Variation at the Glucocorticoid Receptor Gene Locus: Physiological and Pathophysiological Consequences.

By

Guthrie Blackhurst BSc (Hons) MB ChB MRCP

Thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow

Blood Pressure Group,

Department of Medicine & Therapeutics,

University of Glasgow

Western Infirmary,

Glasgow.

November 2000

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Glucocorticoids are important hormones in the regulation of carbohydrate, lipid and protein metabolism, and cardiovascular and central nervous function. The result of the syndrome of glucocorticoid excess is hyperglycaemia, insulin resistance, hyperlipidaemia, central obesity, hypertension, and the accelerated development of atheroma. In rare genetic conditions resulting in glucocorticoid resistance, excess ACTH drive to the adrenal results in excess mineralocorticoid and androgen production and increased cardiovascular morbidity. Recently, an RFLP of the glucocorticoid receptor situated within the promoter or intronic non-coding parts of the gene was found in excess in patients with obesity, insulin resistance, hypertension and hyperlipidaemia. It remains unknown however, whether these findings are due to altered glucocorticoid secretion, altered peripheral glucocorticoid sensitivity, or to linkage disequilibrium with a nearby gene.

The purpose of this project was to examine glucocorticoid receptor gene structure and receptor function in a large cohort of normal subjects with carefully characterised cardiovascular and metabolic variables to assess any association between glucocorticoid receptor function and cardiovascular and metabolic risk factors.

The glucocorticoid receptor gene was screened for common polymorphisms in a panel of 80 normal subjects by the techniques of PCR-SSCP with subsequent sequencing of amplicons suspected of containing mutations. This strategy revealed a complex mutation with 15 nucleotide substitutions in exon 5 in 4 subjects. The mutation resulted in a threonine to alanine substitution in the coded protein.

Receptor binding assays were conducted on 2 large cohorts of subjects from the Scottish Twin Study and the Midspan Study. Both studies demonstrated a previously

unreported seasonal component to glucocorticoid binding with lower Kd for dexamethasone binding in summer months than winter. These studies were extended to serial observations of receptor binding in 9 normal male subjects that confirmed the same seasonal effect on receptor Kd. Furthermore, there was a significant interaction with daylight and environmental temperature in all populations. Further *in vitro* work showed co-incubation of lymphocytes with physiological levels of melatonin resulted in a higher Kd value for dexamethasone binding, which would be consistent with the climatic observations noted.

We conclude that the glucocorticoid receptor gene carries very few mutations in normal subjects, and the effect of an uncommon mutation in exon 5 will require further work to assess its contribution to cardiovascular risk. Glucocorticoid receptor binding is, however, modulated by melatonin presumably through the effect of daylight and this observation may have implications for the wide range of physiological processes controlled by this endocrine system.

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Acknowledgements

This thesis is dedicated to my wife, Lynsay, for her support and patience through the years of the fellowship and ensuing writing.

I would like to thank Professor John Connell for his support and supervision. I am particularly grateful to Dr Wai K Lee for showing me the finer points of manual and automated sequencing and cloning, to Mr S M MacKenzie for his immunohistochemistry expertise and for bringing the Guardian to work every day, and to Dr Eleanor Davies for showing me how to do PCR and SSCP.

I thank Mrs Christine Holloway and Dr Chris Kenyon for showing me how to do the glucocorticoid receptor binding assays, and to Mr Kenny McElroy for performing a large number of these assays when the method was developed.

I am grateful to Miss Mary Ingram and Professor Robert Fraser for performing cortisol assays.

I would like to thank the Wellcome Trust for funding this work as a Wellcome clinical research training fellowship.

Abbreviations

A	Adenine
ACTH	Adrenocorticotrophic Hormone
AF1	Accessory Factor 1
AF2	Accessory Factor 2
AP1	Activator Protein 1
AP2	Activator Protein 2
Arg	Arginine
Asn	Asparagine
AVP	Arginine Vasopressin
bFGF	Basic Fibroblast Growth Factor
C	Cytosine
Ca^{2+}	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
cDNA	Copy Deoxyribonucleic Acid (generated from a Ribonucleic Acid template)
CRE	Cyclic Adenosine Monophosphate Response Element
CRH	Corticotrophin Releasing Hormone
DNA	Deoxyribonucleic Acid
ENaC	Epithelial Sodium Channel
ERE	Oestrogen Response Element
G	Guanine
GABA	Gamma-amino Butyric Acid
GLUT4	Glucose Transporter 4

GM-CSF	Granulocyte/Monocyte-Colony Stimulating Factor
GRE	Glucocorticoid Response Element
GRU	Glucocorticoid Responsive Unit
HDL	High Density Lipoprotein
His	Histidine
Hop	Heat Shock Protein-Organising Protein
11-β HSD	11-β Hydroxysteroid Dehydrogenase
HSP	Heat Shock Protein
5-HT	5-Hydroxy Tryptamine (=Serotonin)
IGF-I	Insulin-like Growth Factor-1
IGF-II	Insulin-like Growth Factor-2
IL-1	Interleukin-1
Ile	Isoleucine
IP_3	Inositol *tris*phosphate
K^+	Potassium Ion
kDa	kilo Daltons
LDL	Low Density Lipoprotein
Lys	Lysine
MCP-1	Monocyte Chemoattractant Protein-1
mRNA	Messenger RNA
MSH	Melanocyte-stimulating Hormone
N-terminus	amino terminus
Na^+	Sodium Ion

NADP	Nicotinamide-Adenine β-Dinucleotide Phosphate
NADP(H)	Nicotinamide-Adenine β-Dinucleotide Phosphate (Reduced form)
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
oxLDL	Oxidised Low Density Lipoprotein
P450	a family of cytochromes, including steroidogenic enzymes
p23	A protein component of the mature glucocorticoid-heat-shock complex
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PEPCK	Phosphoenolpyruvate Carboxykinase
PGI_2	Prostaglandin I_2 (=Prostacyclin)
Phe	Phenylalanine
POMC	Pro-opiomelanocortin
PPAR	Peroxisome Proliferator Activated Receptor
RNA	Ribonucleic Acid
RAR	Retinoic Acid Receptor
RFLP	Restriction fragment length polymorphism
RPMI	Roswell Park Memorial Institute (cell culture medium)
RXR	Retinoic Acid Receptor Heterodimer
T	Thymine

TNF	Tumour Necrosis Factor
Trp	Tryptophan
SAME	Syndrome of Apparent Mineralocorticoid Excess
SCC	Side Chain Cleavage Enzyme
SF-1	Steroidogenic Factor-1
StAR	Steroidogenic Acute Regulatory protein
Val	Valine
VCAM	Vascular Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
Zn^{2+}	Zinc Ion

Steroid Nomenclature and Abbreviations

Cortisol	F
Cortisone	E
Dehydroepiandrosterone Sulphate	DHAS
Corticosterone	B
Dehydroepiandrosterone	DHA
11-Deoxycortisol	S
Androstenedione	AD
11-β-Hydroxyandrostenedione	11βOH-AD
18-Hydroxycorticosterone	18OH-B
Deoxycorticosterone	DOC
Aldosterone	ALDO
18-Hydroxy-11-deoxycorticosterone	18OH-DOC
Testosterone	T
Oestrone	E_1
Oestradiol-17β	E_2

Publications

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2. Blackhurst G, Holloway C, Fraser R, Connell JMC. Characterisation of a Novel Whole Lymphocyte Glucocorticoid Receptor Binding Assay in Man. Abstract and Poster, BES *J Endocrinology* **148 Suppl** P322 1996

3. Blackhurst G, McElroy K, Connell JMC. Seasonal Variation In Glucocorticoid Receptor Binding Characteristics In Human Lymphocytes. Abstract and Poster, BES 1997

4. Blackhurst G, McElroy K, Connell JMC. The Effect of Environmental Temperature on Glucocorticoid Receptor Binding Characteristics, Abstract and Poster, Association of Physicians 1997

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7. Blackhurst G, Davies E, Connell JMC. A Cluster of 15 Point Mutations in the Glucocorticoid Receptor Gene. Abstract and Poster, Endo `98 1998

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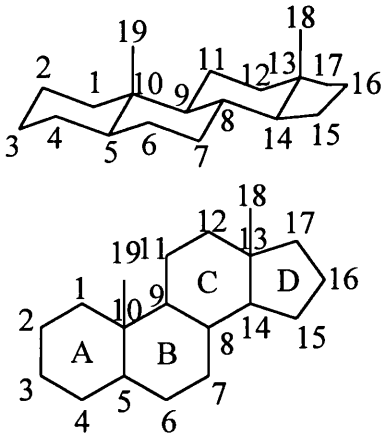
Chapter 1

Introduction

1.1 Steroids

Steroids are derivatives of cholesterol with a tetracyclic structure (Petrow 1999) composed of 3 conjoined 6 membered rings and a single 5 membered ring, (Figure 1.1). Present in all higher vertebrates as hormones and structural components, steroids have profound effects on cellular division and differentiation, organogenesis, morphogenesis, mineral homeostasis and fuel metabolism.

Figure 1.1 The Steroid Nucleus



The basic steroid nucleus is formed from 3 cyclohexane rings joined in the *trans* conformation, a single cyclopentane ring and 2 methyl groups in the β conformation. The carbon numbering convention is shown.
A variable side chain is attached to C_{17}. Modifications at positions carbons 3, 11, 17 and 18 impart differing biological activities.

Steroids exert these effects by interacting with receptors. Although a few rapid steroid effects are due to activation of extracellular receptors (Wehling 1997), the vast majority of steroid actions are mediated by intracellular receptors (Beato 1989, Evans 1988, Fuller 1991). These hormone sensitive transcription factors either greatly increase or

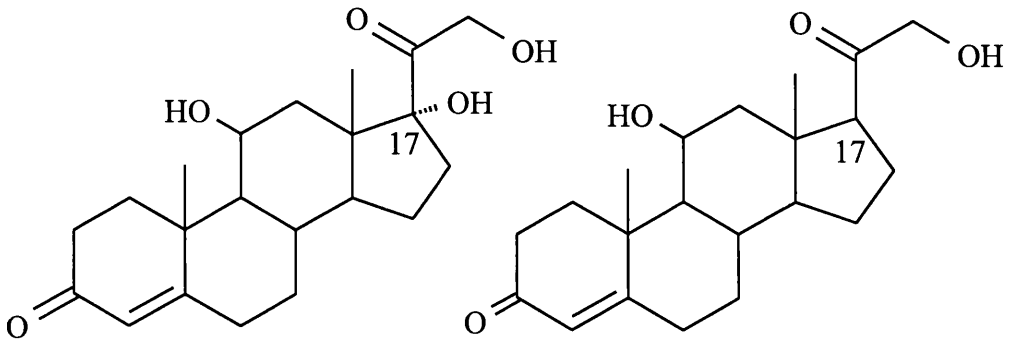
decrease the rate of gene transcription of key intracellular proteins. These target proteins are often rate limiting enzymes or ion channels and altered translation results in changed cell behaviour.

Steroids are traditionally classified into 3 groups: glucocorticoids, mineralocorticoids and sex steroids. Glucocorticoids, together with mineralocorticoids, are synthesised in the adrenal cortex and these two groups of hormones are together known as corticosteroids. This thesis is concerned with the glucocorticoid receptor; the structure and function of the gene encoding this protein, the function of the protein in vitro, and the effect these may have on physiological function in populations of normal human subjects.

Glucocorticoids

In man the principal glucocorticoid is cortisol while in rodents it is corticosterone (Figure 1.2). Synthetic glucocorticoids include dexamethasone, triamcinolone and beclomethasone. No selective glucocorticoid antagonist exists: RU486 is a synthetic compound with glucocorticoid and progesterone antagonistic effects.

Figure 1.2 Glucocorticoids
Cortisol is the main glucocorticoid in man, while corticosterone is the main glucocorticoid in rodents. Corticosterone lacks the β hydroxyl group at carbon 17.

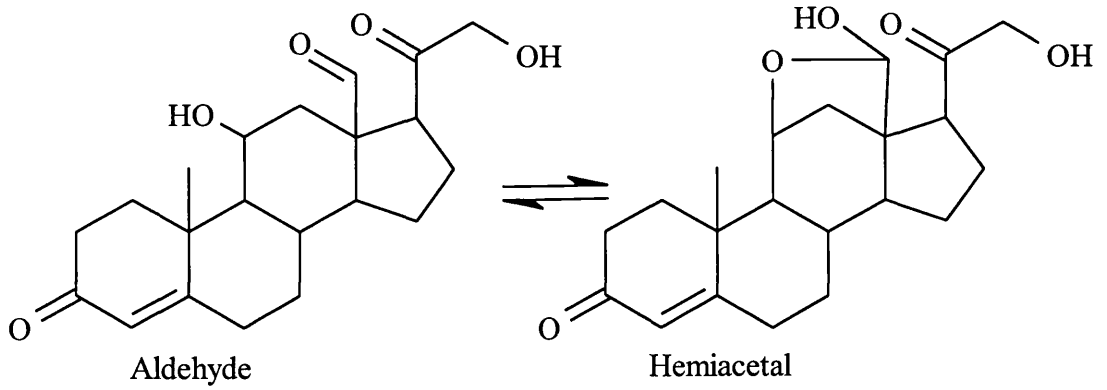


Glucocorticoids have important effects on the regulation of carbohydrate metabolism and also the metabolism of lipid and protein. In addition to these metabolic effects, glucocorticoids have significant roles in the regulation of blood pressure, the ontogeny and response of the immune system, and have an alerting effect on the central nervous system. The main focus of this thesis is on the glucocorticoid receptor and the actions of glucocorticoids will be considered in greater detail in section 1.5.

Mineralocorticoids

The principal mineralocorticoid is aldosterone (Figure 1.3). This exists both as the

Figure 1.3 Aldosterone
Aldosterone, the main mineralocorticoid steroid, exists in the aldehyde and hemiacetal forms shown here.



aldehyde form and the hemiacetal form, which are in free equilibrium. The principal synthetic mineralocorticoid is fludrocortisone. Spironolactone is a synthetic mineralocrticoid antagonist.

Mineralocorticoids principally affect sodium and potassium flux in the distal convoluted tubules and collecting tubules of the kidney, but also increase sodium resorption in the

epithelia of sweat glands and colon. At these sites sodium permeability is controlled by an amiloride-sensitive ion channel, the epithelial sodium channel – type 1 (ENaC-1). Aldosterone, through activation of the mineralocorticoid receptor, increases expression of this channel and enhances sodium resorption. The epithelial sodium channel is a heterotetramer composed of two α subunits (the limiting component of channel formation), one β subunit and a γ subunit (Canessa et al 1994). Aldosterone appears to exert its main effect on sodium channel formation in the kidney by increasing the rate of α and β subunit expression (May et al 1997). Additional induction of the basolateral sodium-potassium ATPase by aldosterone further increase sodium reabsorption (O'Neill 1990).

In vitro glucocorticoids bind to and activate mineralocorticoid receptors (Arriza et al 1987). In vivo, however, the enzyme 11-β hydroxysteroid dehydrogenase type 2 metabolises cortisol to cortisone that is unable to bind to the mineralocorticoid or glucocorticoid receptor, thus protecting the kidney from the large quantities of glucocorticoids reaching it (Edwards et al 1988). Further discussion of the isoforms of 11-β hydroxysteroid dehydrogenase is addressed in section 1.4.

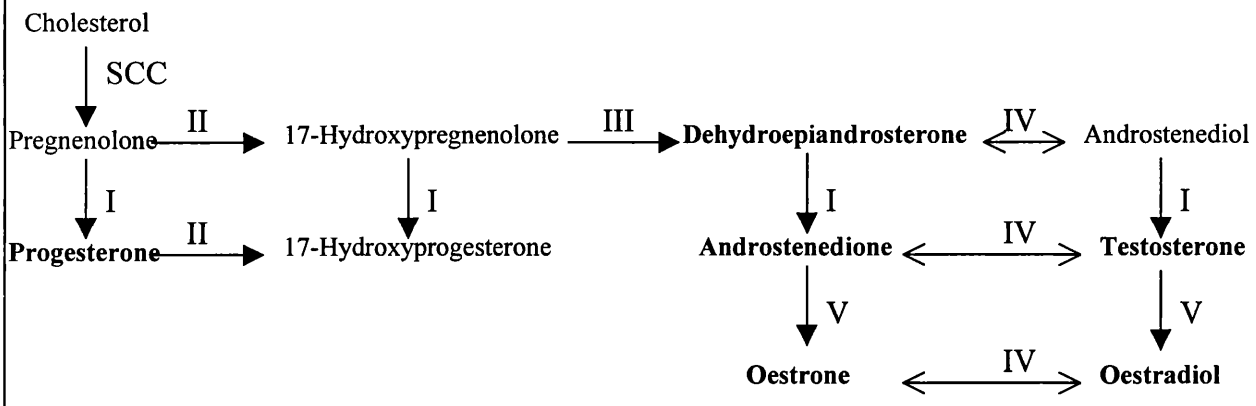
Additional target sites for mineralocorticoid action include the brain and heart. In the hippocampus, which has high levels of mineralocorticoid receptor, and in periventricular grey matter mineralocorticoids are thought to control sympathetic outflow and thus blood pressure regulation. Elegant experiments show intraventricular injection of aldosterone to the brain increases blood pressure, while pre-administration of spironolactone (a mineralocorticoid antagonist) prevents the rise in blood pressure (Gomez-Sanchez et al 1992).

Prolonged exposure to elevated aldosterone, as occurs in Conn's syndrome or in cardiac failure appears to increase cardiac fibrosis and dysrhythmogenesis (Brilla et al 1992). The importance of these findings have recently been underscored by prospective studies showing beneficial effects of spironolactone in chronic cardiac failure (Weber 1999).

Sex Steroids

Sex steroids include androgens, progestogens and oestrogens. The most potent sex steroids are synthesised in the gonads, the placenta and within peripheral tissues (e.g. by conversion of androstenedione to oestrone by aromatase in adipose tissue, Bulun et al 1999). The adrenal also synthesises sex steroids although these possess lower potency. The biosynthetic pathway of sex steroids is shown in figure 1.4. The main actions of sex steroids are on the reproductive tract, muscle and adipose tissue deposition and on hair growth.

Figure 1.4 Sex Steroid Synthesis



The pathways for sex steroid synthesis are shown above.
The enzymes at each step are shown as letters:

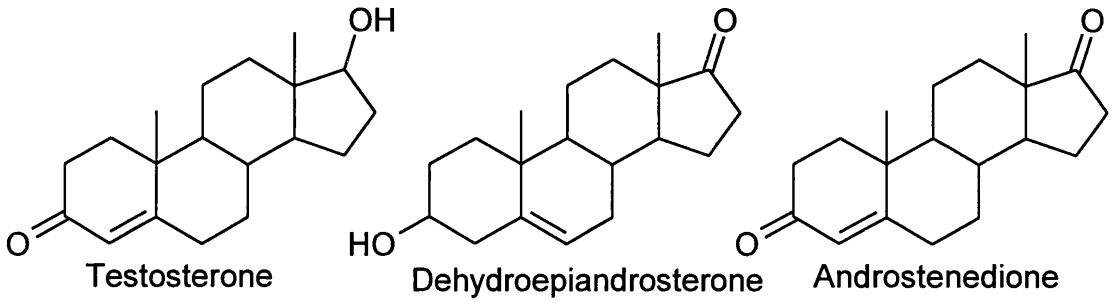
SCC	Cholesterol Side Chain Cleaveage Enzyme
I	3β-Hydroxy steroid Dehydrogenase
II	17α-Hydroxylase *
III	17-20 Lyase *
IV	17β-Hydroxysteroid Dehydrogenases
V	Aromatase

* A single protein is probably responsible for both these enzymatic actions - see 1.3.2.3

Androgens

The main androgen in men is testosterone produced in the testes, with smaller amounts of dehydroepiandrosterone and androstenedione produced in the adrenal, figure 1.5.

Figure 1.5 Androgens
The structure of testosterone and the the weaker androgens dehydroepiandrosterone and androstenedione are shown.



Androgens increase terminal hair growth and sebum secretion, cause deepening of the voice, muscle hypertrophy and maintain libido. In the male, the principal androgen, testosterone, is produced in the Leydig cells of the testis. During embryogenesis testosterone produced from the fetal testis causes development of structures derived from the W[illegible]lffian duct including the epididymis, vas deferens, seminal vesicles and prostate. At puberty testosterone is necessary for penile enlargement and sperm production thereafter. In the female, the adrenal androgens dehydroepiandrosterone, and androstenedione along with small amounts of testosterone from the ovary are produced and may maintain libido.

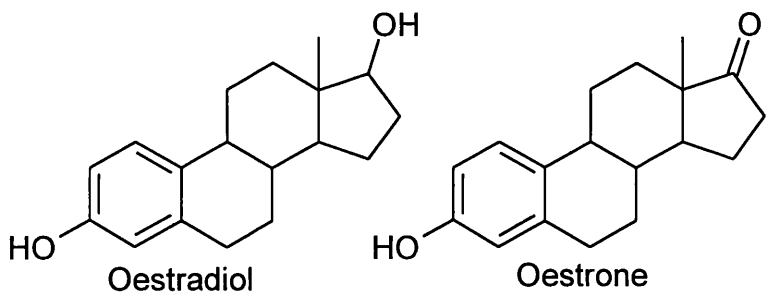
Androgens bind to a specific receptor to modify gene transcription (Hiipakka 1998). The androgen receptor is homologous to the glucocorticoid receptor and other members of the nuclear receptor family but the ligand binding domain is specific for androgens

and the DNA-binding domain recognises a specific androgen response element. Tissue-specific metabolism of androgens occurs in many androgen responsive tissues by the enzyme 5α-reductase that metabolises testosterone to the more potent androgen 5α-dihydrotestosterone. In bone, testosterone is metabolised to oestradiol by the enzyme aromatase where it activates the oestrogen receptor.

Oestrogens

Oestrogens are the main female sex hormones and are responsible for the secondary sexual development of the breast, vagina and vulva and contribute to pubic hair growth. The main oestrogens produced in the ovary in the female are oestradiol and oestrone (figure 1.6). Less potent oestrogens are produced in the adrenals and in adipose tissue by aromatisation of androstenedione in both sexes.

Figure 1.6 Oestrogens
The principal oestrogens, oestradiol and oestrone, are shown below.



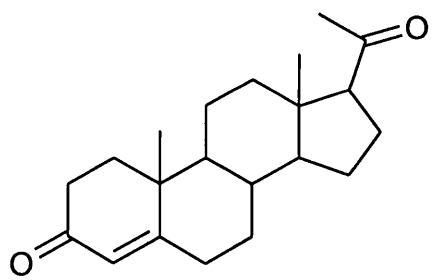
Like the other steroid hormones, oestrogens bind to a specific oestrogen receptor to modify gene transcription. The original receptor was cloned from human ovary (Green et al 1986) and is expressed at high levels in endometrium, ovary, breast and testis. Recently a new oestrogen receptor isoform was identified (Kuiper et al 1996) which

was designated the β-form (ER-β), the original receptor being designated ER-α. This β receptor is strongly expressed in prostate, blood vessels and bone where it may mediate the effects of oestrogens (either secreted from the ovary or aromatised from testosterone within the target tissue).

Progestogens

These hormones are named for their requirement in the maintenance of normal pregnancy. The only important progestogen in the human and most rodent species is progesterone (figure 1.7), although in the rabbit the main progestogen is 20α-hydroxyprogesterone.

Figure 1.7 Progesterone



Progesterone

Progesterone is a normal precursor for all other steroid hormones, but is produced in large quantities, particularly in the ovary in the luteal phase, where it acts as a hormone in its own right. In the luteal phase of the menstrual cycle, progesterone causes differentiation of the epithelia of the uterus and increased tortuosity of the uterine arteries. During pregnancy, progesterone produced from the placenta maintains a viable pregnancy.

Progesterone acts through a specific progesterone receptor (Misrahi et al 1987) to modify gene transcription like its other steroid counterparts.

