite slow. Changes in SHBG production in response to insulin at a transcriptional level by Hep G2 cells after 48 hours have been reported (Plymate et al. 1990). In addition alterations in the glycosylation of the SHBG molecule may affect its clearance (Tardivel-Lacombe and Degrelle 1990) raising the possibility that both mechanisms may play a role in changing circulating SHBG levels. In this study, as in section 4.1. total testosterone concentrations did not alter. The changes were, therefore, most likely to be secondary to alterations in insulin concentrations over the same period.

In this study there was no difference in the fasting IGFBP1 concentrations before and after calorie restriction. This may appear to contradict the findings of the study reported in Chapter 4.1 but in that study the maximum increase in IGFBP1 levels was observed after two weeks of calorie restriction with a decline towards baseline levels after four weeks; the data presented here only includes data after four weeks of calorie restriction. In a longer

term study the levels of IGFBP-1 were similar before and after six months of a 1000 calorie/ day diet in spite of a long term decrease in insulin concentrations (Kiddy et al 1992). The rapidity of the response by IGFBP1 to the rise in insulin confirms the findings of Suikkari et al. 1990). This change may be due to a rapid alteration in hepatic production of IGFBP1 but could also be secondary to an increased peripheral uptake of IGFBP1. The half-life of IGFBP1 is not accurately known but is predicted to be short because its molecular structure includes PEST regions (see Section 1.2.1.) and its mRNA contains sequences found in unstable mRNAs (Julkunen et al.1988, Shaw and Hamen 1986). Recent in-vitro studies have shown that IGFBP1 mRNA production by rat H411E hepatoma cells is inhibited by insulin within three hours (Unterman et al.1991, Orlowski et al.1991) and by glucose in 6 hours (Cotterill et al. 1989, Lewitt et al. 1989). The role of IGFBP1 in glucose counter-regulation has been discussed by Lewitt and Baxter (1990) who suggest that insulin reduces IGFBP1 concentrations in order to increase free IGF-1 concentrations thus allowing IGF-1 to act as a hypoglycaemic agent. The evidence presented here, therefore, implies that IGFBP-1 concentrations may alter quite rapidly in response to acute changes in insulin concentrations, followed by a period of re-equilibration, resulting in a return over time of circulating IGFBP1 concentrations towards their original levels.

In summary the inverse relationship between insulin and SHBG and IGFBP-1 suggests that insulin plays a direct regulatory role in the circulating concentrations of these two binding proteins. The difference in the time course of the response of the two proteins in vivo may be explained by differences in their half-lives in the circulation and by differences in the hepatic regulation of mRNA of the binding proteins. These in vivo studies throw doubt on the concept of glucose as a regulator of IGFBP1 concentrations since the response of serum glucose to a glucose load is unaltered following weight loss, while that of insulin is significantly reduced.

4.3. DIURNAL CHANGES IN SEX HORMONE BINDING GLOBULIN (SHBG) AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 (IGFBP1) IN WOMEN WITH NORMAL AND POLYCYSTIC OVARIES AND THE RELATIONSHIP WITH INSULIN AND INSULIN-LIKE GROWTH FACTOR-1 (IGF-1).

4.3.1. AIMS

The differences between SHBG and IGFBP1 in their response to insulin prompted us to investigate the possible fluctuations in SHBG and IGFBP1 and their relationship to circulating insulin levels during a 24 hour period. Despite the reported long half-life of SHBG a small diurnal variation in SHBG concentrations has been demonstrated in young men (Plymate et al. 1989) in association with the early morning rise in testosterone. There is no similar data available for women. In contrast IGFBP1 levels have been shown to have a clear cut diurnal pattern in both sexes (Holly et al. 1988, Suikkari et al. 1988). The previously presented clinical studies and in-vitro evidence (Chapter 3) suggest that insulin and, possibly, IGF-1 co-regulate the hepatic production of both SHBG and IGFBP1. The hypothesis of this study was that SHBG and IGFBP1 may be coregulated by insulin. The aim of this study was to investigate firstly whether SHBG exhibits a diurnal variation in women as it does in men, and secondly whether there is a significant inter-relationship between SHBG and IGFBP1, insulin and IGF-1. Since physiological concentrations of SHBG may be altered by hyperandrogenaemia the study was conducted comparing ten women with anovulatory PCOS with ten weight-matched ovulatory controls.

4.3.2. METHODS

Twenty women were recruited from the endocrine clinics at St. Mary's Hospital, the Samaritan Hospital for Women and the infertility clinic at the Hammersmith Hospital (with the kind permission of Professor R.M.Winston and Mr R. Margara). Ten had polycystic

ovary syndrome and were anovulatory. They presented with oligomenorrhoea or secondary amenorrhoea. Seven of the women were hirsute (Ferriman-Gallwey score >7). The diagnosis was confirmed by an ultrasound scan, a raised testosterone (>2.6nmol/L -9/10 women) and/or luteinising hormone (LH) (>11 IU/L -7/10 women) concentration (Adams et al. 1986). Ten weight matched women with regular menstrual cycles (cycle length of 28±2 days), normal ovaries on ultrasound scan and a mid-luteal progesterone of >30 nmol/ L on two occasions were recruited as the control group. Each subject signed a consent form and ethical committee permission was obtained from the Parkside Health Authority.

An intravenous cannula was inserted into the antecubital fossa and attached via a three way tap to a slow running intravenous infusion of normal saline. The subjects were given regular hospital meals and drinks at specified times, with the exception of the women with PCOS who were given a 75g (330kcal) oral glucose tolerance test after an overnight fast (during the 24 hours) but did not receive breakfast. The control group were given breakfast (cereal with sugar, tea/coffee and toast) representing a glucose load of approximately 35g (total calories-320kcal). The control and PCOS group all received their calories between 08.00-10.40 in the morning. The two hour period in which they were given the calories is referred to as time 0 in Figure 4.3.2. One study on non-insulin dependent diabetics has suggested that the insulin response to an OGTT or to breakfast is not significantly different (Peterson and Jovanovic-Peterson 1991). Serum samples were collected every two hours. These samples were analysed for SHBG, IGFBP1, insulin and IGF-1. The results are presented as the median (interquartile range) at two hourly intervals, firstly from midnight to midnight in Figure 4.3.1 and secondly from six hours before the OGTT (PCOS group) or breakfast (control group) in Figure 4.3.2.

Differences between the groups were compared using Wilcoxon ranked paired tests of the individual peak and trough concentrations in each group. The variation in insulin, IGFBP1,IGF-1 and SHBG concentrations over 24 hours was tested using two-way analysis of variance with the factors time and subject. Spearmans correlation coefficient was calculated from each subjects median value over 24 hours (Table 4.3.2).

Table 4.3.1. Median (interquartile range) of BMI, LH and testosterone concentrations in the control and PCOS group

	Control group n=10 [Median (interquartile range)]	PCOS n=10 [Median (interquartile range)]
BMI (Kg/m^2)	24.3 (23.2-25.7)	25.2 (22.2-29.3)
LH (IU/L)	4.1 (3.6-4.5)	12 (10-15)*
Testosterone (nmol/L)	1.9 (1.9-2.5)	3.8 (2.9-5.6)**

Wilcoxon ranked signed pairs test * $p<0.05$

** $p<0.001$

4.3.3. RESULTS

The two groups were weight matched (Table 4.3.1.). The serum testosterone (T) and luteinising hormone (LH) levels were significantly raised in the PCOS group compared to the control group ($p<0.001$ and $p<0.05$ respectively).

The results are shown in graphical form in Figure 4.3.1. Each graph shows the median (interquartile range)) of the values for each group at two hourly intervals during the day normalised to midnight. Figure 4.3.2 shows the median (interquartile range) of insulin and IGFBP1 normalised to to the time of the OGTT (PCOS group) or breakfast (control group)..

The individual peak and trough SHBG concentrations were significantly lower in the PCOS group compared with the control group (peak and trough $p<0.01$). Two-way analysis of variance found no variation in SHBG concentrations during the 24 hour period in either group. There was a significant negative correlation between insulin and SHBG (Spearmans correlation coefficient $p<0.05$)

Two way analysis of variance using time and subject as factors showed a significant diurnal variation in IGFBP1 which was similar in the two groups ($p<0.0001$). The peak and trough levels were significantly lower in the women with PCOS ($p<0.03$) compared to controls.