Thus, the sex steroids include a diverse group of steroids with broadly similar mechanisms of action to glucocorticoids, but with different actions determined by different receptors, target tissues and target genes. The remainder of the thesis will concentrate on the function of glucocorticoids and the glucocorticoid receptor and its possible role in disease.

1.2 The adrenal gland

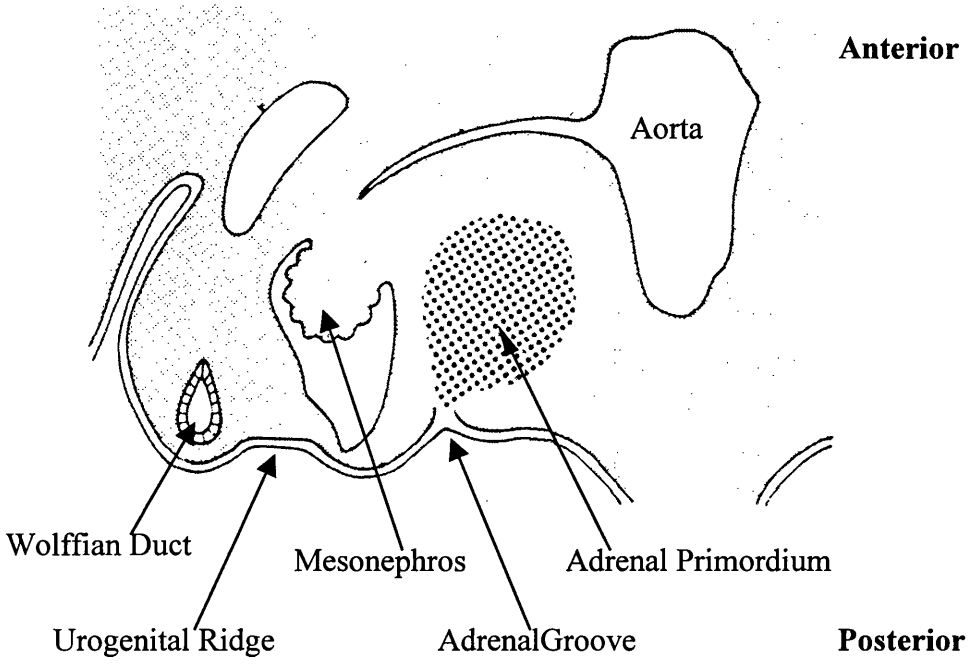
The adrenal glands lie on the upper pole of each kidney. Each gland measures approximately 5cm by 2.5cm by 2.5cm and weighs 4g. The right gland is pyramidal in shape and the left is crescentic.

Each gland is divided into an outer cortex derived from the urogenital mesoderm that produces corticosteroids and an inner medulla composed of chromaffin tissue (named because of its ability to stain with chromic acid salts, Kohn 1902) derived from neuroendoderm that produces catecholamines.

1.2.1 Ontogeny

The adrenal cortex arises from coelomic mesoderm while the medulla arises from neuroectoderm. Adrenal cortical development has been described as occurring in 5 stages (Sucheston & Cannon 1968): condensation, proliferation, differentiation, decline of fetal layer, and development of adult zonation. At around 25 days gestation the coelomic epithelium between the gastric mesentery and mesonephros begins to develop cuboidal/columnar cells that divide rapidly and condense to form the adrenal primordium (figure 1.8).

Figure 1.8 The Adrenal Primordium
The adrenal primordium develops from a condensation of coelomic epithelium between the aorta and the mesonephros. The diagram is of a 4 week old human fetus (modified from Neville and O'Hare)

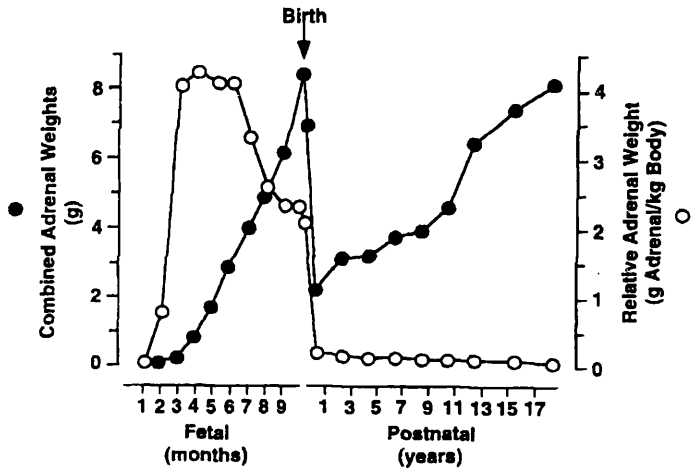


These cells will later form all layers of the adult adrenal cortex. At 30 days gestation cells from the mesonephros migrate towards the primitive adrenal gland. Most of these cells surround the gland forming a loose mesenchymal capsule while others infiltrate the adrenal parenchyme to form a supporting stromal framework. Between 35 and 45 days gestation preganglionic sympathetic nerve tracts and primitive postganglionic sympathetic cells migrate towards the rudimentary adrenal entering the parenchyme of the gland which now surrounds them. These cells will later form the catecholamine synthesising adrenal medulla.

Over the next 4 weeks the cells of the fetal adrenal rapidly proliferate mainly through mitosis of the outer cortical zone. By eight weeks the cells have differentiated into two layers which are clearly visible: the outer definitive cortex and the inner fetal cortex.

During the second trimester the adrenal glands grow rapidly, keeping pace with the increase in fetal body weight. This is largely through expansion of the inner fetal layer of the cortex and at this time the adrenal weighs 4g/kg body weight – 35 times the ratio in the adult (figure 1.9).

Figure 1.9 Adrenal Growth Rate
The absolute total adrenal weight is shown in solid circles and the relative adrenal weight by total body mass, shown in open circles (modified from Mesiano and Jaffe 1997)



Much of the rapid growth occurring in the adrenal at this stage appears to be driven by ACTH-induced insulin-like growth factor II (IGF-II) or basic fibroblast growth factor (bFGF) production within the gland (Mesiano & Jaffe 1997).

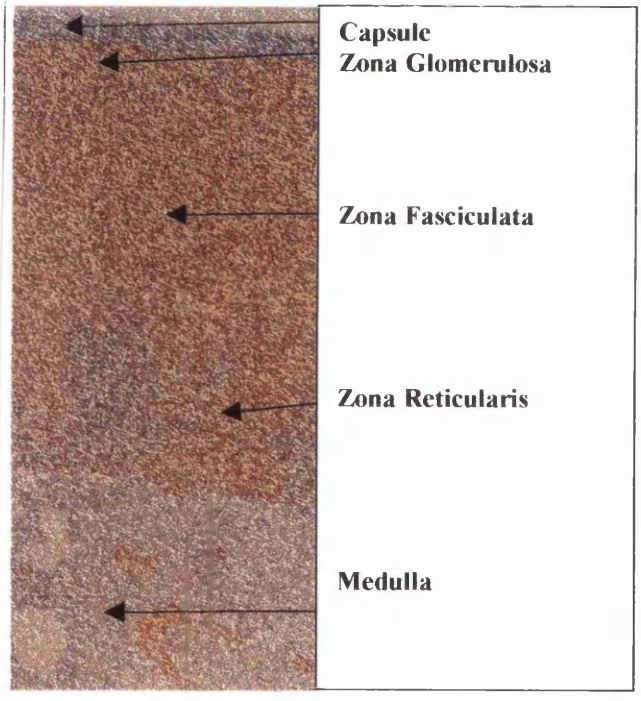
During the third trimester adrenal growth continues but at a slower rate; by this stage the adrenal glands weigh 2.5g/kg. Despite this, the fetal adrenal total weight at term of 8g is equal to that of the adult. Following birth the fetal adrenal cortex rapidly involutes leaving a compact medulla surrounded by a thin layer of adult cortex. Combined adrenal

weight is now only around 2g and this will only gradually increase to the adult weight of 8g over the next 20 years.

1.2.2 Adult Adrenal Histology

The adrenal cortex is composed of three concentric layers of steroidogenic tissue: the outer zona glomerulosa, the zona fasciculata and an inner zona reticularis (figure 1.10). Normally the outer zona glomerulosa, which forms an incomplete layer in the human,

Figure 1.10 Zones of the Adrenal Cortex



contributes around 5% of the cortical mass, while the fasciculata and reticularis contribute 70% and 25% respectively.

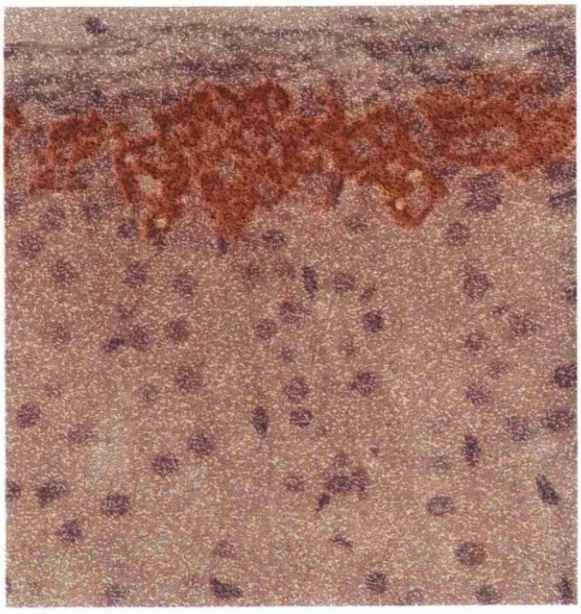
The steroidogenic cells of the adrenal contain conspicuous clear vacuolated cytoplasm that would contain cholesterol droplets in life as the substrate for steroid synthesis.

Mitochondria contain many of the steroidogenic enzymes and provide the energy for many of the oxidation steps, and they too make an important contribution to the structure of steroidogenic cells. The relative number and size of lipid vesicles and mitochondria varies considerably between the zones and allows a rudimentary distinction to be made on light microscopy, and also allows cells to be separated by physical techniques such as density gradient centrifugation.

Zona Glomerulosa

The cells of this zone lie in the outer layer of adrenal cortex and synthesise and secrete aldosterone (figure 1.11). They posses a prominent nucleus surrounded by relatively little cytoplasm that contains small numbers of lipid droplets. On electron microscopy there are numerous mitochondria, which are characteristically elongated with tubular cristae, a prominent Golgi apparatus and normal amounts of smooth endoplasmic reticulum. The cell membranes often posses microvilli which form complex interdigitations with neighbouring cells. The cells of this layer form small clusters (*glomus* – Latin, ball) surrounded by a fine capillary network which compose the incomplete layer of the zona glomerulosa.

Figure 1.11 The Zona Glomerulosa
In this section of a rat adrenal the zona glomerulosa is stained brown with antibodies to aldosterone synthase and forms a continuous layer in the outer adrenal cortex. (kindly provided by Dr S M MacKenzie – see chapter 2.11)

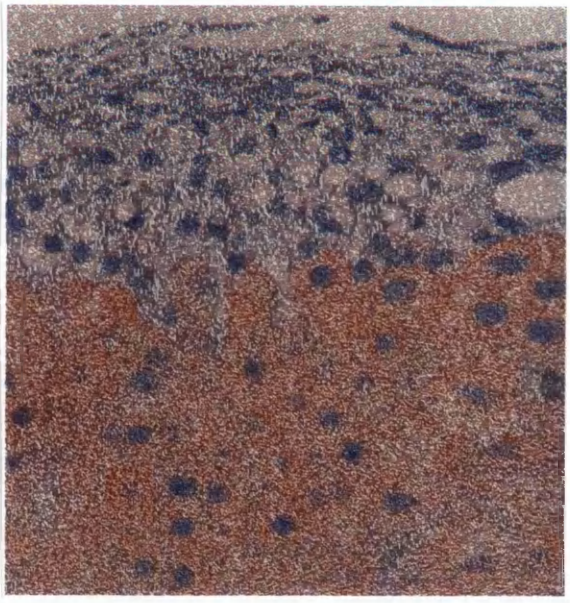


Zona Fasciculata

The zona fasciculata is responsible for the synthesis of cortisol, the principal glucocorticoid in man. It is composed of stacks of steroidogenic cells (*fascis* – Latin, bundle), separated by endothelial-lined sinusoids (figure 1.12). These transport cortisol to the medulla where it plays an important role in inducing and maintaining the enzymes necessary for catecholamine synthesis and in the development of the adrenal medulla (Schmid et al 1995). On gross appearance the zona fasciculata has a buttery yellow appearance due to large amounts of lipid and cytochrome. The cells of this zone are

large with a prominent cell membrane and cytoplasm, and contain a small dense nucleus. Progressing from the outer zona fasciculata to the inner layer, cells contain increasing numbers of lipid-containing vacuoles and spheroid mitochondria with an increasingly lamellar arrangement of cristae.

Figure 1.12 The Zona Fasciculata
The zona fasciculata in this section of rat adrenal is stained brown with an antibody against 11β-hydroxylase. The zone occupies most of the adrenal cortex. (kindly provided by Dr S M MacKenzie – see chapter 2.11)



Zona Reticularis

The inner one-quarter of the adult human adrenal cortex is composed of the zona reticularis. The zone is formed by interconnected limbs of cells separated by coarse sinusoids, thus forming a net-like structure (*rete* – Latin, net). The cells of this zone are small and compact with few lipid vesicles, accumulated lipofuscin pigment and

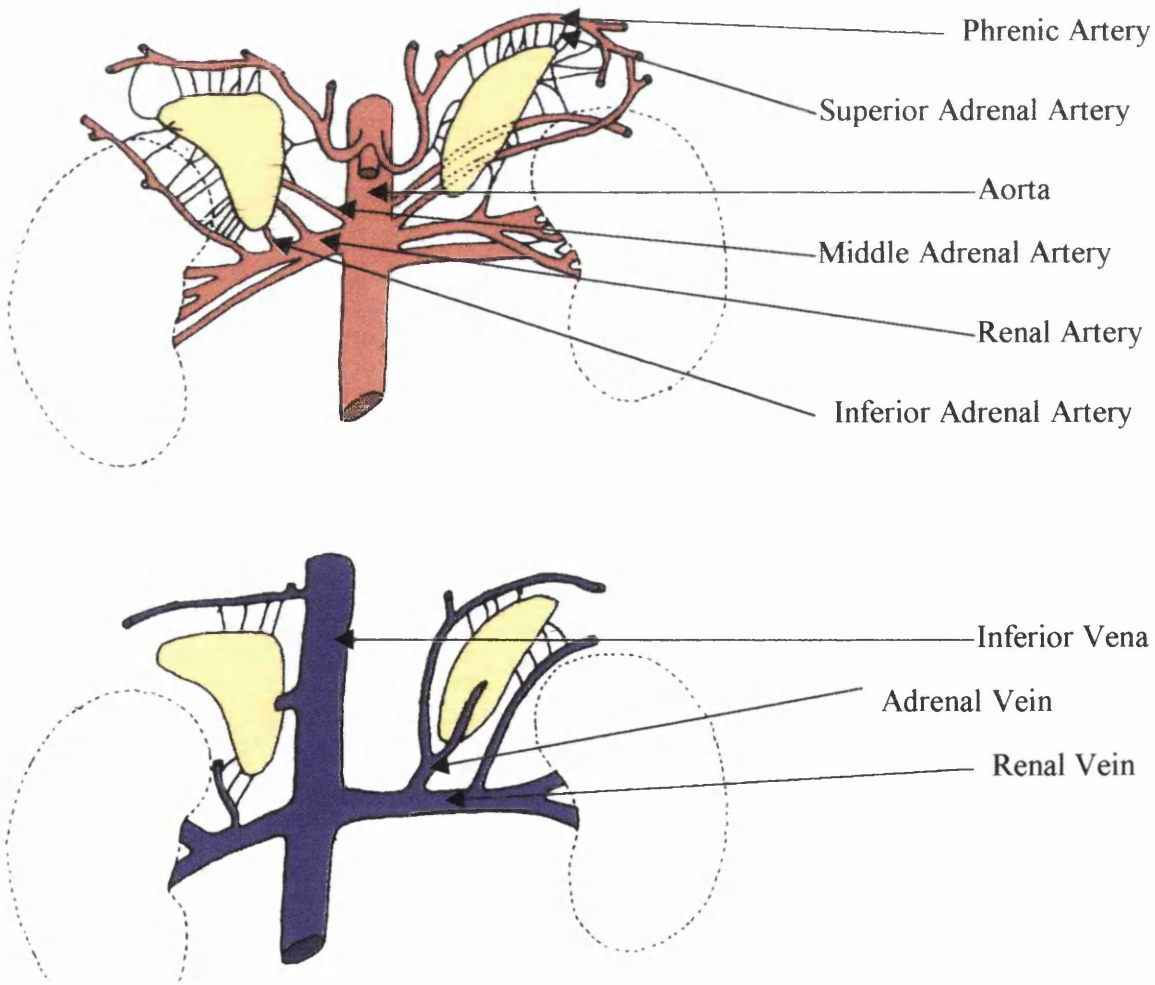
abundant mitochondria. The function of the zona reticularis in humans remains controversial, but it appears to the major source of the adrenal androgens dehydroepiandrosterone (DHEA) and DHEA sulphate.

1.2.3 Adrenal Blood Supply

The adrenal is highly vascular. Much of the blood supply is used to provide the oxygen required to maintain the mitochondrial electrochemical gradients that are necessary for steroidogenesis.

The adrenal cortex and medulla share a common blood supply. Branches of the phrenic artery, aorta and renal arteries form the superior, middle and inferior adrenal arteries respectively (figure 1.13). After penetrating the adrenal capsule, the arteries divide into

Figure 1.13 The Adrenal Blood Supply



a plexus of smaller arterioles supplying blood to the sinusoids of the adrenal cortex. These endothelial-lined spaces carry blood and locally generated corticosteroids to the adrenal medulla. Here the plexus of venules unite and leave the inferomedial surface of the adrenal as a single vein draining into the inferior vena cava on the right, and the renal vein on the left (figure 1.13).

1.2.4 Adrenal Nerve Supply

Preganglionic cholinergic sympathetic fibres from the splanchnic nerve are carried along the penetrating arteries and supply the adrenal medulla. Here the neurones stimulate the medullary tissue to release catecholamines from storage granules. Although some evidence suggests sympathetic neural input to the cortex is required for full steroidogenic and proliferative capacity (Bornstein & Chrousos 1999) the principal regulatory signals for steroid synthesis are humoral.

1.3 Control of Steroid Synthesis

Mineralocorticoid synthesis in the zona glomerulosa and glucocorticoid synthesis in the zona fasciculata are controlled separately. The regulation of adrenal androgen production is less clearly understood and will not be discussed further.

Mineralocorticoid synthesis is stimulated by increases in extracellular K^+ and angiotensin II concentrations. Although stimulated acutely by adrenocorticotrophic hormone (ACTH), cells of the zona glomerulosa rapidly lose responsiveness on prolonged stimulation. In contrast, glucocorticoid synthesis is exquisitely sensitive to ACTH levels.

1.3.1 ACTH

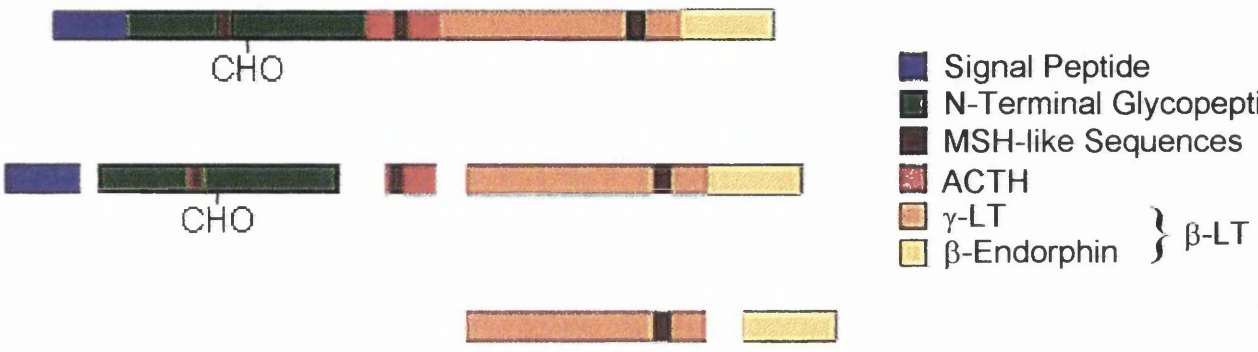
ACTH stimulates corticosteroid synthesis by a cyclic adenosine monophophate (cAMP) dependent mechanism. The hormone binds to the cell-surface receptor of the adrenal

cell and activates the G_s protein and adenylyl cyclase thus increasing cAMP generation. Activation of protein kinase A initiates a cascade of steps that stimulate cyclic AMP response elements (CREs) resulting in increased expression of StAR protein (Kallen et al 1998). This protein increases cholesterol transport to the inner mitochondrial membrane where cholesterol side-chain enzyme begins the process of steroidogenesis. Additional CREs in the promoters of the steroidogenic enzymes stimulate enzyme expression, further increasing steroidogenic capacity of the adrenal. Prolonged stimulation of cAMP by ACTH results in more mitochondria per cell, increased cell size, hypertrophy of the adrenal, and increased steroid-producing capacity. On further prolonged stimulation, ACTH eventually increases the level of active protein phosphates that signal the cell to divide resulting in hyperplasia of the adrenal cortex. This trophic property of ACTH gives rise to its name of adrenocortico*trophic* hormone.

1.3.1.1 Regulation of ACTH secretion

ACTH is synthesised in the anterior pituitary. In response to CRH production from the hypothalamus, the pituitary produces pro-opiomelanocortin (POMC), a glycosylated peptide of 241 amino acids. This subsequently undergoes significant post-translational modification with glycosylation, proteolysis, phosphorylation, N-terminal acetylation and C-terminal amidation. In the anterior pituitary the peptide is cleaved by specific peptidases into ACTH (39 amino acids), N-terminal glycopeptide and β-lipotropin figure 1.14).

Figure 1.14 Pro-opiomelanocortin and ACTH
Pro-opiomelanocortin is synthesised in the anterior pituitary as a peptide of 241 amino acids. Post-translational modification adds carbohydrate groups (shown as CHO in the diagram). Subsequent cleavage releases ACTH and a number of additional peptides (signal peptide, N-terminal glycopeptide and β-lipotropin). β-lipotropin is subsequently slowly cleaved to β-endorphin and γ-lipotropin. Several of these peptides, including ACTH, contain a melanocyte stimulating hormone motif, shown in brown.



Abbreviations: ACTH - adrenocorticotrophic hormone
MSH - melanocyte stimulating hormone
β-LT - β-Lipotropin
γ-LT - γ-Lipotropin

ACTH is released into the inferior petrosal sinus and reaches the systemic circulation. In the adrenal it binds to a specific cell-surface receptor and stimulates adenylyl cyclase initiating the chain of events described above that increases cortisol production. The first 18 amino acids of ACTH contain the sequence required for ACTH-receptor activation. The peptide also contains a tetrapeptide sequence (His-Phe-Arg-Trp) contained in melanocyte stimulating hormones and this sequence is repeated within POMC itself 3 times. This sequence imparts melanocyte stimulating activity to ACTH which binds to the MSH-2 receptor, a closely related receptor to the ACTH receptor (Mountjoy et al 1992). The melanocyte stimulating hormones α- β- and γ- MSH are not thought to circulate in the normal adult, being present only in humans in the fetus and from secretion

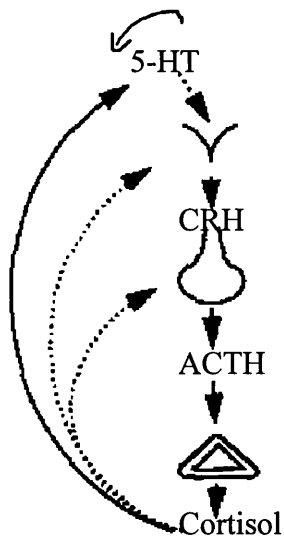
from neoplastic tissue.

N-terminal glycopeptide contains the sequence of γ-melanocyte stimulating hormone (γ-MSH) and may act synergistically with ACTH to stimulate cortisol production by stimulating cholesterol ester hydrolase.

β-Lipotropin contains weak lipolytic activity in man, the significance of which remains uncertain. It is formed from 91 amino acids from the C-terminus of POMC and through a further proteolytic step is slowly cleaved to γ-lipotropin and β-endorphin. The former peptide contains the sequence of β-MSH, although this product is not produced as a free peptide in man. β-Endorphin has potent analgesic activity.

ACTH secretion is under central control at several levels, shown in figure 1.15. The

Figure 1.15 Glucocorticoid Feedback on the Hippocampal-Hypothalamo-Pituitary-Adrenal Axis



Hippocampus

Hypothalamus

Pituitary

Adrenal

Glucocorticoids produced from the adrenal cortex stimulate hippocampal serotonergic fibres (5-HT).
These fibres project to the hypothalamus to inhibit corticotropin-releasing hormone (CRH). This results in reduced ACTH and Cortisol secretion.
Additional levels of negative feedback occur at the hypothalamus where glucocorticoid inhibit CRH secretion, and at the anterior pituitary to inhibit ACTH secretion.
Modified from Panarelli,M. et al 1998

major mechanism is through secretion of corticotropin-releasing hormone (CRH) from the hypothalamus (Rivier et al 1986). This peptide hormone of 41 amino acids is secreted in a circadian rhythm with highest levels around 4 am and lowest levels at 10 pm, and in response to psychological and physical stress. CRH is carried along the portal circulation between the hypothalamus and pituitary, and stimulates POMC transcription and ACTH secretion through a cAMP-Protein Kinase A dependent mechanism. Arginine Vasopressin (AVP) secreted from the hypothalamus and reaching the pituitary increases ACTH secretion synergistically with CRH acting through the generation of inositol *tris*phosphate (IP_3).

Control of hypothalamic CRH secretion appears to be under the control of several brain areas including neurones in the brainstem, paraventricular nuclei and telencephalon. An important controlling pathway appears to be through fibres arising in the hippocampus; a brain structure involved emotion and mood. Serotoninergic fibres inhibit the hypothalamic secretion of CRH, while sparser γ-aminobutyric acid (GABA) containing fibres stimulate CRH secretion. The hippocampus contains glucocorticoid receptors that stimulate firing of the serotoninergic fibres and further reduce CRH and ultimately ACTH secretion (Mulatero et al 1997).

Another signal controlling ACTH release is arginine vasopressin (AVP) released from the paraventricular nuclei. Although a weak stimulus for ACTH release itself, it acts synergistically with CRH to evoke ACTH synthesis and secretion.

The hippocampus appears to be "programmed" by glucocorticoid exposure in late fetal and early postnatal development. Thus the hippocampus postnatally is extremely plastic undergoing neuronal apoptosis and regeneration (McEwen 1999). The rich glucocorticoid receptor levels in this region of brain increase the rate of apoptosis as glucocorticoid

levels rise. Thus, postnatal glucocorticoid exposure provides a mechanism of reducing the sensitivity of the hippocampus to glucocorticoids, and increases the level of stimulation to the hypothalamus increasing CRH production. This feeds into the pituitary to increase ACTH production and adrenal glucocorticoid production. The overall effect is to raise the point where negative-feedback occurs in the hypothalamo-pituitary-adrenal axis.

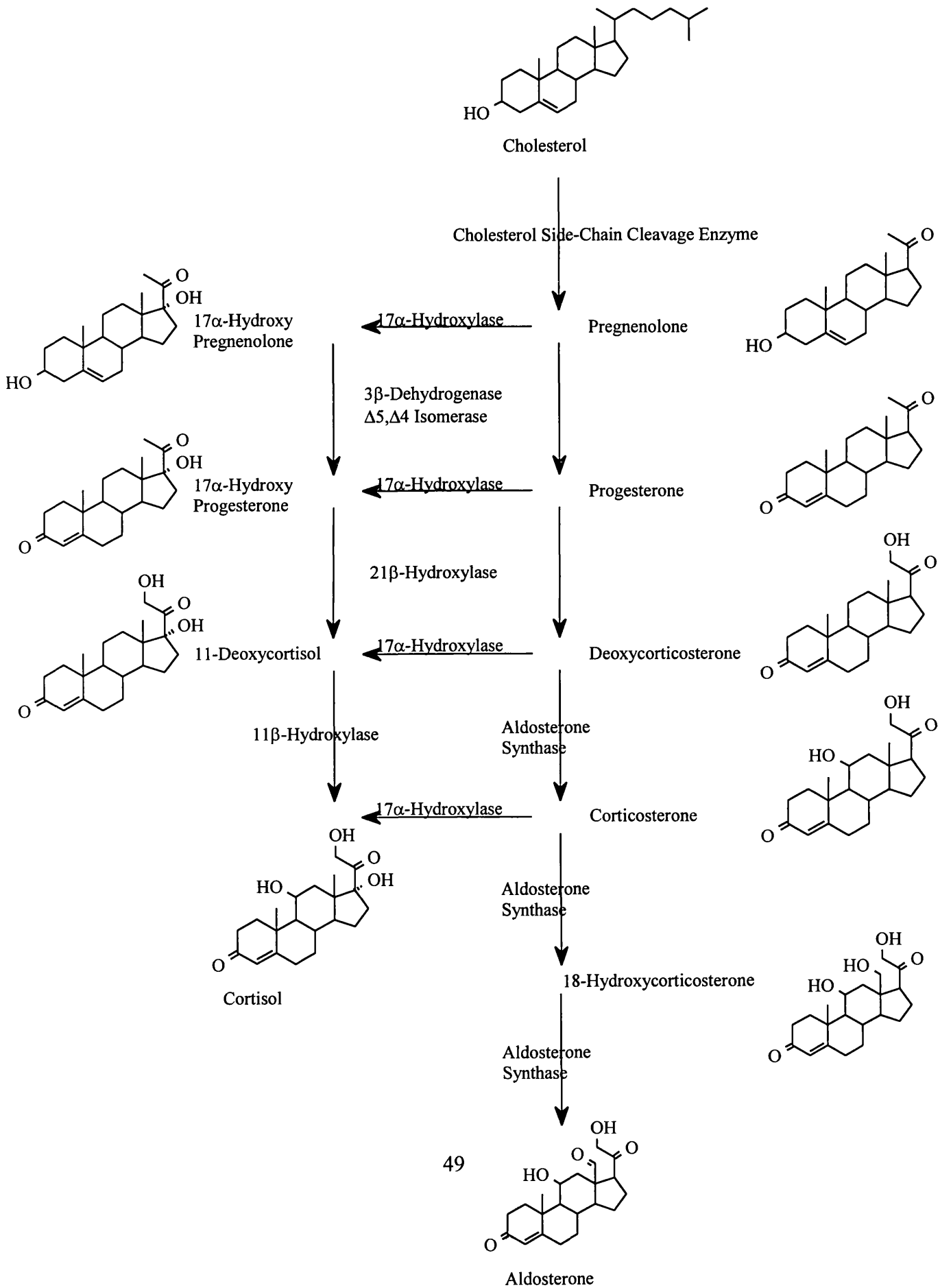
Glucocorticoids reduce ACTH secretion by a direct action on the pituitary. At least 2 glucocorticoid response elements exist in the upstream 700 base pairs of the POMC promoter and glucocorticoid binding to these elements appears to disrupt binding sites for unidentified binding factors normally driving basal and CRH-responsive transcription (Charron et al 1986).

In summary, the control of glucocorticoid secretion is integrated at a number of levels in the central nervous system and under predominant negative feedback by adrenal glucocorticoid production.

1.3.2 Steroid Synthesis – The Steroidogenic Pathway

Several steps are involved in the synthesis of steroids from the initial cleavage of the side-chain from cholesterol to the progressive oxidation to active steroid hormones and these are outlined in figure 1.16.

Figure 1.16 The Steroidogenic Pathway



1.3.2.1 Cholesterol Transport

The initial step in steroid biosynthesis is the transport of cholesterol substrate from the storage vacuoles in the cytosol to the inner membrane of the mitochondrion where the steroidogenic enzymes reside. Recently, the 30kDa phosphoprotein responsible for controlling this transport in adrenal, ovary and testis, but not placenta, has been identified: the Steroidogenic Acute Regulatory protein, or StAR protein (Clark et al 1994). Expression of this protein rapidly follows stimulation of the adrenal cortex resulting in the flux of cholesterol from the outer mitochondrial membrane to the cholesterol-poor inner membrane. The molecular mechanism of the ability of StAR protein to mediate this flux remains to be elucidated.

The promoter of the StAR protein contains 2 steroidogenic factor-1 (SF-1) binding sites and a half oestrogen response element (Sugawara et al 1996). The proximal SF-1 binding site is responsible for the induction of StAR expression following cAMP stimulation (e.g. by ACTH), while the distal SF-1 binding site is necessary for constitutive expression of the factor, but is required for maximal stimulated expression by cAMP.

In the zona glomerulosa K^+ or angiotensin II stimulate steroidogenesis through stimulation of IP_3 and Ca^{2+} release. The induction of StAR protein appears to be under the control of calcium via calmodulin kinase II activation as neither diacyl glycerol nor protein kinase C increase aldosterone production (Cherradi et al 1998).

1.3.2.2 Side-Chain Cleavage Enzyme

The generation of steroids involves several oxidative modifications of cholesterol (fig 1.16). Once cholesterol arrives in the inner mitochondrial membrane, the membrane-

bound side-chain cleavage enzyme removes the aliphatic chain with cleavage of the C_{20}-C_{22} bond. This is achieved by two hydroxylation reactions at $C_{22\alpha}$ and C_{20R} with a subsequent lyase step to complete bond cleavage. As a result C_{20} is oxidised to a ketone generating pregnenolone. The cholesterol side-chain cleavage enzyme is composed of three components: the catalytic protein P450scc, adrenodoxin and adrenodoxin reductase. During the reaction NADPH adds an electron to adrenodoxin and this is used to break the O=O bond of molecular oxygen which is required to oxidise the substrate and break the cholesterol side chain.

The promoter of P450scc contains a binding site for SF-1, responsible for basal expression, and a CRE which provides a mechanism for induction of expression in response to ACTH induced cAMP generation.

1.3.2.3 17α-Hydroxylase, Lyase

This enzyme is found in the endoplasmic reticulum of the zona fasciculata and zona reticularis, but is absent in the zona glomerulosa. In these zones pregnenolone and progesterone are hydroxylated to yield precursors for cortisol and androgen synthesis. The enzyme has additional lyase activity, cleaving the $C_{17\alpha}$-C_{20} bond, in the synthesis of androgens (Buczko et al 1995). The lyase activity appears to require additional cytochrome b5 (Lee-Robichaud et al 1995) for full activity. Expression of this enzyme, as all the steroidogenic enzymes described below, is increased in response to ACTH.

1.3.2.4 3β-Hydroxysteroid Dehydrogenase-5ene, 4ene Isomerase

This enzyme is situated in the endoplasmic reticulum of the adrenal cortex. In contrast to most steroidogenic enzymes, it does not contain a cytochrome P450 moeity. It catalyses the formation of the keto-group on carbon 3, and the shift in the double bond

of cholesterol between carbons 5 and 6 (called the Δ5) to Δ4 (between carbons 4 and 5). Both the 3-keto Δ4-ene groups are characteristic of corticosteroids, and the enzyme is expressed in both the zonae glomerulosa and fasciculata.

1.3.2.5 21β-Hydroxylase

This enzyme is situated in the smooth endoplasmic reticulum of the adrenal cortex and catalyses the 21β-hydroxylation of progesterone or 17α-hydroxyprogesterone. The gene for the enzyme is situated on chromosome 6q, within the HLA complex of genes (New et al 1986) and explains the close linkage of HLA haplotype with congenital adrenal hyperplasia in kindreds with 21-hydroxylase deficiency.

1.3.2.6 11β-Hydroxylase

This enzyme is responsible for the generation of cortisol from deoxycortisol and is therefore characteristic of the zona fasciculata where the protein is located within the mitochondrion. Substrate which has been 17α-hydroxylated and 21β-hydroxylated within the smooth endoplasmic reticulum travels once again to the inner membrane of the mitochondrion where it undergoes 11β-hydroxylation.

1.3.2.7 Aldosterone Synthase

This enzyme is located within the mitochondria of the zona glomerulosa and is responsible for the two oxidation steps of 11β-hydroxylation and 18-oxidation. The biochemical nature of the intermediate steps in the conversion of deoxycorticosterone to aldosterone remain uncertain, as is the physico-chemical regulation of the late stages of adrenal steroidogenesis, but these considerations are outwith the scope of this thesis.

1.4 Glucocorticoid Access - the role of 11β Hydroxysteroid Dehydrogenase

For glucocorticoids to act on target tissues, the steroids must be able to reach their receptor in an active form. The enzyme 11β hydroxysteroid dehydrogenase (11β-HSD) is a major factor controlling this activity.

Two forms of this enzyme are now known to exist, Type I (Tannin et al 1991) and Type 2 (Brown et al 1993). The type 1 enzyme is expressed in most glucocorticoid sensitive tissues, is NADP(H)-dependent, and predominantly converts inactive cortisone to active cortisol (Moore et al 1993). The enzyme is widely expressed, particularly in the liver, adipose tissue and particular regions of the brain (Ricketts et al 1998, Seckl 1997). In the liver, 11β-HSD 1 increases intracellular glucocorticoid concentrations and increases the expression of the rate limiting enzyme in the gluconeogenic pathway; phosphoenol pyruvate carboxykinase (Jamieson et al 1999). Knockout mice lacking this enzyme are resistant to the hyperglycaemic response to stress or obesity suggesting obesity may result in hyperglycaemia by increasing glucocorticoid production or increasing the activity of 11β-HSD type 1 (Kotelevtsev et al 1997).

In the brain 11β-HSD type 1 is expressed particularly in cerebellar granule and Purkinje cells, hippocampal pyramidal and granule cells, hypothalamic cells including the paraventricular nuclei, and in all anterior pituitary cells (Seckl 1997). In the hippocampus, hypothalamus and anterior pituitary the enzyme would be expected to increase the negative feedback has on ACTH secretion, thus amplifying the sensitivity of the system (Kotelevtsev et al 1997).

The type 2 enzyme is predominantly expressed in kidney, placenta and colon (Whorwood et al 1995), has a high affinity for cortisol (inactivating it to cortisone) and is NAD dependent. Thus, the type 1 enzyme tends to regenerate active cortisol, while the type 2 enzyme in contrast inactivates cortisol.

The rare syndrome of apparent mineralocorticoid excess (SAME) was originally described in 1985 (Edwards et al 1985) and has since been found to be due to deficiency of the type 2 enzyme in the distal nephron (Milford et al 1995, Stewart et al 1988). In the distal tubule of the kidney, the mineralocorticoid receptor modulates sodium and potassium balance by directing the expression of subunits of the amiloride-sensitive sodium channel (Canessa et al 1994). In the absence of 11β-hydroxysteroid dehydrogenase type 2 to inactivate cortisol to cortisone the mineralocorticoid receptor, which binds cortisol and aldosterone with equal affinity, becomes activated inappropriately and this causes sodium retention, potassium loss and hypertension. Thus, the role of 11β-hydroxysteroid dehydrogenase type 2 is normally to protect the distal tubule from cortisol and maintain its sensitivity to aldosterone.

In evolutionary terms, the type 2 enzyme may be a relatively novel development. In teleost fish, which produce no aldosterone or other mineralocorticoids, cortisol regulates both mineral balance and fuel metabolism (Ducouret et al 1995) and these fish express no 11β-hydroxysteroid dehydrogenase.

In the placenta, 11-β hydroxysteroid dehydrogenase type 2 metabolises maternal cortisol to cortisone (Benediktsson et al 1995), thus protecting the fetus from exogenous cortisol. Evidence from animal studies in which 11β hydroxysteroid dehydrogenase type 2 is inhibited (Lindsay et al 1996) or by-passed by administration of exogenous

dexamethasone (Nyirenda et al 1998), which is not metabolised by the enzyme, suggest glucocorticoids have a potent effect in reducing the size of the fetus at birth. Furthermore, both these studies showed that in adult life, rats exposed to elevated levels of glucocorticoid *in utero* went on to develop higher fasting glucose levels in adult life. In the human, intrauterine growth retardation has been found in association with reduced placental 11β-HSD activity (Shams et al 1998) suggesting the enzyme may play an important role in determining fetal growth.

These abnormalities have been given clinical import by a series of studies from Barker. The original observation of an epidemiological cohort showed that subjects with low birth weight were more likely to develop coronary heart disease (Barker et al 1989) and a number of cardiovascular risk factors including type 2 diabetes (Hales et al 1991), hypertension and hyperlipidaemia (Barker et al 1993) in adult life. Furthermore, the effect has been found in a number of studies in different countries (Leon et al 1998, Rich-Edwards et al 1976) suggesting it may be an important contributory cause of cardiovascular disease.

Taken together, the observations that *in utero* exposure to excess glucocorticoid results in low birth weight in animal models and humans and impaired glucose tolerance in adult life in animal models are similar to the observations of adult outcome in low birth weight babies. This suggests that excess glucocorticoid exposure in fetal life (through increased fetal production, increased receptor sensitivity or reduced placental 11β-HSD type 2 activity) is a plausible mechanism to explain the above epidemiological observations.

1.5 The Mechanism of Action of Glucocorticoids

Glucocorticoids, in common with other steroid hormones, act through a nuclear receptor that becomes activated on binding ligand, forming a DNA binding form which binds to specific recognition sequences in the genome and through interaction with the transcription apparatus, results in the selective induction or repression of protein synthesis.

1.5.1 The Glucocorticoid Receptor

The glucocorticoid receptor is expressed in most human tissues, in varying concentrations, and has widespread and profound effects on the function of almost all cell types. It plays a role in development: animals lacking the gene have defective lung development and absent adrenal medullae (Schmid et al 1995). It is a member of the superfamily of nuclear receptors that includes receptors for steroids, thyroid hormone, retinoids, vitamin D and a number of other "orphan" receptors for which no ligand has yet been identified. The glucocorticoid receptor has close structural and functional homology with other members of the family (Evans 1988).

In the absence of ligand, the glucocorticoid receptor exists in a complex with heat shock proteins, HSPs (Pratt 1993) in the cytosol. This is in contrast to most other nuclear receptors including the oestrogen, androgen and thyroid hormone receptors which are predominantly nuclear. Following ligand binding, the glucocorticoid receptor translocates to the nucleus binding to specific sequences, glucocorticoid response elements (GREs), in the promoter of glucocorticoid-regulated genes. Interaction of the receptor-DNA complex with transcription factors and other accessory proteins results in increased or suppressed gene expression.

The glucocorticoid receptor is expressed as 2 forms with distinct activities. The α-form of the receptor (Hollenberg et al 1985) is the most extensively studied with ligand-binding and transactivation activities: further discussion here focuses on this form of the receptor. The β-form of the receptor differs only in the C-terminus, where the last 50 amino acids of the α-form are replaced by 15 amino acids unique to the β-form by a differential splicing event (Hollenberg et al 1985, Encio et al 1991). Detailed knowledge of the factors controlling this differential splicing, and the physiological role of the β-receptor remain unclear and this is discussed in a separate section below.

The 94kDa protein of the glucocorticoid receptor is modular in structure with several distinct domains with different functions (Giguere et al 1986). The N-terminus region contains a major transactivation domain, the central portion of the receptor contains the DNA-binding domain and dimerisation domains, and the C-terminus contains the ligand binding domain. Each module is functionally independent, so that molecular transplantation of the DNA-binding domain of the glucocorticoid receptor into the oestrogen receptor imparts oestrogen-sensitivity to the regulation of glucocorticoid responsive genes (Green & Chambon 1987). The function of each domain to the glucocorticoid receptor is discussed in greater detail below.

1.5.1.1 Heat Shock Protein Binding

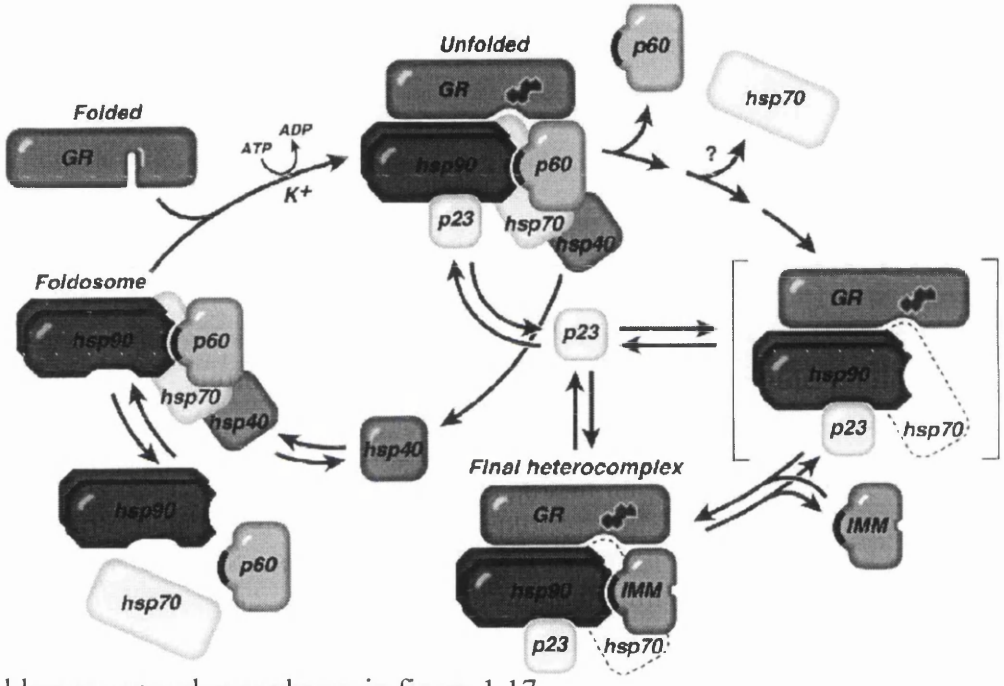
In vitro, the native glucocorticoid receptor protein is unable to bind ligand, and only acquires a high-affinity ligand binding site following binding to a heterocomplex of heat shock proteins (Bresnick et al 1989). Heat shock proteins (HSPs) are an abundant group of proteins that are well conserved throughout evolution and are expressed in increased

amounts following heat and chemical stress. An important function of HSPs is enzymatically to re-fold partially denatured proteins into functional conformations.

Binding of the native glucocorticoid receptor to the heat shock protein heterocomplex (also called a foldosome) imparts ligand binding activity, silences transcription activity and obscures the dimerisation domain of the receptor. The foldosome is composed of several protein components (figure 1.17). The principal component is hsp90. This is an oblong protein that forms a homodimer by antiparallel binding around a domain near the C terminus (Minami et al 1994). This protein has several binding domains including an ATP-binding site, and binding domains for steroid receptors, hsp70, p60, and p23 (reviewed in Toft 1998, Pratt & Dittmar 1998). These binding sites bind hsp70 and p60 (also called Hop; hsp organising protein) to the hsp90 homodimer. An additional heat shock protein, hsp40, binds to hsp70 and Hop to form the foldosome. This then binds the native form of the glucocorticoid receptor converting it to a form with a high affinity ligand binding site. For this structure to be stable, another protein cofactor, p23, is required that binds close to the ATP binding site of hsp90. The mature foldosome-glucocorticoid receptor complex releases hsp40 and Hop. Release of Hop exposes a shared binding site for a group of proteins including the immunophilins FKBP51 (FK506 binding protein of 51kDa – FK506 was an experimental immunosuppressant subsequently marketed as tacrolimus), FKBP52, CyP-40 (Cyclophilin of 40kDa – this is the target for the immunosuppressant cyclosporin) and a serine/threonine protein phosphatase known as PP5. On each foldosome, one of these proteins now binds to the Hop binding site, thus forming a heterogeneous population of foldosome-glucocorticoid receptor complexes. The function of the immunophilin/PP5 subunit is unknown, but it has been suggested this determines intracellular localisation (Czar et al 1995). Under

physiological conditions, the receptor constantly binds to and dissociates from the

Figure 1.17 Formation of the Mature Glucocorticoid Receptor-Foldosome Complex
The foldosome complex is formed from hsp90, p60/Hop, hsp70 and hsp40.
This binds the native glucocorticoid receptor in an ATP/Mg^{2+} and K^{+} dependent manner to generate a steroid binding receptor.
The complex is stabilised by p23.
P60/Hop is released and replaced by an immunophilin (IMM).
Hsp70 may be present in the final complex and is denoted by dashed lines.
Modified from Toft 1998



foldosome complex as shown in figure 1.17.