Figure 4.3.1.
Changes in the concentration of SHBG,Insulin,IGFBP1 and IGF-1 over 24 hours (midnight to midnight)

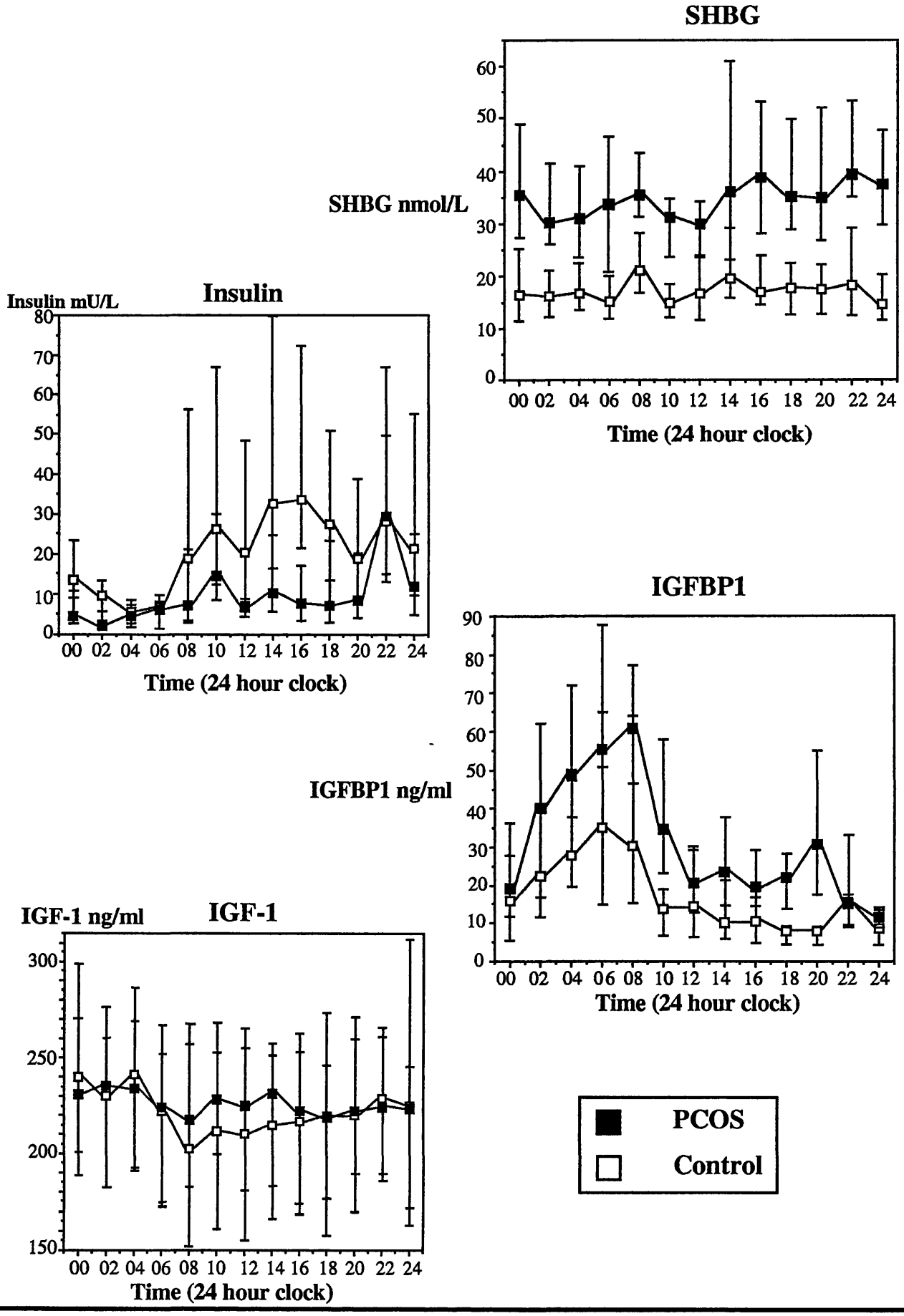


Table 4.3.2. Median (range) concentrations of insulin, SHBG, IGFBP1 and IGF-1 over 24 hours

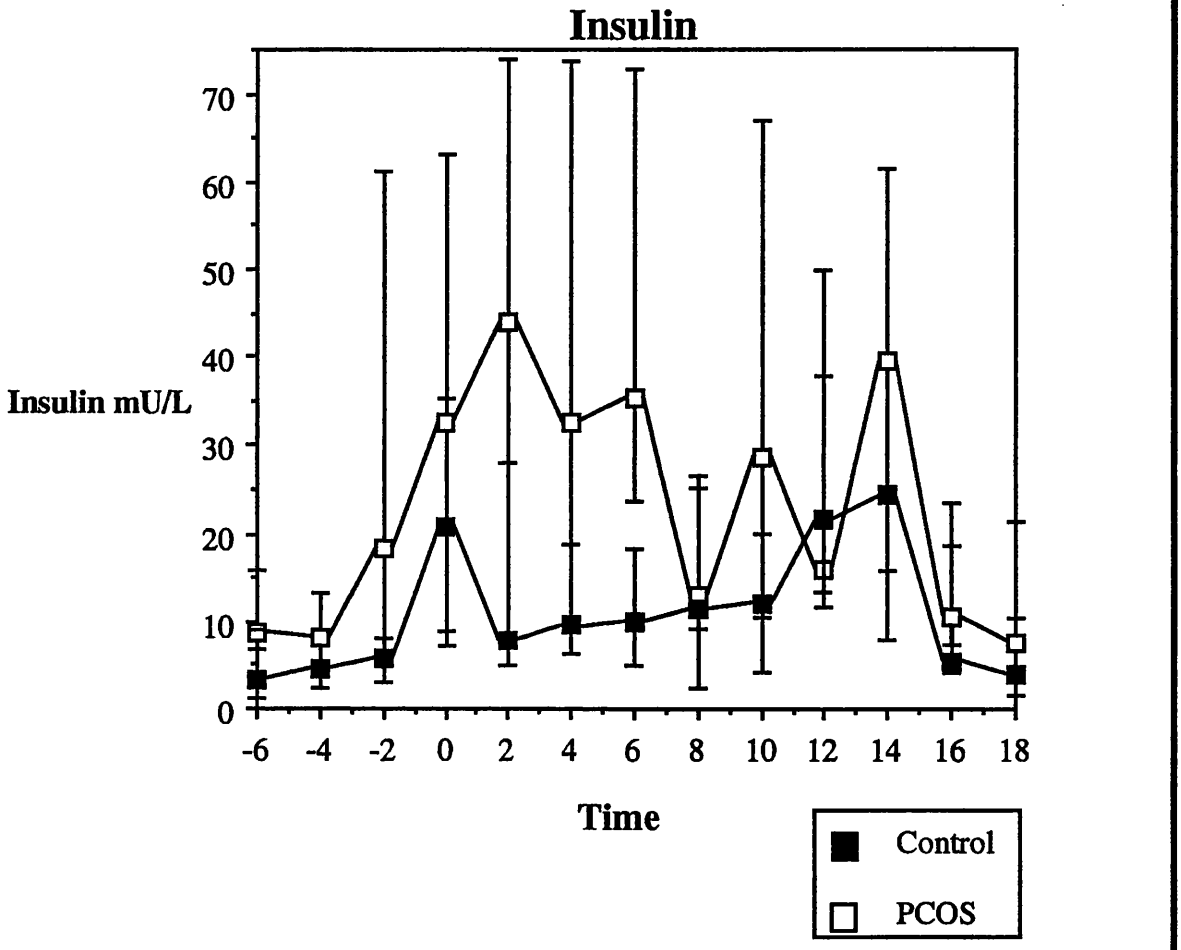
	Control group n=10 Median (range)	PCOS n=10 Median (range)
Insulin (mU/L)	12.8 (5.9-18.1)	27.2 (8.6-91.4)
SHBG (nmol/L)	35.3 (22.8-59.1)	16.7 (6.7-28.3)
IGFBP1 (ng/ml)	34 (15.9-49.5)	17.5 (7.2-43.2)
IGF-1 (ng/ml)	212.4 (162.9-344.2)	212.4 (122.8-387.3)