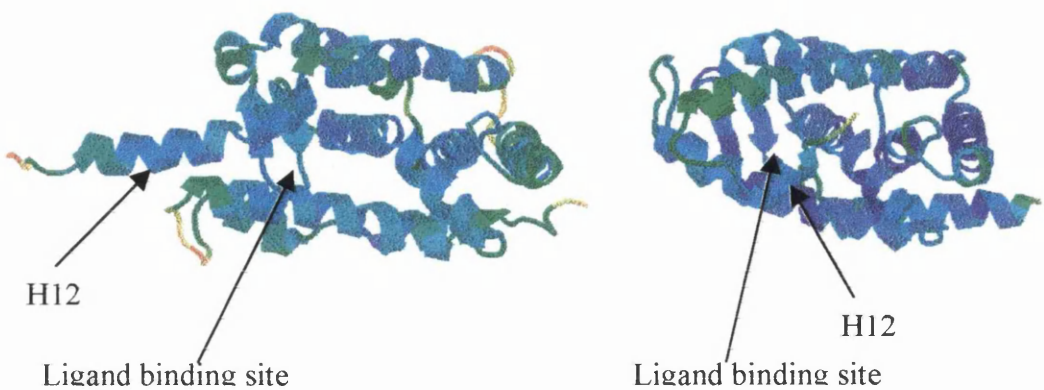
1.4.1.2 Ligand Binding Domain

The endogenous ligand for the glucocorticoid receptor in man is cortisol. Although the detailed crystal structure of the ligand binding domain for this receptor has not yet been resolved, the structures of the retinoic acid-RAR and the apo-RXR receptor have (Bourget et al 1995 and Renaud et al 1995). By comparison with the primary structure of other nuclear receptors, a putative and highly conserved general structure of the

ligand binding domain has been proposed which applies to the glucocorticoid receptor (Wurtz et al 1996). The proposed structure hypothesises that following ligand binding the receptor undergoes major conformational rearrangement. This results in a hydrophobic pocket folding around the ligand, an alpha helix (H12 in the retinoic acid receptor) flipping back to seal the mouth of the pocket thus trapping ligand like a mousetrap: simultaneously this exposes a partial β-sheet and α helix which is proposed to hide the hsp binding domains, while exposing the dimerisation domain and a transactivation surface. The conformational change in the retinoic acid receptor is illustrated in figure1.18

This would explain the dramatic change in the behaviour of the receptor following ligand binding with abolition of hsp-binding activity, and the acquisition of dimerisation activity and transactivation activity. The oestrogen receptor has a similar general structure and proposed mechanism: partial activation of the conformational change, but without proper closure of H12 over the ligand binding pore underlies the activity of the

Figure 1.18 The Unliganded and Liganded Retinoic Acid Receptor
On the left the unliganded RXRα receptor is shown with helix 12 shown in an extended α-helical conformation
On the right, the related RARγ receptor liganded with *all-trans* retinoic acid is shown. Helix 12 has translocated across the mouth of the ligand binding domain. The receptor is now in more tightly packed conformation, with lower thermal energy, shown in darker blue.



receptor modulator, raloxifene (Brzozowski et al 1997). It remains unclear whether the DNA-binding domain becomes exposed during this rearrangement, or whether detachment from the hsp complex and subsequent translocation to the nucleus is sufficient to allow DNA binding.

1.5.1.3 Glucocorticoid Receptor DNA Binding Domain

The glucocorticoid receptor DNA-binding domain has been extensively studied and provides a useful model of the DNA-binding domains of other nuclear receptors. Despite 500 million years of evolutionary divergence (Laudet et al 1992), the domain is extremely well conserved across species and receptor types and the DNA sequence encoding the domain has been used as a probe to find the large number of as yet ligand-less "orphan" receptors.

The DNA-binding domain of the receptor must recognise the specific sequence of the GRE (5'-GGTACANNNTGTTCT) and form a tight bond to DNA. The domain is composed of approximately 90 amino acids in the central portion of the receptor protein. As a purified peptide fragment, the domain folds correctly in solution, binds DNA and increases transcription (Schena & Yamamoto 1988). This has allowed the detailed crystal structure of the ligand-binding-domain-DNA interaction to be defined (Luisi et al 1991) while efforts at the more difficult process of crystallising the intact receptor-DNA complex continue.

Correct folding of the DNA-binding domain requires Zn^{2+} ions as a co-factor, and early studies demonstrated these were bound in a 2 ions:1 protein stoichiometry (Freedman et al 1988). Each Zn^{2+} ion is co-ordinated tetrahedrically by 4 cysteine residues forming the zinc-fingers that hold protein in a more rigid conformation and allow more stable interaction with DNA and neighbouring proteins. Each zinc-finger of the domain has a

different function (Umesono et al 1989). The proximal module, or P-box forms contacts with the phosphate backbone of DNA and specific contacts with the bases of the glucocorticoid response elements and confers the specificity for the GRE sequence. The distal, D-box, is self-complementary and forms contacts with amino acids in the same domain of an adjacent DNA-binding domain. The correct positioning of the D-box relative to the P-box is required for dimerisation to occur and this is aided by the 2 helices packing together around a hydrophobic core formed between the 2 boxes by adjacent phenylalanine residues (Phe_{463}, Phe_{464}). This is further aided by a short region of peptide between the boxes which preferentially forms a short α-helix when the recognition helix binds to DNA. It remains uncertain whether the binding of an activated monomer receptor to DNA then induces the allosteric homodimerisation with another activated receptor, or whether this complex forms in the cytosol and is subsequently transported to the nucleus.

The zinc-fingers of the receptor are crucial features and are highly conserved among nuclear receptors. Although initially described in other transcription factors such as the transcription factor TFIIA of RNA polymerase II (Miller et al 1985) zinc-fingers are generally co-ordinated by 2 histidine and 2 cysteine residues; in contrast 4 cysteine fingers are characteristic of the nuclear receptors. The P-box has an S stereochemical configuration as the protein wraps around the ion, while the D-box has an R configuration. Although the 2 modules are likely to have arisen initially as an intragene duplication event, the fact that the protein wraps around each ion in a different direction suggests that as increasing mutations in amino acids close to one or both of the metal binding sites accumulated the stereochemical preference of one site decreased or became ambivalent. Subsequent mutations in this module then imposed the opposite

stereochemical configuration, and since all nuclear receptors retain this conserved structure, the event is likely to have occurred before the ancestral protein diverged. This sudden change in the structure of a protein which has fundamental developmental and homeostatic consequences is an example of what has been called a "punctuated structural jump" and is thought to be an important factor in defining the erratic rate at which the evolution of species develops: a sudden novel transcription factor allows rapid changes in the way gene expression is directed without requiring large changes in the encoding genome (Luisi et al 1991).

Particular amino acids have been shown to be essential in forming the specificity of the DNA interaction of the P-box. Results from initial work with site-directed mutagenesis, and later confirmed by X-ray crystallography, have shown 3 amino acids, Lys_{461}, Val_{462} and Arg_{466} form direct interactions with the bases of the GRE and are thus responsible for sequence recognition (Luisi et al 1991). Crucial in this recognition process is Val_{462} that interacts with thymine at a position unique to the GRE. The other bases are common to most hormone response elements including the oestrogen-responsive element and substitution of valine by alanine at this position results in a DNA-binding domain which does not discriminate between the response elements of either hormone (Aloy et al 1992). These base-recognition residues are highly conserved with mutations resulting in major defects of hormone action. Additional amino acids interact with the charged phosphate backbone through electrostatic forces or hydrogen bonds. These interactions help anchor the recognition helix in the major groove of the GRE and assist in the deformation of DNA widening the major groove by opening up the distance between the parallel phosphate backbones, thus helping to expose the bases to inspection by the specific recognition residues. Non-conservative substitution of these

amino acids results in loss of DNA binding and loss of hormone-sensitive transcription *in vitro*.

1.5.1.4 Protein Phosphorylation

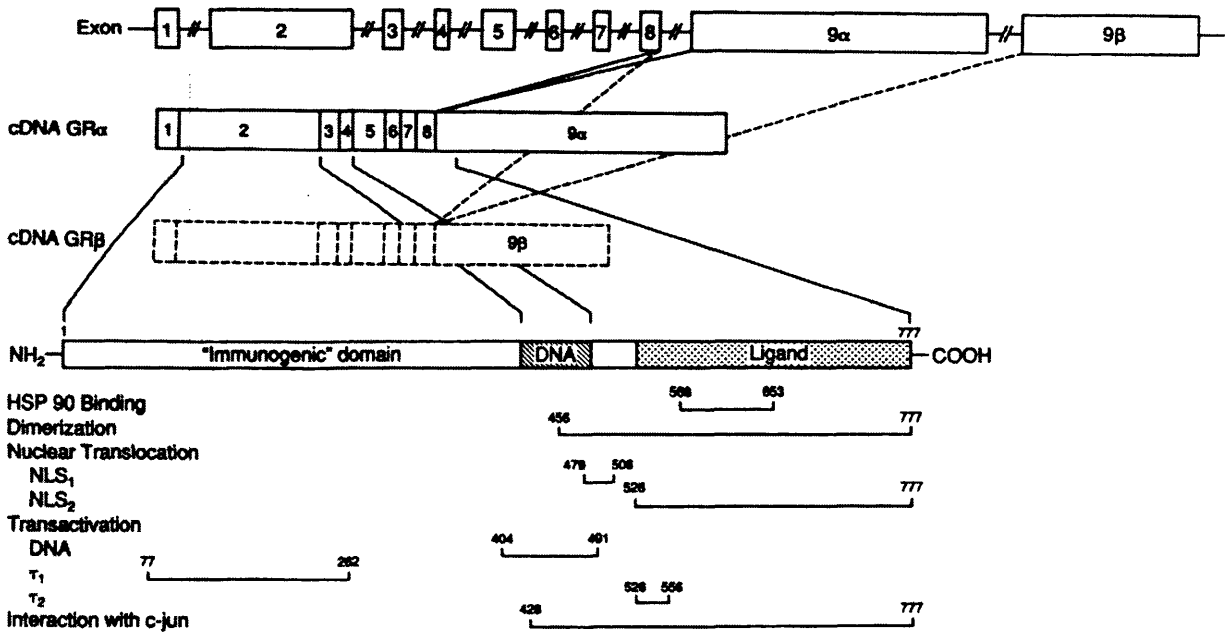
A major mechanism of post-translational modulation of transcription factors is phosphorylation and the glucocorticoid receptor, in common with all other steroid receptors, is capable of being phosphorylated at several sites. The receptor contains 1 threonine and 7 serine residues that have been shown to become phosphorylated (Bodwell et al 1991). The mature receptor is phosphorylated on several serine residues and becomes hyperphosphorylated in response to agonist binding, but not to antagonist binding, suggesting an important functional role (Orti et al 1989). This process appears to occur cyclically with unphosphorylated or modestly phosphorylated receptors becoming more phosphorylated on binding ligand and dissociating from the Hsp complex. On translocation to the nucleus, the receptor binds DNA and may be dephosphorylated before returning to the cytosol again (Orti et al 1992).

The role of phosphorylation in glucocorticoid receptor function remains unclear at present. Manipulation of the phosphorylation state of the receptor by using phosphatase inhibitors (DeFranco et al 1991) has no effect on transactivation activity, although this represents an unphysiological modification. Directed mutagenesis of several potential phosphorylation sites (Webster et al 1997) also does not alter its ability as a transcription factor or its affinity for ligand or for the GRE. However, some evidence suggests that phosphorylation reduces the ability of the receptor to bind to single GREs or partially degenerate GREs and this may explain that phosphorylation reduces the receptors ability to downregulate its own gene product (Webster et al 1997).

1.5.2 Glucocorticoid Receptor Exonic Structure

The glucocorticoid receptor gene was originally cloned in 1985 (Hollenberg et al 1985) and has since been intensively studied as a prototype steroid receptor. It is encoded by a gene on chromosome 5q31 in the human (Theriault et al 1989). It contains a leading promoter, the complete structure of which remains to be determined, 10 exons and 9 intervening introns (Encio et al 1991) as shown in fig 1.19.

Figure 1.19 Structure of the Glucocorticoid Receptor Gene
The structure of the glucocorticoid receptor (GR) gene is shown with a leading promoter sequence followed by the untranslated exon1.
Two receptor isoforms originate from the gene by alternative splicing. Exons 2-8 are common to both, whereas the 9th exons are specific for each isoform, α or β.
The major transactivation domain is encoded by exon 2.
Exons 3 and 4 each encode one of the two zinc fingers.
Exon 5 encodes the nuclear translocation signal sequences, a minor transactivation domain, τ_2, and the beginning of the ligand binding domain.
The remainder of the ligand binding domain is encoded by exons 6-9α.
The protein domains are shown in the legend below.
(Modified from Karl,M. et al 1993)



Exon 1 of the glucocorticoid receptor gene is untranslated and is thought to contain tissue specific enhancer sequences. Messenger RNA for the glucocorticoid receptor shows different 5' sequences suggesting different transcription start sites. In mice, there is evidence for tissue-specific transcription start sites of the GR gene with one promoter activated only in T-lymphocytes (Strahle et al 1992). This may reflect the complement of transcription factors, or the structure of chromatin within tissues. Exon 2 encodes 395 amino acids of the N-terminus of the receptor protein. Exons 3 and 4 separately encode each of the 2 zinc fingers of the receptor protein and are separated by a short 400 base-pair intron suggesting they originated by an intragene duplication event. Exon 5 contains residues involved in transactivation and nuclear localisation of the receptor. Exons 6-8 encode a common region in both the α and β isoforms of the receptor with the C-terminus of the α receptor coded by exon 9α. The β form of the receptor is formed from alternative splicing of exon 9β (Karl et al 1993) but does not bind ligand, and its function remains controversial.

1.5.3 Glucocorticoid Receptor Promoter

The promoter of the glucocorticoid receptor gene is extremely GC rich. Despite the technical problems GC-rich promoters pose for sequencing, clones containing up to 3Kb of the 5' untranslated region of the receptor gene have been characterised (Zong et al 1990). In contrast to the oestrogen and retinoic acid receptors the promoter contains no TATA or CAAT boxes but contains 18 GC boxes (5'-GGGCGG-3'). These are arranged in a complex configuration of 2 pairs of tandem repeats, 1 set of overlapping sites and 8 sites in the reverse orientation. These motifs are the binding site for the Sp1 transcription factor, a factor involved in the basal transcription of housekeeping genes in

many tissues, although other less well characterised transcription factors are also able to bind to these motifs. No consensus GRE sequences are found in the promoter; although 2 half-sites are found at nucleotides –2526 and –2838 these are widely separated and are not associated with degenerate GRE sequences close by and are therefore unlikely to be functional. However, 2 negative GREs similar to the sequences found in the POMC promoter are found at nucleotides –1786 and in the antisense orientation at –1482. These may mediate negative transcriptional control on the gene, but remain to be characterised.

Deletion studies designed to clarify potential regulatory sites within the glucocorticoid receptor gene promoter have shown that some, but not all, of the GC boxes are required for full expression (Nobukuni et al 1995). Using a construct containing 10 GC boxes, it was shown that deletion of the 5' part of the promoter containing 8 of these motifs made no detectable impact on glucocorticoid transcription. Deletion of the 9th motif, however, produced a dramatic fall in promoter activity. This may either reflect the inherent redundancy of multiple GC boxes or may indicate that additional (and as yet uncharacterised) sequences in this segment of the promoter have a vital role in activating the basal transcription machinery.

One consequence of the multiple GC boxes in the promoter is the potential for several different sites for transcription initiation demonstrated by RNAase protection assay (Zong et al 1990). Furthermore, several tissue specific start-sites are found; initiation of transcription at -61bp is specific for T lymphocytes in the mouse (Strahle et al 1992). This suggests the promoter carries tissue-specificity either due to it containing sites for tissue-specific transcription factors or due to tissue-specific concealment of general binding factors by DNA methylation or altered chromatin structure.

In addition to the numerous Sp1 binding sites the promoter contains putative consensus binding sites for a wide range of transcription factors (Nobukuni et al 1995). These include an oestrogen response element (ERE), an activator-protein-1 (AP-1 - the heterodimer of cJun-cFos) binding site, 2 activator-protein-2 (AP-2) binding sites, 2 NF-1 binding sites and one cAMP response element (CRE). Using Dnase I footprinting analysis and gel mobility shift assays to define physiologically relevant sequences, 11 footprints were found in a truncated promoter (-700/+38bp). Although only 7 of these were explored in detail, 6 were found to contain Sp1 binding sites and to bind the factor. Three of the footprints also bound unidentified proteins, but one, footprint 7, contained 2 AP-2 binding sites that bound active AP-2 as demonstrated by shift and supershift analysis. Furthermore, the degree of promoter activity conferred by the AP-2 binding site was found to show tissue-specificity with relatively higher activity in HeLa cell lines (a cell with high endogenous expression of AP-2) than in fibroblast, kidney or liver cell lines.

1.5.4 The β Isoform of the Glucocorticoid Receptor

The glucocorticoid receptor gene encodes 2 distinct proteins, the α and β isoforms of the glucocorticoid receptor, by alternative splicing (Hollenberg et al 1985). The most intensively studied form is the α form that binds ligand and regulates gene expression and is described in detail above. The β form of the receptor remains poorly understood. It is unable to bind glucocorticoid ligand (Hollenberg et al 1985) and appears to be transcriptionally silent. It is widely expressed in brain, hypothalamus, pituitary, thymus, bone marrow, spleen, liver, kidney, lung, fat and muscle, but was not clearly identified in leukocytes (Bamberger et al 1995). At an ultrastructural level, the protein is found in

the nucleus of cells independently of glucocorticoid treatment (Oakley et al 1997) and binds to GREs.

In transient transfection studies, the β isoform of the receptor has been shown to inhibit glucocorticoid-mediated transcription by the α form (Bamberger et al 1995) although the mechanism remains unclear but may involve the formation of non-transactivating α/β heterodimers or competition for a glucocorticoid receptor coactivator.

1.5.5 Transcriptional Control of Glucocorticoid-Responsive Genes

In eukaryotes, all genes are transcribed by RNA polymerase II (Conaway et al 1993) and this is the ultimate target for glucocorticoid-modulation of gene transcription. In a TATA-box (5'-TATAA-3') containing promoter, activation of RNA polymerase II (RNApol) begins by the assembly of a pre-initiation complex composed of RNApol and a number of accessory factors. These are named TFII A to H (the TF stands for transcription factor and the II denotes it is associated with RNA polymerase II). The first factor to bind to the promoter is the TATA-box binding transcription factor TFIID. This complex is stabilised by the binding of TFIIA to TFIID and this complex is recognised and bound to by TFIIB (Maldonado et al 1990). The DAB-TATA complex is critical for the binding of RNApol that binds via a TFIIF factor to the DAB-TATA complex. The complex DAB-RNApol-F is now recognised by TFIIE and TFIIH (Flores et al 1992) and these factors bind to form a mature complex that, in the presence of ribonucleoside triphosphates, directs low level RNA synthesis (Goodrich et al 1994).

As all genes are transcribed by the RNA polymerase II complex, any effect of the glucocorticoid receptor (and indeed all other transcription factors) on gene transcription will be mediated by modulation of the activity of this complex.

The most obvious mechanism would be direct interaction with the polymerase itself or any of the TFII factors to increase or decrease complex assembly. Work on purified in vitro systems show that the glucocorticoid receptor interacts with TFIID through its τ_1 transactivation domain (Ford et al 1997) and similar interactions have been found for the oestrogen receptor with TFIID (Sadovsky et al 1995). Direct interactions between TFIIB and the oestrogen receptor have been demonstrated (Ing et al 1992), but as yet no other TFII factors have been shown to interact directly with the glucocorticoid receptor.

Another mechanism would be indirect interaction between the glucocorticoid receptor and the transcription machinery through a bridging protein. Evidence that this might be an important mechanism initially came from transcriptional interference (or "squelching") studies (Meyer et al 1989). In these experiments a system that expresses a gene in response to one transcription factor is inhibited when another transcription factor is over expressed and is thought to occur through competition between the two transcription factors for a shared bridging protein. Thus the oestrogen receptor interferes with glucocorticoid receptor signalling, and conversely the glucocorticoid receptor interferes with oestrogen receptor signalling (Meyer et al 1989). Similar interactions have since been found between most members of the steroid receptor family.

Direct evidence for the existence of these bridging proteins, or coactivators, has come initially from yeast studies, where candidate proteins can be produced and purified in large scales, with later cloning of homologous genes in mammals. Using this approach, a coactivator of the glucocorticoid receptor has been identified (Hong et al 1996). This protein, known as GRIP1 (glucocorticoid receptor interacting protein 1) binds to the glucocorticoid receptor through its τ_2 domain, and also interacts with all other steroid

receptors and the RAR and RXR retinoic acid receptors, the thyroid hormone receptor and vitamin D receptor. Several other coactivators, including SRC-1 (steroid receptor coactivator), TIF1 (thyroid receptor interacting factor) and RIP 140 (receptor interacting protein 140kDa), have since been identified. What remains to be worked out is whether these coactivators bind to the basal transcription apparatus, and how they increase transcription.

Another potential mechanism to modulate transcription is to control how easily the promoter can be read by the transcription apparatus. Normally, DNA exists in a complex coiled-coiled-coiled double helix structure supported by histones and accessory proteins and known collectively as chromatin. This higher structure hides the promoter from solution making it inaccessible to RNA polymerase and any transcription factors. Recently, enzymes able to open or close this chromatin structure have been identified. Histone acetyl transferase (Grunstein 1997) acetylates basic lysine residues on the histone protein causing it to unwind from DNA and releasing the sequence partially into solution. The corollary enzyme is histone deacetylase (Wolffe 1997) that allows histones to re-wrap DNA into compact coils. Two related proteins, CBP (cyclic AMP response element binding protein binding protein) and p300 have been shown to stimulate histone acetylation activity. These proteins are stimulated by a number of signal pathways. One of these is AP-1 (activator protein 1) a heterodimer of c-jun and c-fos transcription factors, controls expression of the inflammatory mediator collagenase I; another is NF-κB (nuclear factor κB) that controls cytokine expression. The glucocorticoid receptor antagonises the effects of AP-1 and NF-κB possibly by

stimulating histone deacetylase activity (Barnes 1998), although this remains controversial.

Finally, modulation of transcription could be achieved by the glucocorticoid receptor interacting with any other transcription factor either increasing or decreasing its effect on the RNA polymerase complex. Such direct interactions have been found between AP-1 and the glucocorticoid receptor (Yang Yen et al 1990) where the glucocorticoid receptor binds the c-jun subunit of AP-1 in the cytosol preventing it from binding to DNA and increasing transcription. A similar protein-protein interaction has been found between the glucocorticoid receptor and NF-κB (Ray et al 1994) reducing induction of cytokines. An additional anti-inflammatory mechanism whereby the glucocorticoid receptor inhibits NF-κB has recently been proposed. In lymphoid cell lines activation of the glucocorticoid receptor induces a protein known as I-κB that diffuses to the cytosol, binds to NF-kB and forms a transcriptionally silent complex (Auphan et al 1995). The importance of these direct protein-protein interactions in glucocorticoid signalling has recently been underscored by the description of a knock-in mouse with a defect in the D zinc finger preventing cooperative dimerisation to GREs (Reichardt et al 1998). Although unable to bind to classical GRE motifs in promoters, these mice are morphologically and physiologically normal although unable to induce gluconeogenic enzymes in response to hypoglycaemia or stress. This suggests that protein-protein interactions with other transcription factors, without the need for DNA binding, may be the major mechanism of glucocorticoid signalling in vivo.