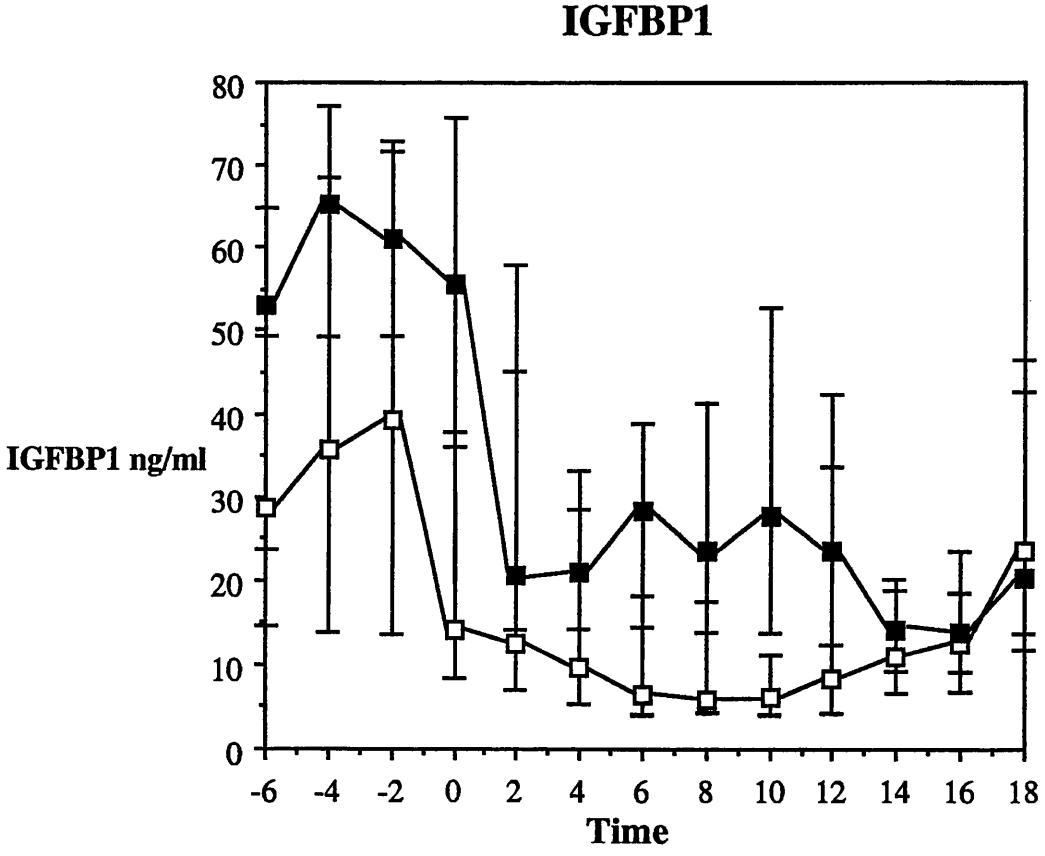
The decline in IGFBP1 concentrations followed the increase in insulin concentrations and there was a significant negative correlation between insulin and IGFBP1 in both groups (Spearmans correlation coefficient $p<0.01$).

Figures 4.3.1 and 2 show how insulin levels fluctuated throughout the day in both groups in response to calorie intake with the greatest rise in the morning. The fasting levels of insulin at 06.00 [median (interquartile ranges)] were not significantly different between the groups; 6.6 (5.4-9.8) and 6.2 (1.9-7.6) mU/L respectively although the individual peak and trough concentrations were significantly different (peak $p<0.05$, trough $p<0.002$). This difference between the groups remained significant even when the four hours following the OGTT/breakfast were excluded ($p<0.007$). None of the women in the PCOS group had an abnormal glucose concentration following their oral glucose load.Only the control group showed a second peak of insulin after their evening meal. The women with PCOS sustained a raised insulin concentration throughout the day before the concentrations declined overnight. Two-way analysis of variance, therefore, showed a diurnal variation in insulin concentrations in the control group ($p<0.001$) but not in the PCOS group ($p>0.1$).

There was no variation in IGF-1 concentrations during the 24 hour period in either group. The individual peak and trough IGF-1 concentrations were similar in the two groups.

Figure 4.3.2.
Diurnal variation of Insulin and IGFBP1 concentrations normalised to the time of an OGTT (PCOS group) or breakfast (control group)





There were significant negative correlations between the 24hr median concentration of insulin and both SHBG ($p<0.05$) and IGFBP1 ($p<0.01$). The correlation between SHBG and total testosterone was not significant ($r=-0.49$). There was no correlation between IGF-1 and insulin, IGFBP1 or SHBG. The subjects in the two groups had similar body mass indices and so these correlations are independent of weight.

4.3.4. DISCUSSION

The absence of a diurnal variation of SHBG in the two groups of women has been reported in a small group of four women (Yie et al. 1990) but there are no previous studies comparing hyperandrogenic women with weight matched controls. A small diurnal variation in males that is thought to be related to the early morning increase in testosterone levels has been reported (Clair et al. 1985, Plymate et al. 1989). The small decrease in men is thought to reflect an increased clearance of SHBG mediated by testosterone rather than a decrease in hepatic SHBG synthesis. This concept is supported by evidence that sex hormones alter the glycan microheterogeneity pattern of SHBG and thence its rate of clearance (Tardivel-Lacombe and Degrelle 1991). The significant difference in SHBG levels between the two groups, which was independent of their testosterone concentrations and their BMI, in association with a significant negative correlation with insulin reported in this study provides additional evidence to support insulin as a more potent inhibitor of hepatic SHBG production than testosterone in women. The inhibition of SHBG production by insulin occurs in the presence of a sustained overall increase in insulin concentrations and is not apparently altered acutely by the daily variation in insulin concentrations.

The diurnal changes in IGFBP1 are similar to those reported by Suikkari et al. (1988). The close correlation of these changes to variations in insulin concentrations provides evidence for a regulatory role of insulin on IGFBP1 levels. The alteration in concentrations is very rapid and the rate of decrease is similar in the two groups despite widely differing insulin levels. It has been suggested that these rapid changes in IGFBP1 concentrations allow an

increased concentration of "free" IGF-1 and that this may be an important mechanism in the maintainance of glucose homeostasis (Lewitt and Baxter 1990). The difference in the 24 hour median secretion of IGFBP1 and insulin between the two groups is to be expected since women with anovulatory PCOS have previously been reported to have lower IGFBP1 concentrations (Suikkari et al. 1989a) and raised fasting insulin concentrations (Dunaif et al. 1988, Sharp 1991) compared to weight matched controls. The rapid decline in IGFBP1 concentrations to changes in insulin is in contrast to that of SHBG and is similar to the responses of the two binding proteins to an oral glucose tolerance test (Section 4.2.3.). This probably reflects the difference in the half-life of the two binding proteins which is approximately 30 minutes for IGFBP1 and 5-7 days for SHBG (Julkenen et al. 1988, Anderson et al. 1976). It was therefore predicted that SHBG would not exhibit any diurnal variation.

Insulin concentrations varied during the 24 hours in both groups although a statistically significant diurnal variation in secretion could only be detected in the control group. The lack of diurnal variation in the women with PCOS demonstrated in this study implies that regardless of the carbohydrate load women with PCOS secrete excess insulin throughout the day. The fact that the two groups did not receive the same glucose load during the 24 hours is a valid criticism of this study. However the finding that the difference in the peak concentrations remains significant after the four hours following the oral GTT have been excluded indicates that these differences in insulin secretion are evidence of true hyperinsulinaemia and not secondary to variations in glucose load. Furthermore, evidence exists that equivalent calorie loads, regardless of carbohydrate content, induce similar insulin responses (Peterson and Jovanovic-Peterson 1991). The finding that the insulin concentrations started rising in the PCOS group prior to the administration of the glucose provides additional evidence of hyperinsulinaemia and probable insulin resistance in women with PCOS (Dunaif et al. 1988,1989, Sharp et al. 1991).