Thus the glucocorticoid receptor may signal to the transcription apparatus through several mechanisms including direct interaction with the basal transcription machinery,

interaction through coactivators, interaction with chromatin structure or by protein-protein interaction with other transcription factors.

1.6 Polymorphisms of the Glucocorticoid Receptor Gene

The original description of familial glucocorticoid resistance (Vingerhoeds et al 1976) described a syndrome with elevated ACTH, mineralocorticoids, androgens and cortisol with no stigmata of cortisol excess, but with hypertension, and in female subjects, hirsutism and infertility. The pathophysiology of the condition is believed to result from global resistance to glucocorticoids (Chrousos et al 1993). In the hippocampus, hypothalamus and pituitary, reduced glucocorticoid sensitivity will increase ACTH secretion from the pituitary. This stimulates the adrenals to synthesise increased concentrations of cortisol, but also deoxycorticosterone, corticosterone and the adrenal androgens DHEA and androstenedione. The elevated concentrations of deoxycorticosterone and corticosterone saturate 11-β hydroxysteroid dehydrogenase type 2 in the nephron causing sodium retention and hypertension. The adrenal androgens produce hirsutism in women, and by inhibiting gonadotrophin releasing hormone and luteinising hormone, disrupt the normal cycling of gonadotrophins and result in infertility.

Subsequently a defect in the ligand binding domain of the glucocorticoid receptor gene was demonstrated in one kindred (Hurley et al 1991). This aspartate to valine substitution at codon 641 (abbreviated as $Asp_{641}Val$ and due to a T to A substitution at nucleotide 2054 of the cDNA, abbreviated as $T_{2054}A$) resulted in reduced affinity for glucocorticoid binding and greatly reduced transactivation of a glucocorticoid responsive reporter construct in transfection experiments.

Several further glucocorticoid receptor mutations have since been described with varying molecular and pathological phenotypes. Karl described a $A_{1220}G$ ($Asn_{363}Ser$) mutation downstream of the transactivation 1 (τ_1) domain in the cDNA of the receptor gene (Karl et al 1993). Transfection experiments showed this cDNA produced a functional receptor. Subsequent analysis of the genomic receptor gene, however, showed a 4 base pair deletion at the splice site between exon 6 and the following intron that resulted in the A to G substitution in the cDNA, and in a rapidly degraded transcript with no expressed receptor protein. Malchoff described a $G_{2317}A$ ($Val_{729}Ile$) mutation in the ligand binding domain that resulted in reduced affinity for ligand binding (Malchoff et al 1990). Karl has also described a $T_{1808}A$ ($Ile_{559}Asn$) mutation (Karl et al 1996). This produces a receptor with absent ligand binding and transactivation activity that also reduced the ability of the wild-type receptor to transactivate a glucocorticoid responsive reporter construct in transfection experiments. In addition to glucocorticoid resistance, the propositus with this mutation subsequently developed Cushing's disease possibly due to prolonged ACTH stimulation of the pituitary.

A further polymorphism, a restriction fragment length polymorphism (RFLP), associated with the glucocorticoid receptor gene has been described (Weaver et al 1992). On digestion of genomic DNA with the restriction enzyme Bcl I, southern blotting detects 2 alleles of 2.3 and 4.5 kilobases, with relative frequencies of 0.45 and 0.55, when probed with the OB7 incomplete cDNA of the glucocorticoid receptor (described by Hollenberg et al 1985). The position of the polymorphism remains unknown, but given the size of the fragments and the lack of polymorphic RFLPs for Bcl I in the cDNA of the gene, it is likely to lie within intronic or promoter sequence.

Several studies have shown an association between this Bcl I RFLP and obesity, hyperinsulinaemia and hypertension. In a study of obese women, subjects homozygous for the larger fragment (AA) had significantly higher fasting and glucose-clamped insulin concentrations (Weaver et al 1992). In a separate study, subjects homozygous for the larger allele (AA) were more likely to have familial higher blood pressure than the mean (Watt et al 1992), although this difference was not statistically significant. In a further study, AA homozygotes had greater sensitivity to vasoconstriction to budesonide applied topically to skin (Panarelli et al 1998). Of note was the observation in this study that urinary corticosteroid metabolites did not differ between groups of contrasting Bcl I genotypes. In contrast to generalised glucocorticoid resistance, therefore, where increased ACTH drive to the adrenal increases the synthesis of a number of corticosteroids, those subjects with increased skin sensitivity did not have reduced rates of corticosteroid production suggesting that any difference in glucocorticoid sensitivity may be restricted to particular tissue types. What remains to be determined is firstly, whether the Bcl I RFLP alters the number of glucocorticoid receptors in tissues, and secondly whether any change is tissue specific.

1.7 Glucocorticoid Excess

When circulating glucocorticoid levels are elevated, the lipid-soluble hormone has free access to all tissues. This increases glucocorticoid receptor activation, alters the balance of gene transcription and has profound effects on every tissue and physiological system. The symptoms and signs resulting from this are collectively known as Cushing's syndrome.

The features of Cushing's syndrome are listed below in table 1.1 and include effects on carbohydrate, lipid and protein metabolism, disturbed immune function and altered neurochemical function.

Table 1.1 Symptoms and Signs of Cortisol Excess

Symptoms	% Prevalence
Weight gain	91
Menstrual irregularity	84
Hirsutism	81
Psychiatric	62
Backache	43
Muscle weakness	29
Fractures	19
Loss of scalp hair	13
Signs	
Obesity	97
Truncal	46
Generalised	55
Plethora	94
Moonface	88
Hypertension	74
Bruising	62
Striae	56
Muscle weakness	56
Ankle oedema	50
Pigmentation	4
Other findings	
Hypertension	74
Diabetes	50
Overt	13
Glucose intolerance	37
Osteoporosis	50
Renal calculi	15

from Ross and Linch 1982

Carbohydrate metabolism

Cortisol excess decreases carbohydrate tolerance and often results in the development of diabetes. The main mechanisms underlying this are increased hepatic glucose production and reduced peripheral tissue utilisation.

In isolated hepatocytes, intact animals and in man, glucocorticoids increase the activity of most of the key enzymes of gluconeogenesis with the greatest effects on the rate-

limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. Most of these effects appear to be due to the increased transcription of mRNA for the respective enzymes due to a glucocorticoid-dependent mechanism. The promoter of PEPCK has an extraordinarily complex enhancer 5' sequence with regions responsive to the dietary, metabolic and hormonal state of the animal (Hanson et al 1997). In particular, it contains sequences that reduce transcription in response to carbohydrate feeding, hypoxia and insulin, while distinct sequences enhance transcription in response to glucagon, through a cAMP-dependent process, glucocorticoids and thyroid hormone. The glucocorticoid-responsive unit (GRU) of the promoter does not contain classical consensus GRE sequences. Instead, activated glucocorticoid receptors bind to 2 relatively weak binding sites in the promoter and through interaction with a number of additional proteins including accessory factors 1 and 2 (AF1 and AF2) stimulate transcription. Many of these additional proteins appear to be tissue-specific transcription factors and transcription is under the control of these proteins rather than the glucocorticoid receptor acting alone, thus in adipose tissue where PEPCK is involved in glyceroneogenesis for the re-esterification of free-fatty acids to triglycerides, glucocorticoids inhibit PEPCK transcription.

A final effect glucococorticoids exert on the PEPCK gene is in imprinting. Elevated fetal glucocorticoid levels have been shown, in rats, permanently to increase the expression of PEPCK in liver, thus programming the liver to increase gluconeogenesis in the adult (Nyirenda et al 1998). The mechanism underlying this effect remains unknown but may involve changes in the methylation state of the 5' region of the gene and altered chromatin condensation, both of which are noted to vary in circumstances affecting gene transcription. Thus, subtle patterns of condensation/decondensation may

permanently alter the sequences capable of interacting with transcription factors and through changes in the methylation state pass this effect to all cells derived from the same lineage in the adult.

In conjunction with increased hepatic glucose production, peripheral insulin sensitivity is reduced particularly in skeletal muscle and adipose tissue. Insulin receptors in most tissues appear to be normal in number and their affinity for insulin, but a post-receptor binding defect results in reduced efficiency of receptor-effect coupling. This is due mainly to reduced translocation of the glucose transporter, GLUT4, from cytosol to cell membrane (Weinstein et al 1998) through a poorly understood glucocorticoid-dependent process. The net result is reduced glucose uptake in response to insulin with a compensatory increase in circulating glucose and insulin secretion.

Lipid Metabolism

Much of the weight gain in glucocorticoid excess is due to deposition of adipose tissue. This is often in a characteristic distribution around the face ("moon-face"), over the nape of the neck ("buffalo hump"), intra-abdominally and in less clinically evident sites such as the epidural space, mediastinum, and retro-orbital space. This is due to increased fat deposition and increased cellularity of adipose tissue.

Glucocorticoid excess increases lipoprotein lipase activity in adipose tissue (Ottosson et al 1994), increasing the uptake of lipid from LDL, and inhibits hormone-sensitive lipase (Samra et al 1998) thus reducing the release of free fatty acids. Together these effects promote net fat accumulation in adipose tissue. Both of these effects are thought to be due to altered expression of the respective genes by a glucocorticoid receptor-dependent mechanism. Increased cellularity of adipose tissue is also found and this further increases the capacity of adipose tissue to accumulate lipid. This effect is partly due to

glucocorticoid induction of the PPARγ2 (peroxisome proliferator activated receptor). This receptor, the endogenous ligand for which has recently been found to be a novel prostaglandin – prostaglandin J, is an adipose-specific gene product that induces the differentiation of pre-adipocytes to mature adipocytes (Vidal-Puig et al 1997).

Of special note is the differential effect glucocorticoids have on various adipose depots. Compared to subcutaneous adipose tissue, the visceral depot is increased more and this may be due to the combination of increased glucocorticoid receptor number, increased 11βHSD type 1 activity (Bujalska et al 1997), increased PPARγ2 receptor and increased local insulin concentrations. This effect of glucocorticoids on visceral fat accumulation is important and has been shown repeatedly to be a potent risk factor for cardiovascular disease, endothelial dysfunction, hypertension, dyslipidaemia, atheroma and diabetes (Carey 1998).

Hepatic triglyceride, cholesterol and lipoprotein production is increased in glucocorticoid excess and this results in increased circulating levels of VLDL, LDL and HDL particles (Brindley et al 1995). When hepatic capacity to produce apo-lipoproteins is exceeded, lipid accumulates within the cell to produce fatty liver disease. Furthermore, glucocorticoids reduce cholesterol clearance in the liver increasing circulating levels further.

In muscle, glucocorticoids promote lipolysis and the liberation of free fatty acids, possibly through their effect to reduce glucose disposal in this tissue, and probably also due to a permissive effect on catecholamine and glucagon action. The overall effect is an increase in free fatty acid and ketone body availability, which is processed in the liver to lipoproteins and to fuel gluconeogenesis.

Protein Metabolism

In peripheral tissues glucocorticoids produce net protein catabolism due to reduced protein formation in adipose tissue, skin, lymphoid tissue and bone and increased proteolysis particularly in muscle, adipose tissue, and lymphoid tissues with free amino acids becoming metabolised to glucose by gluconeogenesis in the liver (Umpleby et al 1996). In muscle the main effect of glucocorticoids appears to be increased protein breakdown (Simmons et al 1984). An additional effect of glucocorticoids is on the growth hormone axis with reduced sensitivity of the pituitary to GHRH resulting in lower growth hormone levels and reduced protein synthesis (Dieguez et al 1996).

In liver, overall protein synthesis is increased by glucocorticoids and much of this effect is due to induction of the enzymes required for gluconeogenesis and lipoprotein formation.

Immune Function

Glucocorticoids produce profound immunosuppression and increase the risk of systemic infection. This effect extends to all the principal cells of the immune system including B lymphocytes resulting in reduced antibody production, T lymphocytes, natural killer cells, monocytes, and eosinophils. Numerous defects in immune modulation have been observed in glucocorticoid excess and it seems likely that to differing degrees and in different circumstances, all make contributions. Glucocorticoids are able to induce expression of an anti-inflammatory glycoprotein, lipocortin-1 (Flower et al 1994), which inhibits the enzyme phospholipase A2, responsible for the generation of prostaglandin and leukotriene mediators. Glucocorticoids have numerous effects on cytokine production including suppressed synthesis of interleukin 1 and 2, interferon γ

and tumour necrosis factor α (Paliogianni et al 1993). This leads to reduced responsiveness to infection and a blunted inflammatory response by B lymphocytes, and T helper and T suppresser lymphocytes. In addition, the production of cytotoxic substances including perforin and granzyme A are reduced in natural killer cells (Zhou et al 1997). A general effect of glucocorticoids is increased apoptosis in lymphocytes and monocytes by poorly understood mechanisms which are, at least in part, due to increased sensitivity to the p53-dependent apoptosis pathway (Owens et al 1991). In contrast, neutrophils have greatly decreased rates of apoptosis in glucocorticoid excess (Cox 1995) and this appears to be the main mechanism underlying the polymorph leukocytosis seen in Cushing's syndrome, and exogenous glucocorticoid treatment.

Leukocyte movement to different tissue compartments is altered by glucocorticoid treatment. Increased glucocorticoid concentrations cause lymphocytes, monocytes and eosinophils to redistribute to bone marrow, spleen, lymph nodes and thoracic duct causing a relative circulating depletion of these cells. In contrast, neutrophils rise in response to glucocorticoid excess due to increased production and release from bone marrow and reduced cell destruction by apoptosis. In addition, through inhibitory effects on chemoattractant factors, the numbers of cells recruited to sites of tissue damage, inflammation or infection is greatly reduced by glucocorticoids.

Neurochemical Function

Psychiatric illness is commonly found in chronic glucocorticoid excess. The commonest illness is depression in around 65% of patients, although euphoria, psychosis and panic disorder are also common findings. Numerous neurochemical disturbances have been described in the brains of subjects suffering from hypercortisolism, the commonest

including increased catecholaminergic activity in the locus caeruleous, and reduced 5-hydroxytryptamine (5-HT) activity in the hippocampus.

The main effects of glucocorticoids appear to be mediated through the mineralocorticoid receptor in the hippocampus, reducing production of 5-HT and reducing expression of 5-HT_{1A} (Joels et al 1991) and 5-HT_{2C} receptors (Holmes et al 1997). The overall reduction in 5-HT activity may be the cause of many of the psychiatric symptoms of glucocorticoid excess. Reduced 5-HT_{1A} receptor levels have been found to be associated with depression and an important effect of antidepressants is to restore levels of these receptors towards normal.

As discussed earlier, these 5-HT pathways also have important roles in the regulation of glucocorticoid production by controlling CRH and ACTH release.

Skeletal System

Glucocorticoids have complex actions on bone that lead to net loss of calcified tissue. The principal mechanisms include reduced intestinal calcium absorption, increased renal calcium excretion, consequent secondary hyperparathyroidism and activation of osteoclast activity (Ziegler et al 1998). Glucocorticoids produce relative hypogonadotrophic hypogonadism by inhibiting gonadotrophin releasing hormone thereby reducing osteoblast production of bone matrix (Reid 1997). Together, these effects produce accelerated bone loss especially in postmenopausal women and older men (Mitchell et al 1990), and greater acute loss of bone is found at the hip than in the spine when measured by dual energy X-ray absorption scanning (Saag et al 1998).

Haemodynamic Function

The majority of patients with glucocorticoid excess have clinically evident hypertension. Glucocorticoids alter cardiac output, circulating volume and peripheral

resistance and could alter blood pressure through any single mechanism, or combinations of these (Whitworth et al 1997).

Cardiac output is increased by glucocorticoids (Mantero et al 1992), although the mechanism is poorly understood. Part of the effect may be due to increased cardiac contractile protein synthesis with an accompanying increase in stroke volume (Clark et al 1986).

Circulating volume increases due to renal sodium retention. This effect is not due to mineralocorticoid activation as renin is rarely suppressed. Furthermore, spironolactone does not reduce blood pressure although the glucocorticoid/progesterone antagonist mifepristone (RU486), does (Mantero et al 1992). As a caveat, however, it is important to acknowledge that although short term administration of cortisol increases renal sodium retention, the effects of chronic cortisol excess may be quite different. Studies in subjects with Cushing's syndrome (and by definition chronic cortisol excess) had normal total body sodium and normal renin-angiotensin systems (Ritchie et al 1990).

Vascular tone is increased in glucocorticoid excess and the reactivity to a number of vasoconstrictors is enhanced including angiotensin II and noradrenaline (Whitworth et al 1986). Systemic vascular resistance is increased, and may contribute to hypertension. Thus, although many mechanisms contribute to glucocorticoid-induced hypertension the relative importance of each remains incompletely resolved.

1.8 Cardiovascular Disease

Cardiovascular disease remains the greatest source of morbidity and mortality in the developed world accounting for 30-50% of all deaths (Murray et al 1997). The epidemic of myocardial ischaemia, infarction and stroke (both ischaemic and haemorrhagic) is estimated to cost around $90 billion per year in the United States alone (Peyser 1997).

As the world becomes more urbanised, cardiovascular disease becomes more common. Countries with no significant incidence of cardiovascular disease in the past 30 years now contribute more to the global cardiovascular burden than all developed countries (Pearson et al 1996). Even as the incidence of stroke, and to a lesser extent myocardial infarction, declines in the West, cardiovascular disease is set to become one of the main health issues world-wide in the next millennium.

The main focus of this thesis is the glucocorticoid receptor and its possible role in determining cardiovascular disease. To consider how glucocorticoids may contribute to cardiovascular disease it is necessary to discuss briefly the development of cardiovascular disease and the mechanisms thought to control these processes.

1.8.1 Pathogenesis

The principal cause of cardiovascular disease is atherosclerosis (Ross 1993). In this condition an atheromatous plaque occludes, partially or wholly, an artery resulting in tissue ischaemia or infarction. Although uncomplicated atheroma may occlude the arterial lumen, more commonly the complications of haemorrhage into the soft core of the plaque or thrombosis formation on the ulcerated surface of a plaque are responsible for disease.

1.8.2 Atheroma

1.8.2.1 Structure of a Normal Artery

Large and medium-sized arteries are composed of several discernible layers; the intima, media and adventitia (figure 1.20).

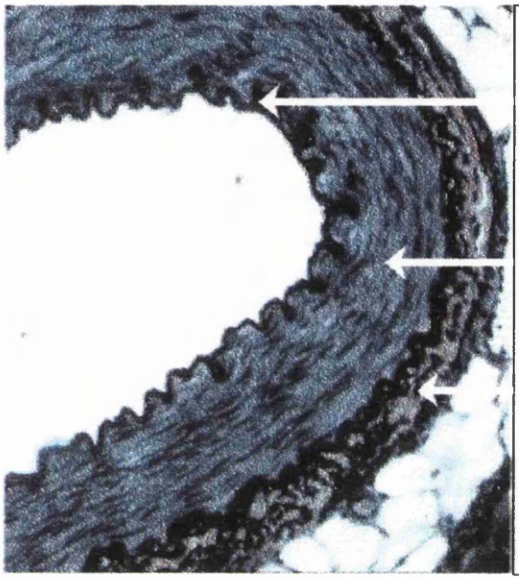


Figure 1.20 Section of Descending Aorta

Intima
The thin layer of endothelial cells is seen in blue, overlying the relatively acellular internal elastic lamina. Normally no smooth muscle cells are present.

Media
The media in this large vessel contains large amounts of matrix and smooth muscle cells

Adventitia
The looser connective tissue of the adventitia surrounds the media, and is separated from it by the external elastic lamina

The intima is composed of endothelial cells joined by tight junctions and overlying an internal elastic lamina. The endothelial layer imposes a physical barrier controlling the passage of macromolecules and cells between the lumen and vessel wall. In addition endothelial cells secrete vasoactive substances which promote vascular dilatation (principally nitric oxide, NO, and prostacyclin, PGI_2) or constriction (mainly endothelin-1), cytokines and adhesion molecules to direct the passage of immune cells, and regulators of the thrombotic pathway (heparin, prostacyclin).

The normal media contains only vascular smooth muscle cells and a matrix of collagen, elastic fibres and glycosaminoglycans. Elastin fibres are arranged to form layers of fenestrated elastic lamina and are more abundant in the capacitance vessels such as the

thoracic aorta. Smooth muscle cells are arranged circularly to the vessel lumen and become more common in the abdominal aorta and medium sized arteries. In health vascular smooth muscle cells display a predominantly contractile phenotype responding to a wide range of vasodilators and vasoconstrictors. Very low rates of secretory activity maintain the normal tissue matrix composition.

At the outer limit of the media is the external elastic lamina that separates the media from the outer adventitia. This layer is composed of connective tissue rich in collagen fibrils that impart high tensile strength to the vessel wall preventing rupture. Contained within the adventitia is a plexus of vessels, the *vasa vasorum*, which provide the blood supply to the adventitia and the media.

1.8.2.2 Development of Atheroma

Atheroma is a process of accumulation of lipid, cells and fibrous tissue within the intima and media of medium and large arteries. Long thought to be a relatively inert lipid mush (Greek root ατηερε describes soft porridge) it is now clear atheroma is a highly organised and dynamic disease process. Progression from normal arterial wall through the earliest lesion of the fatty streak to mature atheromatous plaque is the result of co-ordinated communication between a number of cell types by a variety of mediators (Figure 1.21).

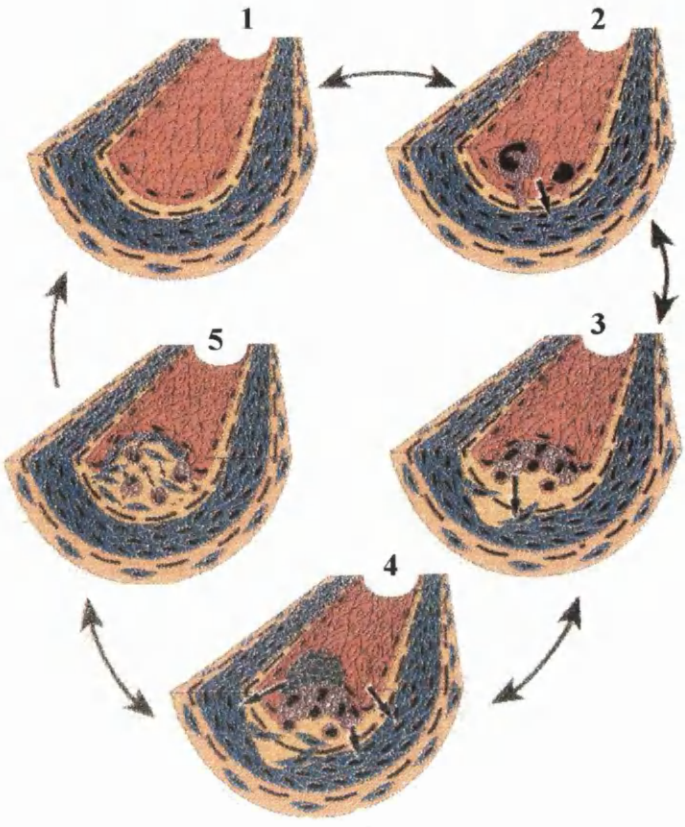


Figure 1.21 Atheromatous Plaque Development

Stages in the development of an atheromatous plaque are shown beginning with a normal artery (1). Endothelial damage results in an adherent surface to which monocytes adhere and insinuate (2). With increasing damage more cells are recruited and imbibe lipid to form a fatty streak (3). Proliferation and lipid uptake by vascular smooth muscle cells follows (4). Complex inflammatory signals are exchanged between cells within the plaque to maintain, increase or reduce the size of the plaque (5).

It is believed that most of the changes in atheroma development are reversible and may be constantly in a state of flux, illustrated by the double-headed arrows from each stage to the next.

(Modified from Ross 1993)

1.8.2.3 The Fatty Streak

The earliest lesion of atheroma is the fatty streak (Faggiotto et al 1984). Commonly found in normal subjects from the second decade onwards these are fatty deposits in the intima of medium and large arteries (Stary 1989). Histologically these lesions are composed of lipid-laden macrophages, known as foam-cells, and T lymphocytes.

Early studies in the animal model of the cholesterol-fed rabbit showed patches of endothelium with adherent leukocytes forming before, and at the same sites, as fatty streaks (Walker et al 1986). The function of the endothelium appeared to be altered resulting in the development of fatty streak, and ultimately atheroma.

A number of injuries are able to increase cell adhesion to endothelium including the mechanical stress of hypertension, toxins from tobacco smoke, elevated homocysteine concentrations and a number of viral and immunological insults. Recently, however, the central role of oxidised low density lipoprotein (oxLDL) in endothelial injury and atherogenesis has become apparent (Yl[redacted]-Herttuala 1999). LDL becoming oxidised in the circulation, or as it passes through the endothelial cell by NO, induces the endothelial cell to express adhesion molecules such as vascular cellular adhesion molecule-1 (VCAM-1). A number of mediators are responsible for this response including lipid peroxides, lysophophatidylcholine, tumour necrosis factor α (TNFα), and interleukin-1 (IL-1). Adhesion molecules act as recognition sites and anchors for circulating monocytes and T lymphocytes. These attach themselves to the endothelial surface and develop surface projections, insinuating them between the endothelial cell junctions allowing the leukocytes to pass into the intima. Here monocytes change their phenotype to macrophages and through a scavenger receptor and a recently cloned oxLDL receptor (Sawamura et al 1997) expressed in endothelial and vascular smooth

muscle cells, begin to endocytose oxLDL. As the macrophages accumulate oxLDL the cytosol becomes stuffed with lipid droplets producing the highly refractile foam cells seen on light microscopy. Initially macrophages are able to export this accumulated lipid by packaging it with apoE lipoprotein forming high density lipoprotein (HDL) which then enters the circulation for peripheral metabolism.

The activated macrophages in the fatty streak secrete cytokines and growth factors that promote plaque enlargement (Ross 1993). Monocyte chemoattractant protein-1 (MCP-1), and granulocyte/monocyte-colony stimulating factor (GM-CSF) promote further cell recruitment to the lesion and maintain cell growth (preventing apoptosis and the discharge of lipid into the intercellular matrix) respectively. The generation of further oxLDL and the elaboration of factors such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TNFα and IL-1 promote further endothelial dysfunction. This results not only in prolonged adhesion molecule expression but also reduced vasodilator generation (principally NO and PGI_2). Meanwhile secretion of factors such as PDGF, basic-fibroblast growth factor (bFGF) promote the proliferation of vascular smooth muscle cells and encourage them to develop a secretory phenotype and to move into the subintima. The overall effect is the accumulation of further macrophages, T lymphocytes and vascular smooth muscle cells in the lesion and the fatty streak grows into the intermediate lesion.

1.8.2.4 Intermediate Lesion

The accumulation of further foam-cells and T-lymphocytes expands the lipid-rich component of the lesion. As cells accumulate some undergo apoptosis discharging their contents into the extracellular matrix (Libby et al 1996). The stimulus for this is unclear but may in part be due to the hostile and ischaemic environment of the core lesion to

extremely metabolically active cells. Alternatively, the accumulation of oxLDL and prolonged stimulation by growth factors and other inflammatory mediators may push the cell cycle into apoptosis. In either event the increased amounts of free oxLDL in the lesion encourage further expansion of the lesion and increased vascular smooth muscle cell proliferation with the elaboration of fibrous tissue around the lipid core and especially at the subintimal cap. As the process advances the lesion develops into the mature plaque. Alternatively, the expanding lipid pool increases the physical instability of the lesion encouraging the development of a complicated plaque by rupture.

It is important to recognise at this point that apoptosis of the cellular components of the lesion may lead to partial plaque regression if the net export of lipid from the lesion exceeds import. Enhanced NO generation may induce apoptosis and lipid export (Wang et al 1999) leading to plaque regression. The source of NO in the active plaque is likely to come from 2 sources; the constitutive endothelial NO synthase (eNOS) and the induced NO synthase (iNOS) enzymes of endothelium. The latter enzyme is widely expressed in endothelium and vascular smooth muscle cells in response to inflammation and produces large amounts of NO over several hours to days. Although its role in vascular physiology remains undefined, this may be the major source of NO in an inflamed plaque. Of note, the expression of iNOS is selectively inhibited by glucocorticoids (Radomski et al 1990).

1.8.2.5 The Mature Plaque

This lesion is composed of a molten lipid core with interspersed surviving foam-cells and T-lymphocytes. Surrounding the lesion is a capsule of secretory vascular smooth muscle cells that produce a fibrous matrix. In the subintima this layer is thickened to form a cap (Stary 1989). The effect of this capsule is thought to stabilise and strengthen

the plaque. As the plaque continues to grow it may narrow the vascular lumen eventually producing a haemodynamically significant stenosis or occlusion. These lesions produce ischaemia or infarction of the dependent tissue, typically myocardium, brain or limbs, resulting in angina, myocardial infarction, stroke, intermittent claudication or peripheral gangrene. However, complications of the stable mature plaque are now thought to be the main cause of these diseases.

1.8.2.6 Complicated Plaque

Vascular Spasm

Frequently the endothelial layer overlying the fibrous plaque becomes denuded. This may be caused by the repeated inflammatory and proliferative stimuli to endothelial cells which eventually results in cell senescence or cell apoptosis. As the protective endothelial barrier is lost NO and PGI_2 secretion are reduced and the vessel is subject to the unopposed vasoconstrictor influences of the sympathetic innervation, circulating adrenaline, and angiotensin II. This may produce vascular spasm around the plaque resulting in repeated episodes of tissue ischaemia precipitated particularly on stress.

Thrombosis

As the cap is damaged the anticoagulant effects of endothelium are lost and platelets adhere to the exposed collagen and matrix of the fibrous cap activating the coagulation cascade and resulting in thrombus formation (Arroyo et al 1999). This thrombus may produce tissue ischaemia which resolves as the thrombus lyses, result in embolisation to the dependent tissue or go on to produce an occlusive thrombus with tissue infarction.

Ulceration

If the protective fibrous cap is breached blood enters the soft core of the lesion producing sudden vascular occlusion by lesion expansion or vascular dissection (Arroyo

et al 1999). Several enzymes secreted by the macrophage may weaken the cap including collagenases, elastase, stromolysin and gelatinases (Mach 1997). The mechanical stress of increased blood pressure may then be sufficient to rupture the weakened fibrous cap. Calcification of the plaque, particularly as lipid soaps, increases the rigidity of the core and raises the intra-lesional stress generated by blood pressure thus making calcified lesion more prone to rupture (Fitzpatrick 1994).

1.8.3 Aetiology

An understanding of the mechanisms through which established risk factors for cardiovascular disease operate includes several important effects on atheroma formation and complication (Lowe et al 1996).

Age

As atheroma is a gradually progressive condition, increased age results in an increased number and severity of atheromatous lesions.

Sex

Premenopausal women have a greatly reduced incidence of cardiovascular disease compared either with postmenopausal women or men of the same age. A large component of this protective effect is thought to be due to oestrogen. Oestrogen increases NO generation in the endothelium (Chen et al 1999) and reduces the LDL cholesterol fraction (O'Brien et al 1997), and both may contribute to cardiovascular protection.

Family History

The influence of family history on an individual's subsequent risk of developing cardiovascular disease is strong. Familial effects include shared environment, including intra-uterine growth rate, diet, infectious agents and pollutants, and shared genes.

Disentangling these influences may be difficult. Nonetheless, there is good evidence for shared risk in parental-offspring (Colditz et al 1991, Watt et al 1992) and twin studies (Marenberg et al 1994). In twins the concordance for premature coronary artery disease, after controlling for conventional risk factors, was increased 8 fold for monozygotic twins and 4 fold for dizygotic twins. This suggests there is an important genetic component to cardiovascular risk.

Dyslipidaemia

In primary hypercholesterolaemia an autosomal dominant defect of the LDL receptor gene results in inactive LDL receptor expression on endothelial cells (Goldstein & Brown 1989). As a result of reduced LDL active uptake, LDL accumulates in the circulation to greatly increased levels and reaches the intima through gaps in the endothelial intercellular junctions. As a result of subsequent LDL oxidation, these subjects have a greatly increased risk of developing cardiovascular disease often in their fourth decade, and in homozygotes in their teens.

More commonly subjects with cardiovascular disease are found to have a mixed dyslipidaemia with moderately elevated LDL and reduced HDL. This profile is thought to increase the likelihood of developing atheroma by increasing the substrate for oxLDL production and, through reduced levels of HDL, reducing the pathway for oxLDL clearance from the developing plaque. Recent studies confirm the likely importance of elevated LDL cholesterol in atherogenesis with reductions in cardiovascular endpoints in established (Pederson 1994) and asymptomatic (Shepherd et al 1995) disease.

Smoking

Smoking remains the greatest avoidable risk factor in developing cardiovascular disease. Despite its importance, the mechanism of vascular damage by tobacco smoke

remains unknown (McGill 1988). A number of tobacco-smoke components have been shown to induce endothelial dysfunction and damage including carbon monoxide, nicotine and polycyclic hydrocarbons. A significant factor appears to be increased fibrinogen and thrombus formation in smokers. This may increase the rate of complicated plaque formation.

Hypertension

Prolonged elevation of blood pressure increases the shear stress across the endothelium and increases turbulence at arterial junctions, favoured sites of atheroma formation (Kolpakov et al 1996). The effect of this is endothelial dysfunction with adhesion molecule expression and reduced NO and PGI_2 production (Traub & Berk 1998).

Diabetes

Both type I and type II diabetes produces metabolic defects that result in increased glucose and lipid in the circulation (Haffner 1998). Prolonged elevation of glucose may result in glycosylation of proteins in the endothelium and vessel wall and reduced NO generation and increased connective tissue generation in the media (Hogan et al 1992). Elevated LDL concentrations increase the substrate for the formation of ox LDL and reduced HDL concentrations reduce the ability of macrophages to export lipid from plaque to peripheral sites for disposal.

Other effects of diabetes include a poorly understood effect on immune cell function that results in mild immunosuppression. This effect is widespread with impairment of function in neutrophils, lymphocytes and macrophages, probably through altered production of cytokines (Pickup & Crook 1998). In an established plaque this may increase the rate of macrophage apoptosis and progression of early fatty streaks to mature atheroma.

Insulin itself has been implicated as a risk factor for atheroma (Stout 1990). In type 2 diabetes where circulating insulin levels are high to compensate for peripheral tissue resistance, insulin may exert growth factor like effects on the cells of the vessel wall or plaque to increase the rate of lesion expansion.

Insulin resistance may reflect a primary alteration in insulin-mediated glucose uptake with a compensatory rise in insulin secretion. This may be a primary abnormality in cardiovascular disease with high correlation between insulin resistance and hypertension, dyslipidaemia (especially elevated LDL, elevated VLDL and low HDL) and obesity. This association of metabolic derangements is known as Reaven's syndrome (Reaven 1988).

Hyperhomocyst(e)inaemia

The rare condition of homozygous homocysteinamia carries a greatly increased risk of premature atherosclerosis and venous thrombosis. This appears to be due to increased auto-oxidisation of homocysteine generating oxygen radicals. These radicals then react to produce lipid peroxides, vascular matrix damage and smooth muscle proliferation increasing atheroma production while endothelial damage presents a prothrombotic surface to the circulation. Recently attention has focused on the effect of modest elevations in homocysteine or homocystine on the development of cardiovascular disease (Malinow 1990). Furthermore, relative deficiencies in folic acid and to a lesser degree vitamin B6 and vitamin B12 (which are required as cofactors for the homocysteine degradation) are commonly found and may thereby contribute to cardiovascular disease (Hankey & Eikelboom 1999).

1.8.4 The Influence of Glucocorticoids on Cardiovascular Disease

Glucocorticoid excess greatly increases cardiovascular risk and the mortality from cardiovascular disease. In subjects with untreated Cushing's syndrome the mortality from cardiovascular causes approached 50% at 5 years (Plotz et al 1952).

A number of risk factors for cardiovascular disease have been derived from epidemiological studies (Anderson et al 1990) and are thought to have mechanistic importance in the development of these diseases. The protean effects of glucocorticoids on physiological and metabolic processes tend to produce a phenotype with many of the established risk factors that resembles Reaven's syndrome, and would be expected to increase the susceptibility to cardiovascular disease. Additional effects of glucocorticoids on non-standard risk factors, such as reduced nitric oxide generation, immunosuppression, central obesity, and depression may further aggravate a poor cardiovascular risk profile.

The purpose of this thesis is to examine whether the wide variation in circulating glucocorticoid concentrations and in glucocorticoid receptor levels contribute to cardiovascular risk in the population.

Chapter 2

Materials and Methods

2.1 Materials

All reagents used were of the highest available quality. Reagents were obtained from the suppliers detailed below.

2.1.1 Basic Chemical Reagents

Absolute ethanol (molecular biology grade) was obtained from University of Glasgow Chemistry store.

Boric acid (molecular biology grade) and digitonin were obtained from ICN Pharmaceuticals Ltd., Cedarwood, Chineham Business Park, Crockford Lane, Basingstoke, RG24 8WD.

Adenosine triphosphate sodium salt, dexamethasone, dimethylsulphoxide (tissue culture grade), forskolin, glycerol, melatonin, mineral oil, 13-*cis* retinoic acid, all *trans* retinoic acid, retinol, *trans* β carotene, 3,5,3' tri-iodo-L-thyronine, reverse tri-iodothyronine (3,3',5' tri-iodo-L-thyronine), Sigmacote ™ (a siliconising solution), sodium chloride (molecular biology grade), TEMED (N,N,N',N' tetramethyl-ethylenediamine), TRIS-hydrochloride (*tris*(hydroxymethyl) aminomethane hydrochloride), Triton X-100 ™, and Urea were purchased from Sigma-Aldrich Chemical Company Ltd., Fancy Road, Poole, Dorset, BH12 4OH.

2.1.2 Radiochemicals

[γ^{32}P] deoxyadenosine 5' triphosphate, [α^{32}P] deoxycytosine 5' triphosphate and [1,2,4,6,7 ^{3}H] Dexamethasone were purchased from Nycomed Amersham plc., Amersham Place, Little Chalfont, HP7 9NA. [α^{35}S] deoxyadenosine 5' thiophosphate

was purchased from New England Nuclear Life Science Products, PO Box 60, Houndslow, TW5 9RT.

Ecoscint ™ scintillation fluid was purchased from University of Glasgow Chemistry stores.

2.1.3 Molecular Biology Reagents and Equipment

Acrylamide, N,N' methylene *bis*-acrylamide and a Prep-a-Gene kit ™ (for DNA purification) were purchased from Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD.

10xTBE buffer and ϕX174 DNA/Hae III Digest molecular marker were purchased from Roche Diagnostics Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG.

Antibody to the glucocorticoid receptor was purchased from Cambridge Bioscience, 24-25 Signet Court, Newmarket Road, Cambridge, CB5 8LA.

Oligonucleotide primers for PCR and sequencing were obtained from Cruachem, Todd Campus, West of Scotland Science Park, Acre Road, Glasgow, G20 0UA.

Agarose was purchased from Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.

Restriction enzymes (BclI, BamHI, EcoRI, FokI, HaeIII, NlaIII, PstI, Sau3AI, ScrFI, and StyI) and T4 polynucleotide kinase were purchased from New England Biolabs (UK) Ltd., 73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY.

A cycle-sequencing kit and sequencing reagents for use in an ABI 373 automated sequencer were purchased from Perkin Elmer Applied Biosystems, 7 Kingsland Grange, Woolston, Warrington, Cheshire, WA1 7SR.

ϕX174 DNA, pGEM-T Easy Vector kit, and Taq were purchased from Promega Ltd., Delta House, Chilworth Research Centre, Southampton, SO16 7NS.

Ammonium persulphate, ethidium bromide and formamide were purchased from Sigma-Aldrich Chemical Company Ltd.

Taqgene precision plus was purchased from Stratagene Europe, Gebouw California, Hogehilweg 15, 1101 CB Amsterdam Zuidoost, The Netherlands.

2.1.4 Photographic Reagents

Autoradiographic cassettes and autoradiographic film was purchased from Nycomed Amersham plc. Polaroid film was purchased from Sigma-Aldrich Chemical Company Ltd.

2.1.5 Cell Culture Reagents

RPMI 1640 cell culture medium, Dulbecco's phosphate buffered saline tablets and lymphocyte separation medium were purchased from ICN Pharmaceuticals Ltd.

GF-C (glass fibre) filter paper was purchased from Whatman International, Whatman House, St. Leonards Road, 20/20 Maidstone, Kent, ME16 0LS.

2.2 Blood Sampling

Blood samples were obtained with the Vacutainer system of needles and evacuated bottles supplied by Becton Dickinson, Between Towns Road, Cowley, Oxford, OX4 3LY. Samples were taken into lithium-heparinised bottles unless otherwise stated and processed immediately. For the biochemical measurements made in the epidemiological studies, electrolytes and glucose were processed on an Olympus 5200 analyser, and lipid determinations were done on a Beckman CX4 analyser.

2.3 Equipment

The following items of equipment were used.

Several micropipettes spanning 0.5-1000µl, polypropylene 96-well cell culture plates, disposable sterile graduated 50ml tubes, 15ml sterile graduated polystyrene tubes and

plastic Pasteur pipettes were purchased from Alpha Laboratories, 40 Parnham Drive, Eastleigh, Hants, SO50 4NU.

A β-particle shielded box and β-particle barriers were purchased from Phillip Harris, E6 North Caldeen Road, Calder Street, Coatbridge, Lanarkshire, ML5 4EF.

Radioactivity was measured using a Packard Tri-Carb 2100TR liquid scintillation analyser, in scintillation counting vials, both from Packard Bioscience Ltd., Brooke House, 14 Station Road, Pangbourne, Berkshire, RG8 7AN.

A Sequegen sequencing cell, 97 well comb, and power packs were purchased from Bio-Rad Laboratories, Ltd.

An Ultraviolet Stratalinker 1800 was obtained from Stratagene Europe.

A 316nm ultraviolet transilluminator was obtained from Ultra-Violet Products Ltd., Unit 1, Nuffield Road, Trinity Hall Farm Estate, Cambridge, CB4 1TG.

A 96-well PCR machine, 96-well PCR plates and lids, and a hybridisation oven were obtained from Techne, Duxford, Cambridge, CB2 4PZ.

A Denley Wellwarm cell incubator and a Titertek A1 cell harvester were obtained from ICN Pharmaceuticals Ltd.

Centrifugation and incubation temperatures were checked with a digital thermometer purchased from Whatman International.

2.4 General Methods

2.4.1 Glassware

All glassware used was rinsed in tap water, then soaked in decon 75 (Decon Laboratories, Conway Street, Hove, Sussex, BN3 3LY) overnight. After rinsing in tap water and then distilled water, glassware was then dried in an oven at 60°C for 4 hours.

2.4.2 Micropipetting

Volumes of fluid were transferred using Alpha pipettes (Alpha Laboratories). These were recalibrated by weighing standard volumes of distilled water and adjusting the set-point of the pipette piston every month. Pipetting errors were never greater than 3% standard error of the mean.

2.4.3 pH Measurement

Measurements of pH were made using a Denver Instrument digital pH meter (Phillip Harris). This meter was regularly calibrated using standard solutions of pH 4.0, 7.0 and 9.0.

2.4.4 Centrifugation

Small samples were centrifuged up to forces of 100g using a Microcentaur benchtop centrifuge (MSE Ltd., United Kingdom).

Larger samples were centrifuged at forces of up to 1500g using a Damon/IEC division DPR-6000 centrifuge purchased from International Equipment Company, Bedfordshire, United Kingdom.

Ultracentrifugation of plasmid preparations was performed in a Beckman Ti70.1 centrifuge and rotor.

2.5 Cell Culture Methods

2.5.1 Lymphocyte Extraction from Whole Blood

Lymphocytes were separated from whole blood using a ficoll/hypaque density gradient (Boyum 1968). Whole blood was taken into a lithium-heparinised vacutainer bottle and immediately transported for processing. Normally, 25ml of whole blood was taken and diluted to 50ml with 25ml of Dulbecco's phosphate buffered saline in a 50ml sterile polypropylene tube. This diluted blood was then divided into 3 equal aliquots and

layered over a ficoll/hypaque gradient (lymphocyte separation medium, ICN, UK) in 3 sterile universal containers (Alpha, UK) each containing 10ml of lymphocyte separation medium. These were centrifuged at 400g for 40 minutes at 25°C with the centrifuge brake switched off to reduce turbulence. The buffy-coat from each tube was carefully aspirated and combined in a fresh 50ml sterile polypropylene tube and the volume made up to 50ml with Dulbecco's PBS. This tube was centrifuged at 400g for 20 minutes at 25°C to wash cells of any remaining plasma, and reduce contaminating platelet debris. After centrifugation, the supernatant was carefully poured off and the remaining cell pellet re-suspended in 15ml of Dulbecco's phosphate buffered saline in a sterile graduated 15ml polystyrene tube. After a further centrifugation step at 400g for 10 minutes at 25°C the supernatant was carefully discarded and the washed cell pellet re-suspended in a total volume of 2ml of RPMI 1640 cell culture medium.

The cell concentration of this suspension was measured using a Sysmex NA 8000 haematology counter and adjusted to 10 million cells/ml with RPMI 1640 cell culture medium. An aliquot of this mixture was rechecked by counting in the same haematology counter and the number of cells/ml obtained used in subsequent binding calculations.

2.5.2 Preparation of Receptor Binding Plates

Receptor binding incubations were performed in 96 well flat-bottomed polystyrene plates. These were prepared in batches in advance with varying concentrations of dexamethasone in each well.

Stock dexamethasone was prepared by dissolving dexamethasone in dimethylsulphoxide to a concentration of 1.6384 mmol/l. This standard was further diluted 100 fold in RPMI 1640 to produce a concentration of 16.384 μmol/l. An aliquot

of this solution was diluted 2 fold in RPMI 1640 to 8.192 μmol/l and subsequent serial dilutions to a concentration of 1 nmol/l achieved. To each well 50 μl of various concentrations of dexamethasone was added as shown in table 2.1. Well 1 contained RPMI 1640 medium alone (i.e. without any added dexamethasone). It should be noted that the highest concentration of dimethylsulphoxide was 1% vol/vol.

Table 2.1 Dexamethasone Concentrations (in nmol/l) in Receptor Binding Incubation Plates

Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
0	1	2	4	8	16	32	64	128	256	4096	16384

The concentrations of dexamethasone in each well are shown above in nmol/l. Well 1 contained cell culture medium alone with no added dexamethasone, providing conditions for maximal radiolabel binding. Wells 2-10 contained increasing concentrations of competing dexamethasone and wells 11 and 12 concentrations of dexamethasone sufficient to block radiolabel binding to receptor, thus providing a measure of non-specific binding.

2.5.3 Preparation of Radiolabelled Dexamethasone

[1,2,4,6,7 ^{3}H] Dexamethasone obtained from Amersham International, UK, was used as the radiolabel for glucocorticoid receptor binding assays. Stock radiolabel was used without further purification steps as advised by the supplied datasheet. A 1:1000 dilution of radiolabel in RPMI 1640 cell culture medium was used with measurements of activity of the solution counted on a Packard Scintillation Counter and the final

concentration of the solution adjusted by adding appropriate volumes of RPMI 1640 medium to achieve a final concentration of 8nmol/l [1,2,4,6,7 ^{3}H] Dexamethasone.

2.5.4 Glucocorticoid Receptor Binding Assay

Glucocorticoid receptor binding was measured using a whole cell homologous displacement assay using dexamethasone as the displacement ligand, and [1,2,4,6,7 ^{3}H] dexamethasone as the radiolabel. Lymphocytes prepared from whole blood and adjusted to 10 million cells per ml (see 2.5.1) were used to assay whole cell receptor binding activity. To suspensions containing fresh lymphocytes, half the volume again was added of freshly prepared radiolabel solution. The suspension was carefully mixed and 150µl of the cell/label mixture added to each well of a 96 well plate. Assays were performed in quadruplicate for each subject. In the final incubation plate each well contained 1 million lymphocytes, 2nmol/l [1,2,4,6,7 ^{3}H] dexamethasone and the concentration of dexamethasone in each well was reduced to one quarter, as shown in table 2.2.