The lack of variation in IGF-1 concentrations is consistent with other studies of diurnal variation (Holly et al. 1988). Changes in IGF-1 levels have been reported following fasting

(Clemmons et al.1982, Kiddy et al. 1989) and exercise (Suikkari et al. 1989b). The similarity in the levels of IGF-1 is interesting and the absence of any correlation with insulin, SHBG and IGFBP1 is in contrast to the findings of other studies (Conway 1990) when IGF-1 was found to be positively correlated with insulin and IGFBP1.

The increased insulin concentrations in the women with PCOS support the concept that these women are at greater risk of developing non-insulin dependent diabetes (Dunaif and Graf 1988). All the women in the PCOS group were anovulatory and there is evidence that hyperinsulinaemia only occurs in this group and that ovulatory women with PCOS are neither hyperinsulinaemic nor insulin resistant (Sharp et al. 1991, Robinson et al. 1993). In vitro evidence in cultured ovarian stromal cells has demonstrated that insulin increases the production of testosterone (Barbieri 1986). In granulosa cells from normal and polycystic ovaries, insulin and IGF-1 increase FSH stimulated oestradiol production (Erikson et al. 1990) and decrease IGFBP-1 production (Mason et al. 1993). It is not known whether the ovary is insulin resistant in women with polycystic ovary syndrome but it is possible that hyperinsulinaemia may play a role in the mechanism of anovulation in these women.

In summary these data indicate that there is no diurnal variation in SHBG concentrations in women. The previously reported diurnal variation in IGFBP1 and the close relationship between insulin and IGFBP1 is confirmed. The median concentration of SHBG correlated more strongly with insulin than testosterone or BMI implying that the persistently raised insulin concentrations found in obese and annovulatory PCOS women acts as a principal inhibitor of SHBG concentrations. This finding explains the increased incidence of significant hirsutism in thin anovulatory and obese women with PCOS as a decrease in SHBG concentrations leads to an increase in free ("biologically active") testosterone concentrations. This study does not provide evidence for the coregulation of hepatic SHBG and IGFBP1 production by insulin. The increased insulin concentrations and the lack of diurnal variation in women with PCOS confirms the tendency to insulin resistance in these women which is independent of obesity and suggests that hyperinsulinaemia is sustained over a 24 hour period.

CHAPTER 5
SUMMARY AND CONCLUSION

The clinical studies and in vitro experiments reported in this thesis provide evidence that insulin is likely to be an important regulator of circulating SHBG and IGFBP1. The results from the in-vitro experiments, while showing no significant inter experimental differences, should be interpreted with caution since the Hep G2 cells are a transformed immortal cell-line and therefore exhibit important cellular differences from normal hepatocytes. The most important of these with respect to ts thesis is the presence of IGF-1 receptors on their surface which are not present in adult hepatocytes. In addition experimental factors such as the binding of insulin to the plastic flasks, the difference in the distribution of the cells could not be quantified. The clinical studies although done on small numbers of women provide additional and supportive evidence for this finding. There is little evidence in the literature or in this thesis,however,that the two binding proteins are truly co-regulated. The production of the binding proteins by the liver is inhibited by insulin, yet their responses to insulin-like growth factor-1 (IGF-1), growth hormone (GH), thyroxine and the sex steroids shows important differences. These are emphasised by the in vivo response to changes in insulin concentrations; SHBG concentrations change over a longer period but remain stable while IGFBP1 concentrations change rapidly. In this summary I shall therefore consider the regulation of the two binding proteins separately.

5.1. SEX HORMONE BINDING GLOBULIN

The evidence that insulin may be an inhibitor of SHBG synthesis by the liver is supported by the clinical studies in which a significant negative correlation was found both before and after weight loss, during an oral glucose tolerance test and over a 24 hour period. The relationship was strengthened by the finding of a reciprocal change after weight reduction with an increase in SHBG and a decrease in insulin concentrations.

The consistent and highly replicable finding of insulin inhibition of SHBG production by Hep G2 cells further supports the conclusion that insulin is a primary regulator of hepatic SHBG synthesis. The in-vitro studies did not find a dose-related inhibition of SHBG by

insulin. There was an apparent threshold concentration of insulin before significant inhibition occurred which is hard to explain. Non-dose dependent insulin-mediated SHBG inhibition has been confirmed at the molecular level with a decrease in SHBG mRNA levels on Northern blots from Hep G2 cells incubated with various concentrations of insulin following stimulation with thyroxine (Plymate et al. 1990).

The effects of IGF-1 as an inhibitor of SHBG concentrations found in the in-vitro study are difficult to interpret. They have been confirmed in another study which demonstrated a reduction in SHBG mRNA by Hep G2 cells after incubation with IGF-1 (Plymate et al.1990). In the short term diet study an inverse correlation between IGF-1 and SHBG was found. The changes in IGF-1 concentrations during calorie restriction are short-lived, however, since in a longer term study this relationship was not confirmed (Kiddy et al. 1990). Alterations in IGF-1 concentrations during fasting are well reported and are consistent with the changes detected in the diet study. In addition, the lack of response to growth hormone despite an increase in IGF-1 production may confirm that these results represent a pharmacological response rather than a physiological one. The decreased serum concentration of SHBG found in acromegaly is, therefore, more likely to be mediated by the hyperinsulinaemia associated with this condition than directly by growth hormone.

The effect of thyroxine on SHBG production by Hep G2 cells is in accordance with clinical findings (Vermeulen 1969-see Table1.2.) and reports of studies using Hep G2 cells (Rosner et al.1984). It has been shown that thyroxine acts at the nuclear level with an increase in SHBG mRNA. This effect is reversed by insulin and blunted by IGF-1 (Plymate et al.1990). The clinical significance of this relationship is obscure. In pregnancy, circulating thyroxine concentrations remain constant although thyroxine binding globulin (TBG) and SHBG concentrations increase significantly. The increase in TBG and SHBG, therefore, may not be mediated by a direct effect of thyroxine on hepatic production but by a separate mechanism.

The role of the sex steroid hormones in the regulation of SHBG concentrations is not clarified by this data. In the clinical studies only a weak inverse correlation between SHBG

and total testosterone concentrations was found. In addition, total testosterone concentrations did not alter during calorie restriction although SHBG concentrations rose significantly. The possibility that the sex steroids affect SHBG production concentrations by a mechanism other than modulation of hepatic production is supported by the absence of a response to oestradiol and an increase in SHBG with testosterone in the in-vitro experiments (Chapter 3). The important role of insulin in regulating hepatic SHBG production is emphasised by the inhibition of SHBG production by insulin in the presence of oestradiol and testosterone in-vitro. The previously reported findings that oral administration of oestrogens and testosterone affect the circulating concentration of SHBG may, therefore, be mediated by post translational events and this may play a role in physiological conditions such as pregnancy. The post translational events that have been postulated include the alteration of the glycosylation of SHBG (Tardivel-Lacombe and Degrelle 1990) and this idea is supported by similar evidence for TBG (Ain and Refetoff 1987). The clinical and physiological importance of these findings may improve our understanding of the changes in SHBG concentration during puberty and in hirsute and anovulatory women - the two symptoms that are most commonly found in women with PCOS.

The more direct role which insulin plays on SHBG production has obvious consequences for sexual maturation as it will increase the concentration of free testosterone or oestradiol being produced by the gonads so that they can exert their peripheral effects in the development of secondary sexual characteristics. Several studies have reported increases in insulin concentrations during puberty (Smith et al. 1988, Holly et al. 1989). A decrease in SHBG concentrations in both sexes is well reported (Apter et al. 1984, Bartsch et al. 1980, Blank et al. 1978, Belgorosky et al.1986, Lee et al. 1984, Holly et al. 1989). It is interesting to note that boys who are hypogonadotrophic but have normal growth hormone function have SHBG levels that are similar to post-pubertal girls, implying that testosterone does play a role in further lowering SHBG concentrations in normal boys (Vermeulen et al. 1969, Plymate et al. 1983,).