Table 2.2 Concentrations of Dexamethasone (in nmol/l) in Receptor Binding Incubations

Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
0	0.25	0.5	1	2	4	8	16	32	64	1024	4096

After diluting cells and adding radiolabel, the final concentrations of dexamethasone are shown in nmol/l above.

Each 96 well plate containing lymphocytes for incubation was sealed with a water tight plastic film to prevent evaporation. Cells were incubated for 20 hours at 25°C in a

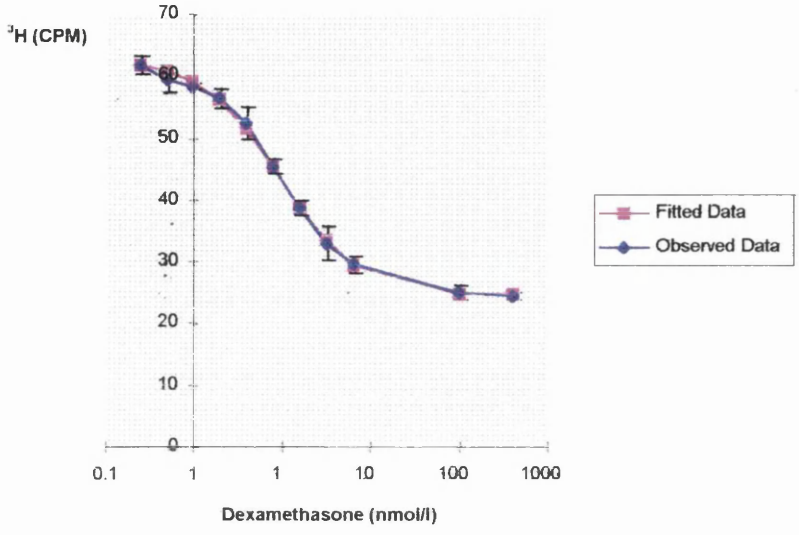
thermostatically controlled cell incubator, and were gently agitated to maintain the cells in suspension.

At the end of the incubation period, cells from each well were harvested onto GF-C glass fibre filter paper using a ICN Model A1 cell harvester. This model has a 12 well manifold which allows a row of 12 wells to be harvested simultaneously and reduces the time taken to harvest an entire plate. At the end of each harvesting run a blank row of wells was harvested onto GF-C paper and 1 disc was added to each of 8 scintillation vials. To 4 of these vials 50µl of radiolabel solution was added. Thus the final 8 vials provided a measure of tritium contamination, and a measure of radiolabel activity to ensure consistency between harvesting batches. Each disc containing the harvested cells, or blanks, was placed in a scintillation vial containing 200µl of 1% Triton X-100 and gently agitated for 1 hour to ensure all cells were solubilised. Each vial then had 3 ml of Ecoscint scintillation fluid added and was left for a further 3 hours to allow the glass fibre discs to become saturated with scintillation fluid and become transparent. Finally, scintillation vials were counted on a Packard scintillation counter for tritium at 66% efficiency for 5 minutes each.

2.5.5 Analysis of Glucocorticoid Receptor Binding Curves

For each binding assay tritium activity was measured 4 times at each concentration of dexamethasone. The mean of these was taken and a graph of tritium activity for each concentration of dexamethasone obtained as shown in figure 2.1.

Figure 2.1 Glucocorticoid Receptor Binding Curve
A sample binding curve is shown below with experimental data shown in blue and a non-linearly fitted binding curve shown in pink. Errors are standard error of the mean.



Binding curves were fitted using a non-linear regression algorithm written in a Microsoft Excel spreadsheet (for details of the mathematics see Appendix 2). Using this application, Kd and the number of binding sites per cell were obtained for each binding experiment.

2.6 DNA Extraction from Whole Blood

Genomic DNA was extracted from whole blood by a variation of the method described by Sambrook (Sambrook et al 1989). In the first step red blood cells are lysed while fresh white blood cells, or frozen white cell nuclei, are left intact. Ten millilitres of either fresh or frozen and thawed whole blood collected in potassium EDTA was added to 40 ml of cell lysis buffer (see Appendix 1) on ice. After ten minutes, the tubes were

centrifuged at 2000 g for 10 minutes at 4°C. The supernatant containing cytoplasmic debris and haemoglobin was disposed of into 1% sodium hypochlorite solution, and the pellet containing intact nuclei re-suspended in 3 ml of nuclear lysis buffer (see Appendix 1), 200µl 10% SDS and 100µl proteinase K (10mg/ml) and incubated at 37°C for 16-20 hours. Following incubation, 1ml of 6 mol/l sodium chloride was added and gently mixed by inversion to precipitate cellular proteins leaving genomic DNA in solution. Five ml of phenol/chloroform (see Appendix 1) was added and the tubes centrifuged at 2000 g for 20 minutes at 4°C. The upper aqueous phase of the supernatant containing DNA was transferred to a fresh universal container and 2 volumes of absolute ethanol added. DNA then formed a white thread-like precipitate and was spooled out of solution using a sterile glass rod, washed in 70% ethanol and dried in air. The dried DNA was finally dissolved in either 100µl of sterile distilled water, or 100µl of TE buffer (see Appendix 1). DNA solutions were then stored at either 4°C or frozen at –20°C.

2.7 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a well described method for specifically amplifying target DNA sequences from complex templates (Saiki et al 1988). For each specific target sequence optimisation of the polymerase chain reaction was required to achieve optimal product specificity and yield. A general PCR master-mix was usually a useful starting point and is detailed in table 2.3.

Table 2.3 Reaction Mix for Polymerase Reaction

Reagent	Concentration
Magnesium Chloride	1.5 mmol/l
Taq Buffer	1 x
dATP	50 µmol/l
dCTP	50 µmol/l
dGTP	50 µmol/l
dTTP	50 µmol/l
Sense Primer	10 nmol/l – 1 µmol/l
Anti-sense Primer	10 nmol/l – 1 µmol/l
Taq	1 U
DNA	1 µl
Distilled Water	To make final volume to 25 µl
Mineral Oil	50 µl

Generally, the PCR solution (i.e. without added DNA) was prepared freshly in a batch. DNA was then aliquoted into the tubes, or plates used for PCR and aliquots of the PCR master-mix added to each before finally adding a layer of mineral oil to prevent evaporation.

PCR was generally performed using an initial step of 94°C for 5 minutes to ensure denaturation of genomic DNA. Thirty cycles of annealing (this temperature was specific for each primer pair and was optimised empirically) for 30 seconds followed by extension at 72°C for 30 seconds and denaturation at 94°C for 30 seconds, and a final step at 72°C for 5 minutes to ensure all products were fully extended. All PCR products were either freshly analysed on agarose gel electrophoresis, or stored at 4°C until analysis.

2.8 Single Strand Conformational Polymorphism (SSCP) Analysis

Single strand conformational polymorphism analysis (Orita et al 1989, Grompe 1993 for review) was performed using either end-labelled primers, or incorporation-labelled amplicons.

2.8.1 End-labelling

Sense and anti-sense primers for the region of interest were end-labelled at the 5' position using T4 nucleotide kinase (an enzyme that catalyses the transfer of the γ-phosphate from ATP to the 5' terminus of polynucleotides or mononucleotides) and [γ^{32}P]-dATP. The composition of the reaction mixture is shown in table 2.4.

Table 2.4 End Labelling Primers

Reagent	Concentration
Primer	50 μmol/l
[γ^{32}P]-dATP	Specific Activity 1.85 MBq
T4 Polynucleotide Kinase	5 U
TRIS-HCl	70 mmol/l, pH 7.6.
Magnesium chloride	10 mmol/l
Dithiothreitol	5 mmol/l
Distilled water	added to final volume 50 μl

The 50 μl mixture was carefully mixed and incubated at 37°C for 1 hour and finally denatured at 94°C for 5 minutes to inactivate the enzyme. PCR was performed on the region of interest using genomic DNA using conditions previously optimised. Each PCR reaction contained 1 μl of the labelled sense and antisense primers, i.e. contained 1 μmol/l of each primer.

2.8.2 Incorporation Labelling

As an alternative to end-labelling of primers, PCR amplicons were labelled by incorporation using [α^{32}P]-dCTP. Each PCR reaction was prepared as described in section 2.7 above, with the addition of 1 μl of [α^{32}P]-dCTP per 50 PCR reactions (specific activity 30.7TBq/mmol) and a compensatory reduction in the volume of distilled water added by 1 μl. The increase in total concentration of dCTP was less than 0.001% and was not corrected for by altering the volume of dCTP added. This provided

more radioactive amplicons with clearer bands on autoradiography and an overall simplification of method.

2.8.3 Polyacrylamide Gel Preparation

A 6%, 29:1 acrylamide: N,N' methylene *bis*-acrylamide gel (Sanger et al 1977a), with or without 5% glycerol, was prepared by mixing the reagents in the order listed in table 2.5.

Table 2.5 Polyacrylamide Gel Mix

Reagent	Volume added	Final Concentration
30% 29:1 Acrylamide:Bisacrylamide	30ml	6%
(Glycerol	7.5ml)	5% if added
TBE Buffer x 5 (see Appendix 1)	7.5ml	0.5%
Distilled Water	105ml(112.5ml if no glycerol added)	
0.5 M EDTA	0.3ml	
Temed	150µl	
25% Ammonium Persulphate	150µl	
Total	150ml	

Sequecell ™ sequencing plates were prepared by siliconising one plate with Sigmacote ™, and cleaning and drying the other with absolute ethanol. The sequencing cell was then assembled and the gel solution injected through the injection port. After allowing to set for 1 hour, the cell was loaded into the buffer system and pre-run at 30 watts for 1 hour to ensure buffer equilibration throughout the gel. Finally, a 97 well comb was inserted into the gel.

2.8.4 Sample Preparation and Loading

For each PCR reaction, 5 µl was added to 10 µl formamide/dye (see Appendix 1) in a fresh PCR tube or well and denatured at 94°C for 5 minutes to allow the stable

formation of single stranded labelled DNA. Samples were then immediately placed on ice and loaded onto the gel.

2.8.5 Electrophoresis Conditions

To increase the sensitivity of the method, each sample was run at 4 conditions: 25°C with and without glycerol, and 4°C with and without glycerol. Generally, running the gel at 25°C with 5% glycerol produced the greatest resolution: no further polymorphisms were detected in gels run at other conditions. Gels were run at a constant temperature at 30 watts normally for 4 hours at 25°C, or 60 watts for 3 hours at 4°C.

2.8.6 Autoradiography

Following electrophoresis, the sequencing cell was dismantled and the gel blotted onto Whatman 3M filter paper, covered in cling-film and dried at 65°C in a vacuum oven for 1 hour. The dried gel was then placed directly against autoradiographic film for 12-48 hours in a light-tight autoradiographic cartridge. Autoradiographs were developed using a Kodak X-Omat automated film developer.

2.9 DNA Sequencing

2.9.1 Manual Sequencing

Manual sequencing of DNA was performed using a modification of the 2',3'-dideoxynucleoside-5'-triphosphate (dideoxy-, dd-) termination chemistry originally described by Sanger (Sanger et al 1977b). This original sequencing method works best with single stranded DNA template that requires time-consuming preparation by subcloning into single stranded bacteriophages such as M13. To overcome this, a cycle-sequencing kit (supplied by Perkin Elmer, Buckinghamshire, United Kingdom) was used. Briefly, this technique uses one primer end labelled with [γ^{32}P]-dATP to elongate

double stranded template using a recombinant Taq polymerase. In the presence of one of each of the dideoxy-terminators (ddATP, ddCTP, ddGTP, ddTTP), four identical reactions are prepared each terminating at positions corresponding to the respective nucleotide (A,C,G,T). After resolution of the products from each reaction ("sequencing ladders") on a polyacrylamide gel, and autoradiography as described above, the sequence as a whole can be deduced. As a final refinement, we used 7-deaza-dGTP in place of dGTP in each of the sequencing reactions – this has the advantage of producing product with more uniform resolution on polyacrylamide electrophoresis, preventing the artefact called "G-compressions" seen when dGTP is used as the elongation nucleotide.

2.9.2 Automated Sequencing

Manual sequencing as described has several disadvantages: the use of radioactivity, limited reading frames (around 300 nucleotides on a normal gel), time consuming preparation, and limited numbers of sequences per gel (each sequence requires 4 lanes). To overcome these disadvantages, an automated sequencing protocol was adopted. This involved the same dideoxy-termination chemistry and cycle sequencing method as described above, but in place of a radiolabelled primer, labelling is achieved by attaching fluorescent probes to each of the dideoxy-nucleosides. Thus as each elongating chain incorporates a dideoxy-nucleoside that terminates further extension, it is also labelled with a fluorescent tag. If each of the dideoxy-nucleosides are tagged with fluorochromes with different spectra of fluorescence, the sequence ladder can be read by scanning the colour of the each band with a laser and photodetector as it elutes from the bottom of a polyacrylamide gel. This is the basis of the ABI 373 automated DNA sequencer which uses the dyes 6-FAM, HEX, NED and ROX as fluorochromes. This method has the advantages that no radioactivity is involved, greater numbers of nucleotides can be read in a single run (up to 600 bases), a complete sequence can be

prepared in a single reaction, and since each sequence occupies only a single lane, more sequences can be run on a single gel.

2.10 Statistical Analyses

Unless otherwise stated, comparisons between paired datasets were performed by Student's 2 tailed t test. Associations between 2 variables were tested by linear regression, and between several continuous variables with multiple regression. All analyses were performed using the Minitab computer program (Mintab Inc, USA).

2.11 Immunohistochemistry

Sections of rat adrenal stained for aldosterone synthase or 11β-hydroxylase were kindly provided by Dr S.M.MacKenzie and performed as detailed in MacKenzie et al 2000. Briefly, primary antibodies were raised against hydrophilic peptides corresponding to epitopes in aldosterone synthase or 11β-hydroxylase with minimal homology. For aldosterone synthase the peptide was MAPKVRQNARGSLTMDVQQ representing residues 175-190 and for 11β-hydroxylase the peptide was KNVYRELAEGRQQS corresponding to residues 272-285. Each antibody was of the IgG_1 class and was monoclonal having been generated by cell fusion with an SP-2 myeloma cell line. Primary antibody-antigen complexes were detected by using a secondary antibody coupled to horseradish peroxidase and developed with 3,3'-diaminobenzidine tetrachloride producing a brown colour. Slides were then counterstained with haematoxylin.

Chapter 3

Development of Glucocorticoid Receptor Binding Assay

3.1 Introduction

The glucocorticoid receptor is a ubiquitous nuclear receptor that behaves as a hormone sensitive transcription factor. It is present in all nucleated white blood cells with highest concentrations in lymphocytes where it is believed to play important roles in modulating immune function and controlling the balance between T suppressor and T helper activity, and in reducing antibody production from B cells.

Previous glucocorticoid receptor binding assays have used lymphocytes freshly prepared from whole blood as a convenient tissue for study. What is unknown, however, is whether receptors in this tissue are regulated similarly to receptors in other key tissues such as liver, adrenal, hippocampus, hypothalamus and pituitary.

In developing this assay I started with a modified version of a previously reported receptor binding assay using fresh lymphocytes (Schlechte et al 1982). Progressive alterations were made to the assay, verifying that it performed at least as well at each stage, to optimise the method for high throughput in the study of large populations. The final assay method, after refinement, is detailed in 2.5.

3.2 Initial Assay

3.2.1 Venepuncture

Sixty millilitres of blood was taken into a sterile plastic container to which sodium citrate to a final concentration of 10 mmol/l was added as anticoagulant. The blood was centrifuged at 800g at 20°C for 10 minutes and the plasma layer discarded.

3.2.2 Cell Preparation

The buffy coat overlying the erythrocyte pellet was transferred to a fresh tube and diluted 1:2 with sterile phosphate buffered saline (PBS). This was carefully mixed and layered over lymphocyte separation medium. After centrifugation at 400g at 20°C for 40 minutes, the buffy coat was transferred to a fresh tube and diluted 1:2 with sterile PBS. This was centrifuged at 400g at 20°C for 10 minutes to remove platelet debris and the supernatant discarded. The cell pellet containing lymphocytes and monocytes was re-suspended in 10 ml PBS and centrifuged at 200g at 20°C for 10 minutes. The supernatant was again discarded and the cell pellet was re-suspended in 10 ml PBS and centrifuged at 100g at 20°C for 10 minutes. This final step was repeated and the supernatant discarded and the cell pellet left undisturbed.

Meanwhile fresh cell culture medium was prepared in a sterile laminar flow hood using RPMI 1640 medium (without glutamine and without bicarbonate, Flow Laboratories) to which was added 100 IU/ml penicillin, 100 μg streptomycin, 2 μmol/l L-glutamine and 10mmol/l sodium bicarbonate. Finally, freshly thawed fetal calf serum was heat inactivated at 56°C for 2 hours and added to the cell culture medium to a final concentration of 10%.

The cell pellet was then re-suspended in 1 ml of the freshly prepared cell culture medium and a 10 μl aliquot taken for cell counting. This was done by diluting the cells 1:20 in a dye solution containing 1% acetic acid and 0.01% crystal violet. Cell concentrations were assessed by counting the number of cells seen within the 4 large outer squares of a standard haemocytometer (a total volume of 0.4 μl) and multiplying

by 50,000 to give the number of cells per millilitre in the initial solution. From this, the total number of cells yielded was given by:

Number of cells yielded=number of cells per ml x 0.990.

3.2.3 Incubation

Cells were incubated with a constant concentration of [^{3}H 1,2,4]-labelled dexamethasone and varying concentrations of unlabelled dexamethasone.

Labelled dexamethasone was prepared by drying down 100 µl of stock dexamethasone (supplied as a solution in ethanol) under a nitrogen stream. This was reconstituted in 5 mls of RPMI 1640 medium and adjusted to 300,000 counts per minute per ml.

Concentrations of unlabelled dexamethasone were prepared in the following concentrations (in nmol/l): 20,000, 2,500, 156, 78, 39, 19, 9.6, 4.8, 2.4, 1.2, 0.6 and finally RPMI 1640 medium was used as 0 nmol/l dexamethasone.

To each well of a 96-well plate 50 µl of labelled dexamethasone (resulting in a final concentration of approximately 2 nmol/l in each well) and 100 µl of the cell suspension (containing approximately $3x10^6$ cells) was added. To each row, decreasing concentrations of unlabelled dexamethasone was added in 50 µl aliquots.

The plate was then incubated at 24°C for 3 hours, and terminated by aspirating the cell suspensions onto glass fibre (grade GF/C, Whatman) using a wash solution of ice cold PBS containing 0.1% polyethylenimine, using a cell harvester.

Finally the cells adherent to the glass fibre discs were lysed in 1% Triton X 100 and counted using a scintillation counter calibrated for [^{3}H] β-particles.

3.3 Optimisation of the Receptor Binding Assay

Several problems with the existing assay made it unsuitable for direct application to large populations. The volume of blood was excessive when additional samples were required as part of the epidemiological studies. Furthermore, the centrifugation steps were time consuming and labour intensive, the preparation of cell culture medium was time consuming and prone to inter-assay variation, the incubation times and temperatures were unoptimised and it was unknown whether the binding had reached equilibrium at harvesting. Finally, the number of detected scintillations was only two orders of magnitude greater than background and was therefore prone to significant counting error.

For these reasons, each step of the binding assay was investigated for simplifications or refinements to increase overall efficiency and reliability.

3.3.1 Venepuncture

Blood Volume

Blood taken from six individuals was prepared and mononuclear cells incubated at a cell density of 1 and 3 million cells per well. No difference for Kd or sites per cell was found between incubation conditions (2-tailed paired T-test, $p=0.96$, $p=0.99$ respectively) as shown in figures 3.1 and 3.2. This allowed the volume of blood taken to be reduced from 60 to 30 mls in subsequent assays.

Figure 3.1 Kd assayed with 1 million or 3 million cells per incubation

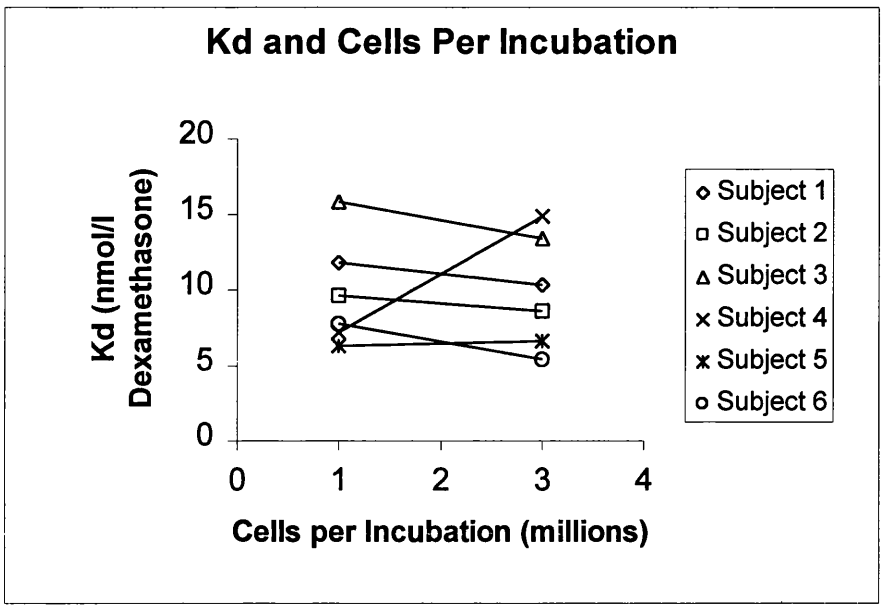
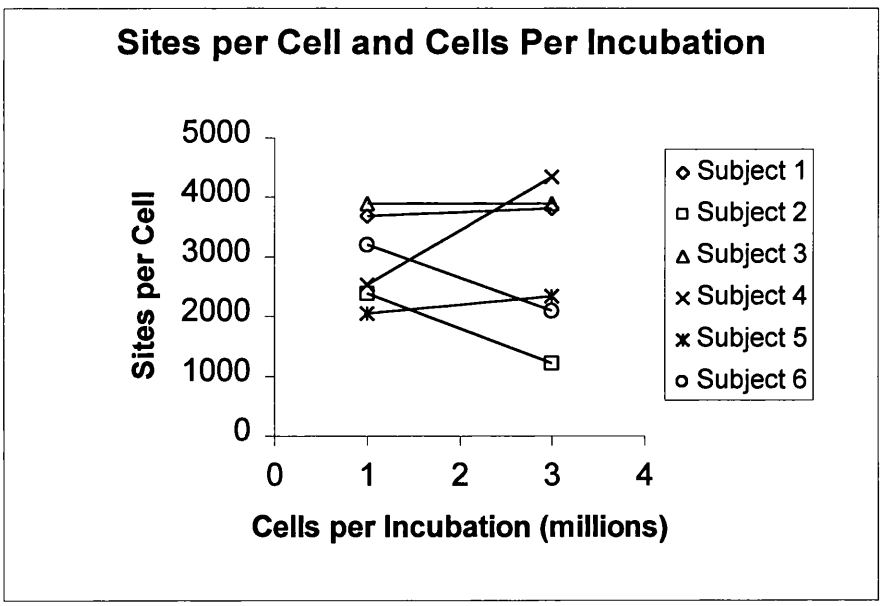


Figure 3.2 Sites per cell assayed with 1 million or 3 million cells per incubation



Anticoagulant

To allow blood to be taken directly into commercially available sterile bottles (vacutainer ^{TM}, Beckton Dickson, UK), the anticoagulant was changed from sodium citrate to lithium heparin. No difference was found in Kd or sites per cell between samples prepared with either anticoagulant (2 tailed T-test, p=0.33, p=0.51 respectively), figures 3.3 and 3.4.