The degree of hirsutism suffered by a woman with hyperandrogenaemia is determined by two main factors; firstly her genetic predisposition to developing hirsutism and secondly the

concentration of free testosterone in her circulation. Free testosterone is considered to be the biologically active fraction of testosterone and is that which is not bound to the circulating proteins, SHBG and albumin. The idea that free testosterone is available to tissues only by diffusion is currently under review, as increasing evidence emerges that SHBG may bind to specific cell receptors. Circulating testosterone or oestradiol binds to the SHBG and the whole complex enters the cell and activates adenylate cyclase (Hyrb et al. 1985,1990, Rosner 1991). It is unclear whether this system plays a significant role in androgen metabolism. A reduction in the concentration of SHBG will, under either of the above systems, increase the biologically available testosterone concentration and so further predispose the individual to the development of hirsutism. Women with PCOS are both hyperandrogenaemic and hyperinsulinaemic compared to weight matched controls (Chang et al.1983, Jialal et al.1987, Dunaif et al. 1989) although a recent study has shown that hyperinsulinaemia in lean women with polycystic ovaries is confined to those who are anovulatory (Sharp et al. 1991). Hyperinsulinaemia occurs in both obese and lean anovulatory women with PCOS although it is more marked in the obese. Obese women with PCO are more likely to have menstrual irregularity and hirsutism than lean women (Kiddy et al. 1991) and this is thought to be related to their raised insulin concentrations.

The increased prevalence of hirsutism suffered by obese women with PCOS may reflect reduced SHBG concentrations secondary to increased insulin concentrations. The precise role that insulin and androgens play in anovulation has yet to be elucidated. Insulin and IGF-1 have been shown to increase androgen production by ovarian stroma cells from women with PCOS in-vitro (Barbieri et al. 1986). It is therefore possible that women with PCOS who are also hyperinsulinaemic have a mechanism for further increasing ovarian androgen production. The association of hyperinsulinaemia and anovulation is well established (Dunaif et al.1988,1989, Sharp et al. 1991) but the role that insulin may play in the mechanism of anovulation is unclear.

In conclusion, insulin is a primary inhibitor of sex hormone binding globulin production by the liver. This has important implications for our understanding of hirsutism and, to a lesser extent, anovulation in women with PCOS. The effect of obesity on women with this

disorder is to further increase fasting insulin levels and increase relative insulin resistance. This leads to a decrease in SHBG concentrations and an increase in free testosterone. Weight loss is associated with a decrease in insulin concentrations and an increase in SHBG which can have a pronounced effect on the clinical picture with an improvement in hirsutism and a restoration of ovulation (Kiddy et al.1991). Lean women with PCOS who are anovulatory are also hyperinsulinaemic compared to weight matched controls and this is reflected in their significantly lower SHBG levels.

5.2. INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 1

The hepatic production of IGFBP1 is also regulated by insulin. The inverse relationship between IGFBP1 and insulin in the clinical studies is a significant one. The half-life of IGFBP1 in plasma is very short as shown by the rapid decrease in circulating levels seen after an oral glucose tolerance test in obese women before and after calorie restriction (Section 4.2.). The rapid decrease in IGFBP1 concentrations in response to an increase in insulin concentration both during an OGTT and following the early morning increase in insulin concentrations, may be a mechanism for maintaining euglycaemia by releasing IGF-1 locally (Lewitt and Baxter 1991). The finding that both SHBG and IGFBP1 levels decline while insulin concentrations increase during puberty and fasting supports the view that these two binding proteins are co-regulated. The changes observed both in the short and long term, however, show that while hepatic production may be co-regulated in the short term, IGFBP1 levels return to their pre weight loss levels within a few weeks despite insulin concentrations remaining lower than before. This is in contrast to SHBG concentrations, which remain higher. The possibility of the existence of compensatory mechanisms was suggested by Suikkari et al. (1988) in their study of women following hysterectomy. The uterus is an important producer of IGFBP1 in women and following its removal IGFBP1 levels fell significantly. Five weeks post operatively circulating IGFBP1 concentrations had returned to pre-surgery concentrations suggesting that the liver or another tissue had increased production to compensate for the loss of the uterus.

IGF-1 does not seem to play a direct role in IGFBP1 regulation although it is an inhibitor of SHBG production in vitro. The alteration in both IGF-1 and IGFBP1 after fasting is shortlived. This may, again, represent an alteration in the homeostatic mechanism to maintain euglycaemia until a new equilibrium is reached. The lack of diurnal variation in IGF-1 may be thus explained since its availability as a hypoglycaemic agent could be regulated by IGFBP1 concentrations while the majority of the circulating IGF-1 concentration remains bound to IGFBP3.

Hepatic production of IGFBP1 is not, according to our studies, regulated by sex steroids although progesterone has been shown to modulate endometrial IGFBP1 production (Rutanen et al.1986). This indicates that the regulation of IGFBP1 (and the other IGFBPs) may differ from one tissue type to another and evidence for this has been presented by Dr. D. Clemmons at the International Conference of Diabetes in Washington (1991). In most tissues insulin has been found to be an inhibitor of IGFBP1 production in fibroblasts (Conover et al.1990a,c), endometrium (Rutanen et al. 1988) as well as Hep G2 cells.

During puberty, sexual maturation and growth need to be coordinated. The insulin mediated decrease in SHBG and IGFBP1 may play an important role by increasing circulating free testosterone/oestradiol and IGF-1. Growth hormone acts as a further regulator of free-IGF-1 concentrations by altering the concentration of the principal IGF-1 binding protein IGFBP3. Insulin may act as a secondary regulator of tissue growth by altering IGFBP1 concentrations at a cellular level. IGFBP1 levels may therefore decline in order to increase the availability of IGF-1 as a mitogen.

The role of IGF-1 and IGFBP1 in ovulation is uncertain. IGFBP1 has been shown to be produced by granulosa and luteal cells in the human ovary, and it inhibits the DNA amplification normally induced by IGF-1 so reducing progesterone production (Angervo et al. 1991). IGF-1 augments the response of granulosa cells to FSH so increasing oestradiol production. More recently IGFBP1 has been shown to be produced by stromal and thecal cells in addition to granolosa/luteal cells. The addition of IGF-1 or insulin at very low doses (0.1ng/ml IGF-1) inhibits the production of IGFBP1 (Mason et al.1993). The contribution

of the ovary to circulating IGFBP1 concentrations is not known but the relative hyperinsulinaemia found in women with PCOS may reduce ovarian production of IGFBP1. The way in which this paracrine system affects ovulation is unclear and it is hard to understand how this may fit into the mechanism of anovulation in women with PCOS who have reduced levels of IGFBP1 and normal circulating levels of IGF-1, a combination which might predict an increased response to FSH rather than a reduced one.

5.3. CONCLUSION

Insulin inhibits hepatic production of SHBG and IGFBP1. The apparent co-regulation of SHBG and IGFBP1 in physiological or pathological conditions associated with changes or abnormalities in insulin secretion is probably coincidental, since they do not share other regulating factors. The role of insulin in regulating these two binding proteins may be important for our understanding of puberty, hirsutism and anovulation in women with PCOS. The role of SHBG in androgen metabolism is well established and the studies in this thesis provide evidence that insulin and possibly IGF-1 play an important role in the regulation of this protein. The evidence that simple measures such as weight loss can significantly alter SHBG concentrations may prove to be clinically significant, particularly in the treatment of hirsutism. The role of the sex steroids in the regulation of SHBG is unlikely to be within the hepatocye and they probably act by altering the clearance of the protein.

The rapid response of IGFBP1 during an OGTT and in response to daily fluctuations in insulin supports the idea of Lewitt and Baxter that IGFBP1 plays a role in glucose homeostasis. Its role in ovulation is less clear. IGFBP1 was originally called "Growth Hormone Independent" and this is supported by the evidence reported here. IGF-1 does not seem to play a role in the hepatic regulation of IGFBP1. The sex steroids do not alter hepatic IGFBP1 production but progesterone has been shown to increase IGFBP1 secretion in endometrial cells, implying that different cells have different regulatory mechanisms.

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APPENDIX A
ASSAYS

A.1. TESTOSTERONE ASSAY

Performed by:	Dr Francis Short
Place:	St.Mary's Hospital Steroid Biochemistry Laboratory
Type of assay:	Radioimmunoassay (RIA)
Source of STRIA kit:	Dr M. Wheeler, St.Thomas' Hospital, London.
Source of testosterone antiserum:	Guildhay (University of Surrey, Guildford, UK).
Inter and intra assay coefficient of variance:	6.3% and 4.2 % respectively.
Reference:	Wheeler and Luther 1983

A.2. FREE TESTOSTERONE ASSAY

Performed by:	Dr Michael Reed
Place:	Department of Chemical Pathology, St.Mary's Hospital Medical School
Type of assay:	Ultrafiltration
Materials and Source :	Sepharose columns-Amersham International, Amersham, Bucks Tritiated testosterone: In house.
Calculation of results:	The background count was subtracted from the free and total counts. The percentage free testosterone was determined by dividing the free counts by the total counts.
Inter and intra assay coefficient of variance:	<10%.
Reference:	Vlahos et al. 1982

A.3. INSULIN ASSAY

Performed by:	Dr Victor Anyaoku
Place:	Unit of Metabolic Medicine St.Mary's Hospital Medical School
Type of assay:	Radioimmunoassay (RIA)
Reagents and source:	[^{125}I] Insulin-Amersham International,Amersham, Bucks.
Polyclonal Antiserum:	MF/GP/9-Guildhay Ltd, Guildford, Surrey
Separation reagent:	Donkey anti-guinea pig antiserum-Guildhay Ltd.
Standards:	WHO reference standards-Porton Down, Wilts., U.K.
Inter and intra assay coefficient of variance:	2.6 % and 4.8 % respectively.
Reference:	Hampton et al. 1986

A.4. IGF-1 ASSAY

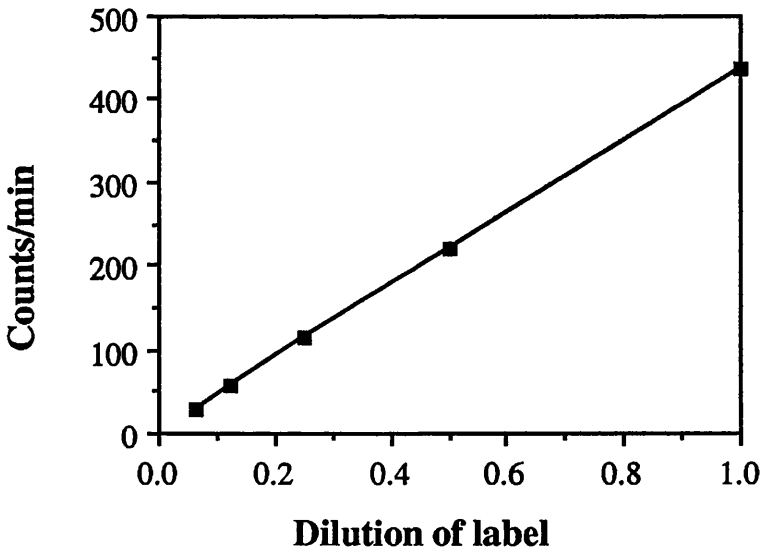
Performed by:	Dr Victor Anyaoku
Place:	Unit of Metabolic Medicine St.Mary's Hospital Medical School
Type of assay:	Radioimmunoassay (RIA)
Reagents and source:	[^{125}I] IGF-1 from Dr D. Teale, St.Lukes Hospital, Guildford, Surrey.
Antisera antiserum:	Rabbit and second donkey anti-rabbit from Guildhay Ltd, Guildford, Surrey
Standards:	WHO reference standards-Porton Down, Wilts., U.K.
Inter and intra assay coefficient of variance:	1.8 % and 2.5% respectively.
Reference:	Daughaday et al 1980

APPENDIX B
LABORATORY TECHNIQUE

B.1. SAMPLE ACCURACY

In order to ensure that the pipetting of samples was accurate 10µl of ^{125}I-FSH was pipetted using a Gilson 20µl pipette into 20 consecutive test tubes and the radioactivity was counted for 10 minutes. The CV for this was 3.8% (SD/mean x 100). The dilution of the samples was checked as accurate by serial dilutions from 1:2 to 1:16 (fig B.1.).

Figure B.1. Serial dilution of ^{125}I-FSH.



B.2.1. ACCURACY OF CELL PASSAGE TECHNIQUE.

Hep G2 cells were grown from stock to 70% confluence and then passaged. The cells were washed in 5 mls phosphate buffered saline (PBS) for 5 minutes and the PBS removed. 2 mls trypsin EDTA was added, the flask was allowed to stand for two minutes, and the trypsin was removed. The cells were placed in the incubator for 10 minutes and when they were seen to be floating off the surface of the flask, were resuspended in 7 mls of supplemented medium. The cells were equally divided between 7 flasks (1 for stock) by pipetting 1ml of

medium into each flask with thorough mixing in between each flask. The flasks were placed in the incubator for 48 hours after making the volume of medium up to 5mls in each flask. After 48 hours the cells were lysed and counted in the Coulter counter as described in Chapter 2.3. Each flask was counted three times and the mean of these three values was taken as the number of cells in millions. The coefficient of variation was calculated and found to be 5.8%. The overall CV% (controls and flasks containing non-mitogenic agents only) was 4.9% (range 0.65-10.25%) in 14 experiments.

B.2.2. CELL VIABILITY BEFORE AND AFTER THE EXPERIMENTS.

The results in chapter 3 are presented per million cells. In order for this to be the correct way to express the results the number of viable cells before and after an experiment was performed using the trypan blue method. Hep G2 cells were grown in four $25cm^3$ flasks in 5mls EMEM supplemented with 10mls of serum. The cells from two of the flasks were then passaged as normal and resuspended in 1ml of unsupplemented medium. 10µl of this suspension was added to 10µl of trypan blue and mixed thoroughly. The cells were then counted on a haemocytometer. The cells that took up the trypan blue were considered non-viable (mean 6.5%) while those that were white were considered viable. The medium in the other two flasks was changed to phenol free, serum free medium and the flasks were incubated for a further 48 hours. The cells were then passaged, mixed with trypan blue and counted. In 10 squares counted, the mean % of non-viable cells was 9% (range 0-16%). This was considered to be within reasonable limits.

APPENDIX C

DATA APPERTAINING TO CHAPTER 3

C.1. DATA FOR FIGURE 3.1.1, 3.1.2 , 3.1.3, 3.1.4, 3.1.5.

Effect of insulin, IGF-1 and Growth Hormone on SHBG and IGFBP1 production by Hep G2 cells. Mean (SD) of triplicate values for SHBG and IGFBP1.

Mean (SE) of triplicate values

	SHBG fmol/million cells					IGFBP1 ng/million cells	
Reagent/ concentration	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 1	Exp 2
Insulin µmol/L							
Control	59 (3.2)	72.2 (3.1)	50 (4.0)	55.4 (5.2)	66.7 (2.8)	2.2 (0.2)	3.1 (0.3)
1x10-9	59.0 (1.9)	56.3 (4.8)	62.9 (3.9)	59.4 (3.3)			
1x10-8	56.2 (4.3)	63.8 (1.5)	48.8 (6.2)	56.2 (7.5)	57.4 (1.8)		
1x10-7	38.5 (2.7)	57.4 (1.8)	36.0 (1.5)	46.9 (4.1)	45.0 (3.9)	1.72 (0.11)	2.1 (0.21)
1x10-6	34.1 (2.6)	45.4 (5.1)	38.0 (5.1)		48.8 (0.2)	1.65 (0.17)	2.4 (0.16)
1x10-5	42.1 (4.3)	45.4 (4.8)	39.0 (1.5)			1.75 (0.18)	2.5 (0.24)
IGF-1 nmol/L							
Control	71.0 (1.5)	65.0 (7.2)	66.7 (2.8)			3.2 (0.52)	
1	58.3 (4.0)	61 (0.2)	63 (1.5)			3.1 (0.16)	
10	57.3 (5.1)	43.9 (1.8)	54.3 (2.7)			3.0 (0.37)	
20	51.0 (7.0)		52.1 (3.5)			3.0 (0.48)	
100		39.6 (2.2)	45.6 (1.1)				
GH ng/ml							
Control	107.8 (2.9)	72.4 (1.4)	41.9 (1.7)			1.7 (0.29)	4.0 (0.25)
0.1	98.2 (9.4)	75.6 (1.5)	44.9 (1.2)			2.0 (0.13)	3.9 (0.12)
1	87 (3.9)	75.2 (1.5)	37.6 (2.9)			1.9 (0.16)	3.7 (0.2)
10	71.9 (1.9)	67.4 (5.8)	44.8 (5.8)			1.1 (0.4)	4.2 (0.18)
100		62.2 (3)	45.9 (2.0)				3.9 (0.11)

C.2. DATA FOR FIGURES 3.2.1,3.2.2, 3.2.3, 3.2.4, 3.2.5.

Effect of steroid hormones and thyroxine on the production of SHBG and IGFBP1 by Hep G2 cells.

Mean (SE) of triplicate measurements

Reagent Concentration	SHBG fmol/10^6cells Experiment 1	Experiment 2	IGFBP1 ng/10^6cells Experiment 1	Experiment 2
Testosterone (T)				
Control	35.3 (4.6)		3.70 (0.65)	
$1x10^{-8}$	47.1 (9.2)		3.54 (0.25)	
$1x10^{-7}$	51.9 (10.9)		2.86 (0.59)	
$1x10^{-6}$	70.2 (2.6)		3.20 (0.37)	
T plus Δ-4-OHA				
Control	25.4 (5.7)	101.3 (11.9)	2.54 (0.48)	4.10 (1.10)
T $1x10^{-7}$	33.7 (6.4)	111.6 (9.8)	2.93 (0.46)	4.84 (0.50)
D-4-OHA $1x10^{-8}$	36.8 (2.0)	101.6 (23.9)	2.04 (0.62)	4.61 (0.81)
T + D-4-OHA	38.1 (6.6)	116.7 (10.4)	2.05 (0.62)	4.57 (0.81)
T^{-8} +D-4-OHA	33.2 (8.5)	92.0 (0.7)	3.31 (0.77)	5.29 (0.26)
T^{-9} +D-4-OHA		105.1 (1.9)		5.04 (0.39)
Oestradiol (E2)				
Control	53.4 (5.1)	96.5 (27)	1.81 (0.23)	6.15 (1.26)
E2 $1x10^{-9}$	54.5 (1.5)	69.9 (6.0)	1.87 (0.21)	6.17 (0.36)
E2 $1x10^{-8}$	64.3 (2.4)	78.5 (6.0)	1.85 (0.10)	6.01 (0.18)
E2 $1x10^{-7}$	62.3 (1.1)	83.6 (2.9)	1.40 (0.36)	6.17 (0.56)
E2 $1x10^{-6}$	63.1 (4.1)	81.8 (1.6)	1.05 (0.16)	5.94 (0.61)
Progesterone (P)				
Control	98.7 (4.3)	45.0 (4.4)		0.79 (0.09)
P $1x10^{-6}$	166.5 (4.6)	47.7 (9.7)		0.88 (0.13)
P $1x10^{-7}$	111.8 (2.5)	41.4 (6.0)		0.83 (0.06)
P $1x10^{-8}$	118.2 (8.8)	56.6 (4.8)		0.97 (0.19)
P $1x10^{-9}$	104.7 (4.2)	40.6 (2.3)		1.01 (53.7)
Thyroxine				
T_4 $1x10^{-8}$	134.5 (4.7)	103.7 (13.9)	3.6 (0.75)	
T_4 $1x10^{-7}$	131.8 (2.2)	130.7 (22.8)	3.7 (0.10)	
T_4 $1x10^{-6}$	123.5 (5.2)	164.1 (13.7)	3.2 (0.29)	

C.3. DATA FOR FIGURES 3.3.1,3.3.2

Effect of insulin on the production of SHBG and IGFBP1 by Hep G2 cells in the presence of oestradiol or thyroxine

Mean (SE) of triplicate measurements

Reagent Concentration	SHBG fmol/10^6 cells Experiment 1	Experiment 2	IGFBP1 ng/ml
E2 + Insulin Control	51.6 (3.8)	72.2 (3.4)	26.1 (2.8)
E2 $1x10^{-6}$	55.7 (6.9)	74.6 (7.8)	20.3 (2.4)
Ins $1x10^{-6}$	30.2 (1.8)	45.4 (4.6)	32.9 (2.8)
E2 +Ins	35.2 (1.0)	47.7 (2.8)	20.1 (2.2)
Thyroxine + Insulin Control	104.8 (9.8)	72.2 (3.4)	
Thyroxine 1x10-7	123.5 (5.3)	81.6 (9.3)	
Insulin 1x10-5	65.3 (1.4)	45.4 (4.6)	
T4 + Ins	104.6 (5.8)	63.1 (1.4)	

APPENDIX D

DATA APPERTAINING TO CHAPTER 4

D.1.1. INDIVIDUAL DATA FOR THE FIGURES 4.1.1 AND 4.1.2.

	T nmol/L	SHBG nmol/L	Free T pmol/L	Insulin mU/L	IGF-1 nmol/L	IGFBP1 ng/ml
PCOS group						
1. 0 weeks	3.9	38.8	78	21.1	114	22.4
2	1.9	46.4	38	6.1	141	36.4
4	3.3	64.5	49	4.1	71	43
2. 0 weeks	2.8	12.7	81	7.8	68	27.2
2	3.3	42.4	66	4.2	43	46
4	2.4	39.1	53	3.9	30	65.4
3. 0 weeks	4.4	10.1	120	24.2	118	25
2	3.6	38.7	94		132	36.6
4	4.5	28.2	110	10.4	154	33.4
4. 0 weeks	2.5	25.4	45	8.9	193	37
2	3.9	60.4	43	3.3	21	64
4	3.0	165.0	33	5.5	40	20
5. 0 weeks	2.4	74.4	62	4.7	102	29
2	3.0	92.3	48	2.7	47	61.4
4	2.5	156.7	40	0.4	67	37.8
Controls						
6. 0 weeks	1.4	46	43	8.9	219	40
2	1.2	90	14	5.4	50	86.2
7. 0 weeks	0.96	34	20	12.0	215	8.6
2	0.76	90	10	2.2	64	36.8
8. 0 weeks	1.3	34	42	4.4	156	
2	1.4	56	19	0.1	48	
9. 0 weeks	2.6	25	49	14.4	156	24.8
2	3.2	45	42	3.3	79	34.8
10. 0 weeks	2.5	44	35	12.9	69	14.6
2	2.6	64	34	8.3	56	22.6
11. 0 weeks	3.3	77	33	2.5	69	45
2	2.2	173	12.3	5.9	24	56

D.1.2. DATA FOR FIGURE 4.1.3.

Daily changes in SHBG and IGFBP1 during a two week low calorie diet in a single subject

	SHBG nmol/L	IGFBP1 ng/mL
Day 0	25.3	4.8
1	25.9	15.6
2	28.1	16.8
3	27.1	25.6
4	28.2	13.8
5	26.9	33.6
6	29.0	39.0
7	25.1	28.2
8	24.9	22.4
10	32.8	33.0
12	35.2	32.8
14	38.8	28.2

D.2.1. DATA FOR FIGURE 4.2.1.

Table showing the mean (SE) values of SHBG, Insulin, Glucose and IGFBP1 in 6 obese women with PCOS during an OGTT before and after dieting

Time (minutes)		SHBG nmol/L	Insulin mU/L	Glucose mmol/L	IGFBP1 ng/ml
0	Pre	21.4 (2.1)	13.8 (4.6)	3.7 (0.2)	27.3 (4.3)
	Post	27.1 (3.6)	3.3 (3.9)	3.9 (0.2)	28.4 (4.9)
30	Pre	18.9 (2.7)	69.8 (21.6)	6.8 (0.8)	22.3 (3.9)
	Post	26.3 (5.3)	24.5 (5.6)	6.4 (1.5)	7.9 (5.6)
60	Pre	18.2 (4.1)	76.1 (31.8)	7.1 (0.6)	16.0 (3.8)
	Post	24.8 (4.0)	47.6 (16.5)	6.3 (0.6)	17.8 (3.9)
90	Pre	18.1 (2.6)	98.1 (34.7)	7.0 (0.4)	11.3 (1.9)
	Post	24.4 (4.2)	40.4 (11.3)	5.8 (0.4)	13.1 (3.1)
120	Pre	20.0 (2.8)	88.8 (31.3)	5.5 (0.4)	8.3 (1.4)
	Post	26.2 (3.4)	44.1 (16.3)	5.8 (0.5)	9.1 (2.0)
150	Pre	19.2 (1.9)	65.2 (33.7)	5.1 (0.6)	6.5 (1.9)
	Post	28.6 (2.6)	23.2 (7.9)	5.1 (0.7)	7.0 (1.1)
180	Pre	18.8 (3.6)	30.0 (13.9)	4.3 (0.5)	8.9 (1.7)
	Post	27.7 (2.3)	18.0 (5.7)	4.5 (0.5)	6.2 (0.9)
Total	Pre	129.9 (16.5)	415.6 (142.5)	39.4 (1.2)	99.8 (38.1)
	Post	164.3 (28.8)	201.1 (52.8)	38.0 (1.6)	96.2 (43.5)

Total: Mean (SE) of the total values during an OGTT

D.3.1. DATA FOR FIGURE 4.3.1.

24 hour values (median (interquartile range))) of insulin, SHBG,IGFBP1 and IGF-1 in 10 women with anovulatory PCOS and 10 ovulatory controls

Time 24 hr clock	Insulin Control mU/L	Insulin PCOS mU/L	SHBG Control nmol/L	SHBG PCOS nmol/L	IGFBP1 Control ng/mL	IGFBP1 PCOS ng/mL	IGF-1 Control ng/mL	IGF-1 PCOS ng/mL
00.00	4.6 (2.5-10.7)	13.4 (9.1-23.7)	35.6 (26.9-48.7)	16.4 (12.7-25.7)	19.3 (12.4-36.6)	15.8 (6-28.2)	230.6 (200.6-270.2)	239.8 (189.4-298.2)
02.00	2.2 (1.5-5.5)	9.6 (3.6-14.4)	30.3 (26.1-41)	16.3 (13.1-21.9)	40.2 (14-62.2)	22.3 (12.4-40)	235.8 (182.7-276.2)	230.5 (1824-.260.4)
04.00	4.4 (6.2-8.7)	5.5 (6.2-8.7)	31.1 (26.1-41)	16.8 (13.1-21.9)	48.9 (37.4-72.8)	27.7 (19.6-48.6)	234.1 (191.5-269.2)	241.7 (192.2-285.6)
06.00	6.2 (1.9-7.6)	6.6 (5.4-9.8)	33.7 (20.1-46.9)	15.1 (12.5-19.6)	55.3 (50.2-86.6)	34.9 (14.2-65)	223.9 (188.3-267.8)	222 (185.5-252.7)
08.00	7.0 (3.6-20.3)	18.6 (3.8-56)	35.6 (31.4-43.4)	21.1 (17.2-27.9)	60.6 (46.8-77.2)	30.2 (14.8-63)	247.7 (184.1-257.6)	202.4 (141.8-266.7)
10.00	14.4 (8-29.4)	25.9 (12.4-67.2)	31.2 (23.7-34.8)	15 (12.4-17.9)	34.8 (22.6-57.8)	13.9 (6-18.2)	228.3 (200.6-267.4)	211.6 (161-252.7)
12.00	6.7 (4.3-9.2)	20.4 (7.6-48)	29.9 (22.9-33.4)	16.6 (12.3-22.8)	20.6 (13.6-29.2)	14.6 (5.8-30)	224.9 (181-254.5)	210 (153-262.2)
14.00	10.4 (5.4-24.6)	32.6 (16.6-80.1)	36 (22.6-60.1)	19.7 (15.3-29.2)	23.4 (14.4-37.6)	10.3 (5.6-21)	231.2 (182.4-256.6)	215.2 (167.3-250.6)
16.00	7.8 (2.9-17.5)	33.3 (21.9-71.6)	38.9 (27.6-52.4)	16.9 (14.9-24)	19.5 (14.8-28.4)	10.4 (4.5-17.8)	222.5 (174.3-251.9)	216 (158-260.4)
18.00	7.2 (2.8-22.8)	27.4 (13.9-50.6)	35.3 (29.5-49.8)	17.7 (13.3-22.7)	22.2 (13.8-30.6)	7.8 (4.5-8.8)	218.7 (177-245)	219.4 (160-266.7)
20.00	8.3 (3.5-19.7)	18.5 (9-38.8)	34.9 (26.3-51.6)	17.6 (13.1-22.2)	30.7 (17-54.8)	7.9 (4.5-8)	222.3 (190.1-265.1)	220.3 (172.2-254.8)
22.00	29.1 (14.5-48.7)	27.9 (12.6-66.1)	39.4 (35-53.5)	18.2 (13.1-29.1)	15.3 (9.6-17.8)	15.6 (8.6-32.6)	224.3 (187.1-257.7)	229.1 (190.4-261.8)
24.00	11.8 (5.1-24.8)	21.3 (10-54.9)	37.7 (29.5-47.5)	14.7 (11.9-20.8)	11.6 (8.6-14.2)	8.8 (4.5-13.8)	223.7 (174-242.2)	225 (163.2-313.7)

normalised to midnight.

D.3.2. DATA FOR FIGURE 4.3.2.

24 hour values (median (interquartile range)) of insulin and IGFBP1 in 10 women with anovulatory PCOS and 10 ovulatory controls normalised to the time of an OGTT (PCOS group) or breakfast (control group).

Hrs	Insulin Control	Insulin PCOS	IGFBP1 Control	IGFBP1 PCOS
-6	3.4 (1.7-9.1)	8.7 (6.3-15.9)	53 (23.8-64.9)	28 (14.7-49.0)
-4	4.6 (2.1-7.0)	8.0 (5.3-13.9)	65.1 (43.4-76.7)	35.6 (13.9-68.6)
-2	5.6 (3.2-7.0)	18.4 (6.6-62.1)	61.2 (49.4-72.7)	39.1 (13.6-71)
0	20.8 (7.4-35.5)	32.5 (6.6-62.1)	55.7 (35.8-76.1)	14.1 (8.0-37.4)
2	7.8 (4.5-27.8)	44.1 (7.6-74.1)	20.5 (13.9-57.8)	12.4 (6.5-44.8)
4	9.7 (6.1-18.7)	32.5 (8.9-73.7)	20.9 (13.8-28.6)	9.5 (5.0-33.3)
6	9.8 (4.9-17.5)	35.1 (23.1-73)	28.4 (14-38.6)	6.3 (4.5-18.6)
8	11.4 (2.1-25)	13.1 (8.6-26.3)	23.4 (13.7-40.9)	5.8 (4.5-17.7)
10	11.9 (3.2-19.9)	28.5 (10-66.8)	27.7 (13.8-53.3)	6.1 (4.5-10.4)
12	21.6 (12.9-37.9)	16 (11.4-49)	23.4 (12.7-42.2)	8.4 (4.5-34.0)
14	24.4 (8.1-37.5)	39.4 (15.2-61)	14.0 (9.1-18.2)	10.9 (6.0-20.2)
16	5.5 (4.0-18.1)	10.4 (7.3-23.1)	13.9 (9.2-18.4)	12.5 (6.6-17.4)
18	3.8 (1.3-9.6)	7.6 (3.5-20.30)	20.2 (12.7-42.2)	23.3 (11.3-46.4)