Figure 3.3 Kd from cells prepared from blood with sodium citrate or lithium heparin

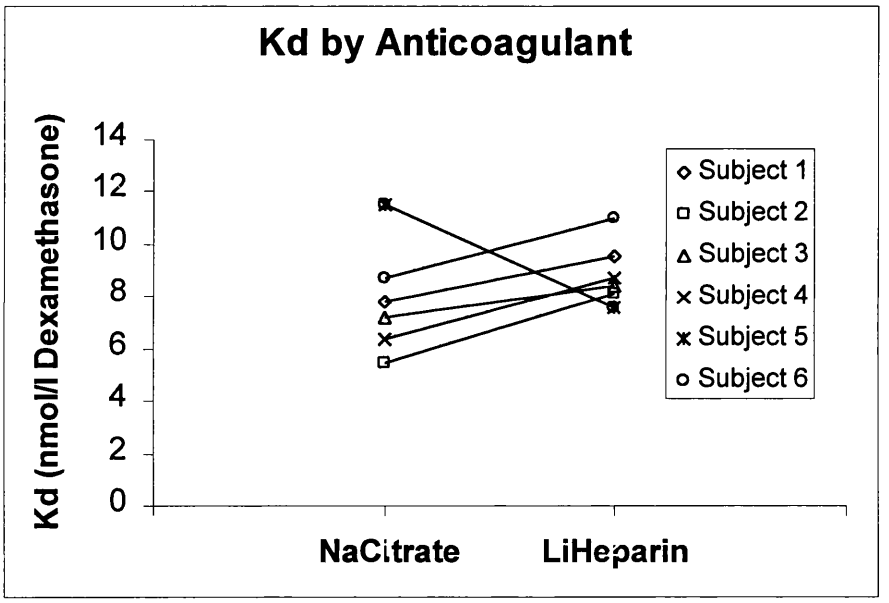
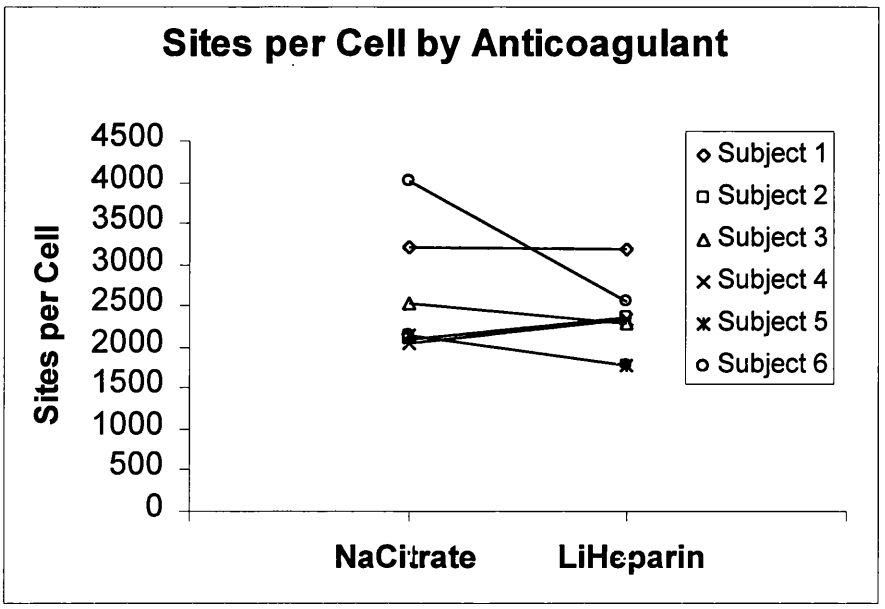


Figure 3.4 Sites per cell from cells prepared from blood with sodium citrate or lithium heparin



3.3.2 Cell Preparation

Cell Isolation

The steps outlined in the original assay involved several centrifugation steps that were time consuming and resulted in a significant loss of mononuclear cells. An attempt was made to reduce the number of centrifugation steps required while maintaining overall cell yield and purity.

In place of using the centrifuged cell pellet from 60 mls of whole blood as the source of white cells, 30 mls of blood was diluted 1:1 with sterile PBS and layered directly over a ficoll/hypaque gradient and centrifuged at 400g for 40 minutes at 20°C. The buffy coat was transferred to a fresh tube washed by centrifugation steps as described in the original assay. The final cell pellet was suspended in a volume of 2 mls of RPMI 1640 cell culture medium without additives.

This greatly improved the speed of cell separation and increased the yield by approximately 30%. It was important, however to ensure that the cells contained no significant cortisol as this would reduce the assayed Kd for dexamethasone binding. Cells were therefore prepared from 3 subjects before and 30 minutes after intravenous administration of 250 μg of the ACTH analogue, Synacthen. The cell pellets from the final centrifugation step were snap frozen in liquid nitrogen for later assay for cortisol. This showed no detectable cortisol in any of the cell pellets with a threshold for detection of 2 nmol/l as shown in table 3.1.

Table 3.1 Cortisol Concentrations in Cell Preparations

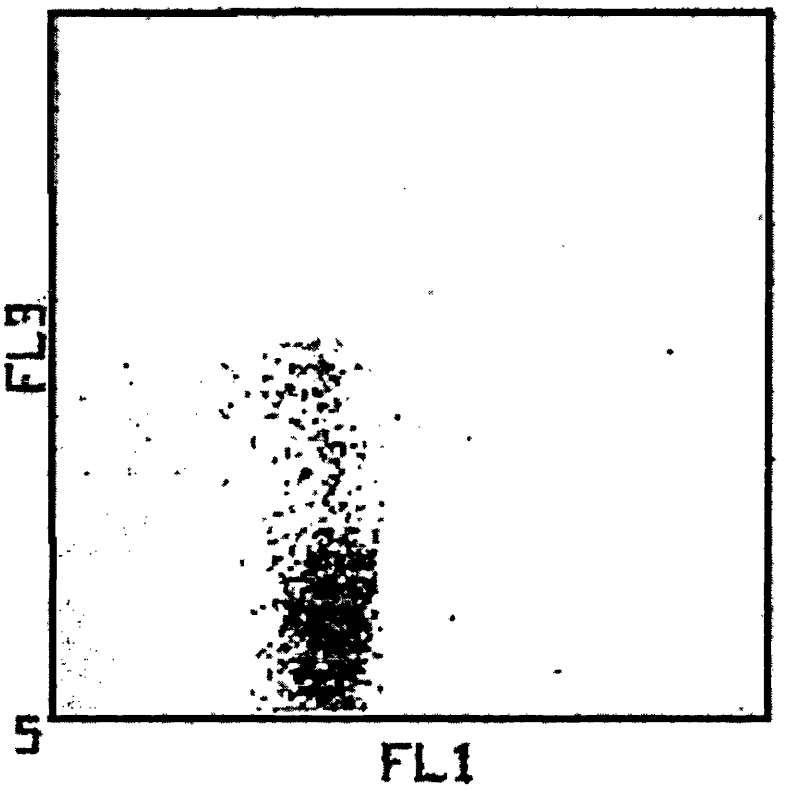
Subject	Basal/Post Synacthen	Plasma Cortisol (nmol/l)	Cell Cortisol (nmol/l)
1	Basal	469.2	<2
	Post-synacthen	607.2	<2
2	Basal	193.2	<2
	Post-synacthen	662.4	<2
3	Basal	207	<2
	Post-synacthen	634.8	<2

Trypan blue exclusion was used to ensure the cells were viable at the end of the centrifugation steps. Using 0.1% Trypan blue and microscopy of a haemocytometer cell-film, >98% of cells showed Trypan blue exclusion. Pre-treatment of cells with 0.1% Digitonin was used to provide a positive control and confirmed this abolished Trypan blue exclusion in 100% of cells.

Direct microscopy of a Geimsa-stained cell film showed >95% of the film was composed of mononuclear cells with a smaller number of neutrophils and platelet clumps. To ensure the cell population was relatively homogeneous flow cytometry was performed using as labels phycoerythrin conjugated anti-CD14 (directed against the lipopolysaccharide receptor expressed mainly on monocytes and macrophages) and fluorescein-isothiocyanate (FITC) conjugated anti-CD45 (directed against the protein tyrosine phosphatase expressed mainly by lymphocytes and also by monocytes). This allowed the relative numbers of lymphocytes and monocytes to be determined as shown in figure 3.5.

Figure 3.5 Flow Cytometry Analysis of Cell Preparation

Phycoerythrin conjugated anti CD14 is shown in the FL3 channel in red, and FITC conjugated anti-CD45 is shown in the FL1 channel in green. The majority of cells are CD14-, CD45+ suggesting they are lymphocytes.



Using this method, cells were prepared from 4 subjects on different days and showed >85% of cells stained with either PE-anti-CD14 or FITC-anti-CD45 suggesting they were monocytes or lymphocytes. The proportion of (CD14-ve and CD45+ve):(CD14+ve or CD45+ve) was between 0.92 and 0.96 suggesting at least 92% were lymphocytes.

Culture Medium Preparation

As described in the initial protocol, preparation of culture medium normally took around 1 hour. Furthermore, it was likely there would be significant variation in the composition of the medium between assays due to variation in batches of fetal calf serum, in particular this contains corticosterone which varies between batches and may interfere with dexamethasone binding. Since the lymphocytes did not require to divide, and since incubations were short I reasoned that growth factors and antibiotics may not be necessary.

Using RPMI 1640 medium untreated, and with all the described additives I conducted 5 receptor binding assays in parallel. There was no visible sign of infection (acidification of medium or turbidity) and cells remained in excess of 95% viable at up to 36 hours. There was no significant difference in measured Kd or sites per cell ($p=0.26$ and $p=0.52$ respectively, 2 tailed T-test), as shown in figures 3.6 and 3.7

Figure 3.6 Effect of Cell Culture Medium on Kd

Receptor binding assays were performed in parallel with unmodified RPMI 1640 medium "RPMI" or with RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 µg streptomycin, 2 µmol/l L-glutamine, 10mmol/l sodium bicarbonate and 10% heat inactivated fetal calf serum "RPMI + additives".

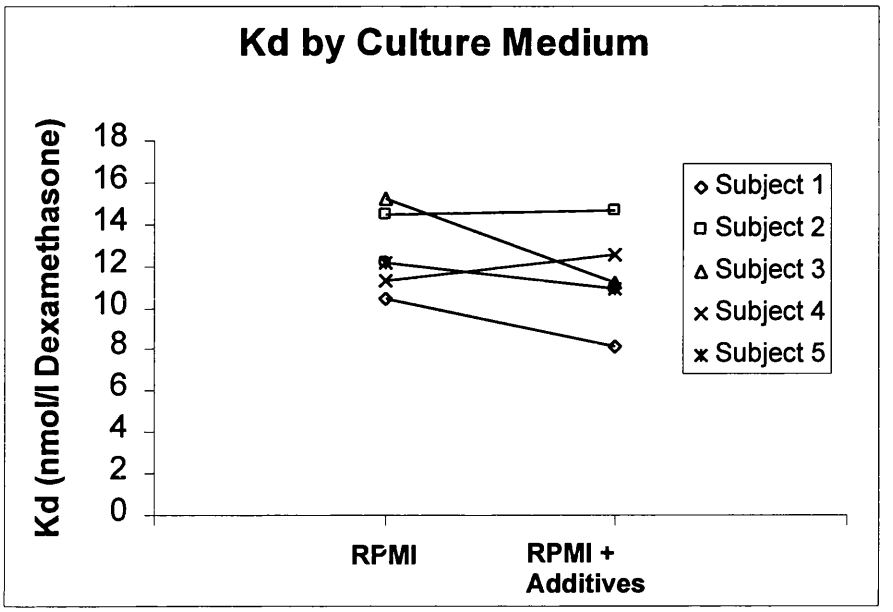
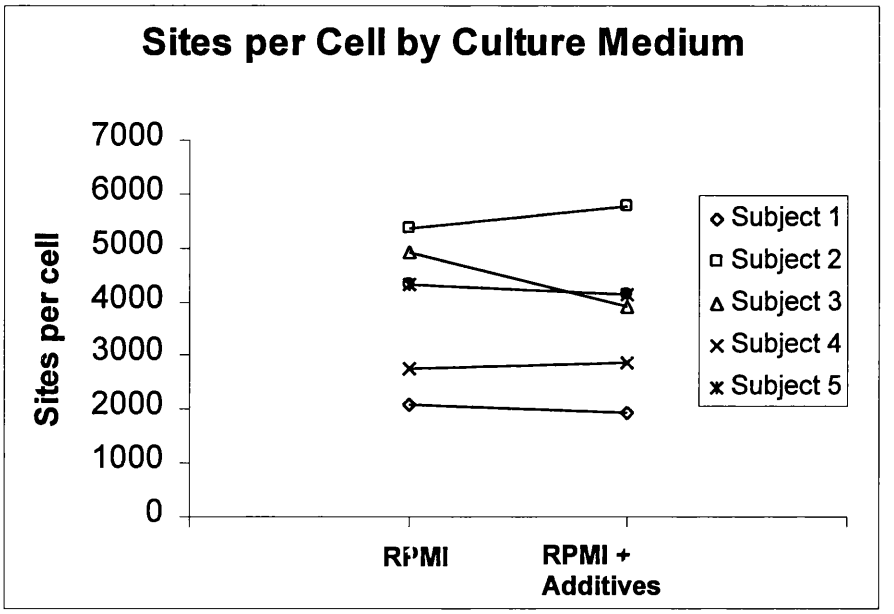


Figure 3.7 Effect of Cell Culture Medium on Receptor Sites per Cell



Cell Counting

Counting the cell concentration was done manually as described above and diluted to 10 million cells per ml. As the final cell concentration per incubation was crucial to determining the number of sites per cell, an aliquot of the final cell suspension was taken for counting in an automated haemocytology counter (Sysmex NE 8000) and calculations made on the basis of these results. The cell distribution curves obtained from this apparatus confirmed the flow cytometry results that an average of 90% of the cells were lymphocytes.

3.3.3 Incubation Conditions

Incubation conditions are the most likely to alter receptor binding characteristics and it was important, therefore, to ensure they were well controlled and validated. Vital components of the incubation are to find the optimal temperature for binding, to ensure

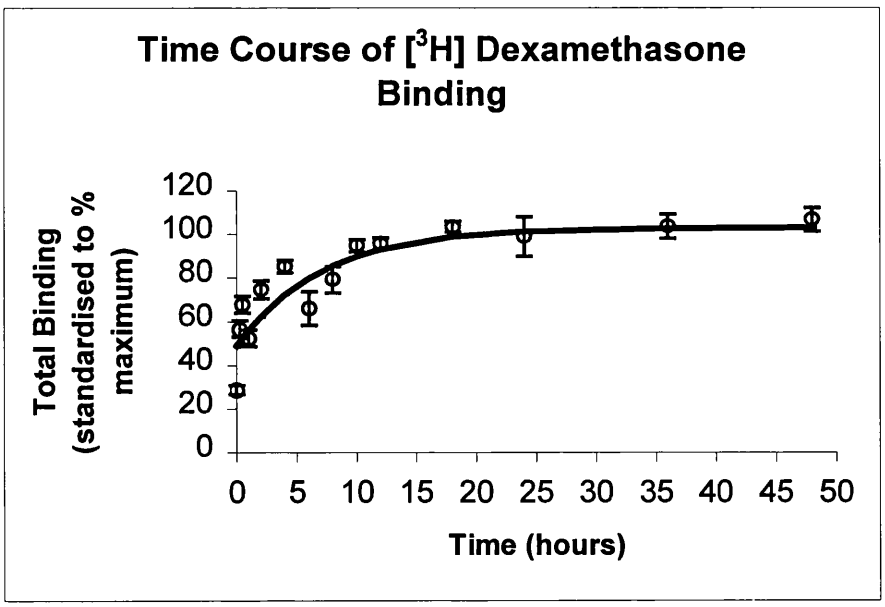
the binding has reached equilibrium and in a homologous displacement assay to ensure that the range of concentrations of competing ligand span the Kd with the logarithmic median close to the expected Kd.

Incubation Time

Receptor binding assays were initially terminated at 3 hours, although it remained unknown whether binding equilibrium had been reached. Incubations of cells at a concentration of 1 million per well with 2 nmol/l of radiolabelled dexamethasone and no competing dexamethasone were performed and terminated by harvesting at a number of time points. Using the CurveExpert curve fitting program, the time course at 20°C fitted with an exponential curve of form $y=52.2*(1.92-e^{-0.15*hours})$ as shown in figure 3.8.

Figure 3.8 Time-course of Dexamethasone Binding

No significant difference in binding was found between 18 hours and 24 hours, or 36 hours or 48 hours.

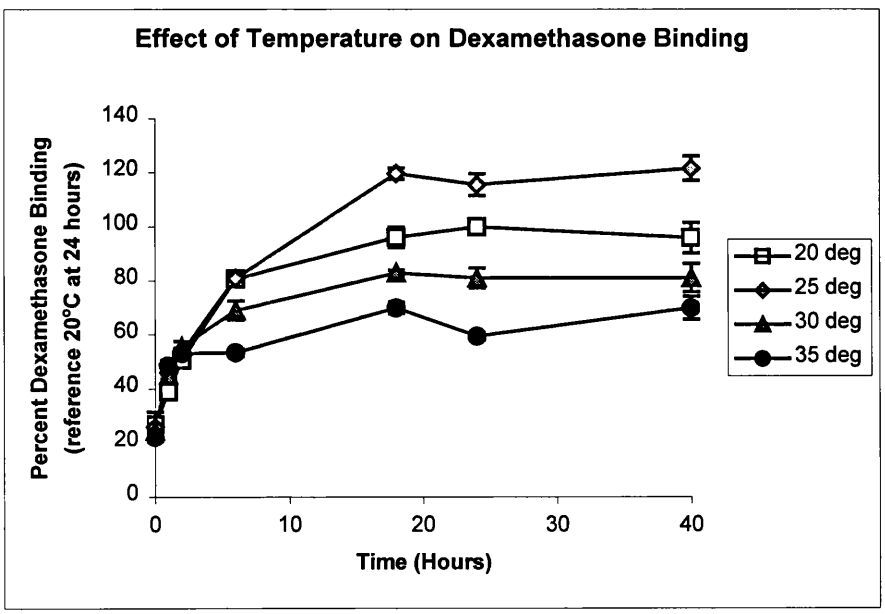


Incubation Temperature

The glucocorticoid receptor is known to be sensitive to temperature with reduced binding at higher temperatures. To explore the optimal temperature for assaying receptor binding, time course experiments were performed from 3 subjects at 4 different temperatures. Binding was adjusted to 100% of the maximum achieved at 20°C. There was little difference in binding at times before 3 hours but thereafter there was an increasing disparity with maximal binding occurring at 25°C. At 18 and at 24 hours there was a significant difference in binding between 25°C and all other temperatures as shown in figure 3.9. The most important finding in this series of experiments is that equilibrium is reached by 18 hours and remains stable for up to 40 hours.

Figure 3.9 Effect of Temperature on Receptor Binding

From this series of experiments it was decided to use 25°C as the standard incubation temperature in subsequent experiments using a thermostatically controlled cell incubator.



Radioligand Concentrations

The radioligand used in the initial assay was [^{3}H 1,2,4]-labelled dexamethasone. This tended to produce high non-specific binding and scintillation data only 1 order of magnitude higher than background and was therefore prone to counting error. To avoid increasing the concentration of radioligand and non-specific binding further I used an alternative label, [^{3}H 1,2,4,6,7]-labelled dexamethasone, with double the specific activity, but also with higher chemical purity (as discussed with Amersham). This was used at a concentration adjusted to 2 nmol/l which was within 1 order of magnitude of the expected Kd (2-16 nmol/l) and produced scintillation data with 2 orders of magnitude between maximum binding and background.

To minimise pipetting error, batches of radioligand were prepared weekly, stored at 4°C and diluted in 2 volumes of cell suspension before aliquoting into the incubation wells of a 96-well plate.

Competing Dexamethasone Concentrations

The concentrations of unlabelled competing dexamethasone were modified for simplicity to final incubation concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 1024, 4096 nmol/l. These concentrations ensured 4 concentrations spanned the expected Kd and that the highest concentrations (which would determine non-specific binding estimation) were in 500-2000 fold excess of radioligand.

Cell Harvesting

As the number of receptor sites per cell were determined from the cell count prior to incubation, it was essential to ensure the number of cells remaining viable at the end of incubation were similar. Cells were prepared as described above and diluted with either

RPMI medium alone or with dexamethasone to a final concentration of 4.96 μmol/l (the highest concentration in the receptor binding assay). At the end of a 24 hour incubation at 25°C the cells suspension was agitated and transferred to a fresh tube for counting on a Sysmex NE 8000 haematology counter, and for staining with Trypan blue to assess viability. Cell counts were extremely reliable with a coefficient of variation of <1%. Furthermore, cell viability assessed by Trypan blue exclusion was in excess of 96%.

To ensure there was no significant cell adhesion to the bases of the wells, normal incubations were performed (without radiolabel), cells harvested and the 96-well plates allowed to air dry. Each well than had 20μl of Giemsa stain added and any traces of adherent cells counted under a microscope. All wells were clear with no evidence of cell residue.

Summary

In summary the assay was simplified at each step to improve throughput and to attempt to improve reliability. The modifications described reduced cell preparation time from an average of approximately 3 hours to under 2 hours with improved cell yields.

3.4 Assay Reliability

Intra-assay Variation

Blood was taken from 1 individual with sufficient volumes to prepare cells for 8 receptor binding assays in parallel. From these, the intra-assay coefficient of variation for Kd and sites per cell was calculated as standard deviation/mean*100%.

Using the original assay, the coefficients of variation were 15% for Kd and 18% for sites per cell (table 3.2). With the refinements made to the assay described above these were reduced to 10% and 11% respectively. This reflects greater precision in the refined

assay. However, as a standard preparation of live cells was not available, no conclusions on the accuracy of either assay method can be drawn.

Table 3.2 Intra-assay Coefficient of Variation for Kd and Sites per Cell

	Original Assay		Refined Assay	
	Kd	Sites per cell	Kd	Sites per cell
Mean	6.56	1988	8.46	1191
Standard Deviation	1.01	361	0.83	136
Coefficient of Variation	15.3%	18.1%	9.8%	11.4%

Inter-assay Variation

Receptor binding assays were performed on 4 consecutive days for 1 subject. Performing these assays using the original and refined protocols provided estimates of the coefficient of variation for Kd and for sites per cell (table 3.3).

Both the original and refined assays showed greatly increased variation compared to intra-assay variation, and as will be discussed in the following chapters, this may have been a combination of assay error and environmental variation. However, the absolute differences in Kd and in sites per cell between the original and refined assays may reflect that these were performed in different months under different climatic conditions as will be discussed in section 6.6.

Table 3.3 Inter-assay Variation in Kd and Sites per Cell

	Original Assay		Refined Assay	
	Kd	Sites per cell	Kd	Sites per cell
Mean	14.1	3747	10.0	10412
Standard Deviation	3.13	584	2.07	1164
Coefficient of Variation	22.2%	15.6%	20.7%	11.1%

3.5 Summary

Modification of an established whole-cell receptor binding assay produced a simpler and more precise method suitable for use in larger population-based studies. In terms of the percentage coefficient of variation, this was reduced in both intra-assay and inter-assay versions of the new assay compared with the original assay reflecting greater precision.

Chapter 4

Glucocorticoid Receptor Binding in Twin Pairs

4.1 Introduction

As discussed in the sections 1.7 and 1.8.4 several lines of evidence suggest that glucocorticoids increase cardiovascular risk. This may be mediated by increased circulating levels of glucocorticoids, relative differences in access to various tissues by altering local metabolism, or to relative differences in glucocorticoid receptor binding characteristics between tissues.

Previous studies have demonstrated that a restriction fragment length polymorphism of the glucocorticoid receptor gene, which probably maps to the promoter or proximal intron of the gene, is associated with increased blood pressure and insulin resistance (Watt et al 1992, Weaver et al 1992). However, there are very few data on the relationship between glucocorticoid receptor binding characteristics and traditional cardiovascular risk factors. Furthermore, apart from the rare syndromes of glucocorticoid resistance due to mutation in the receptor gene and the polymorphism described above, there are no studies that have assessed the importance of genetic and environmental factors in determining glucocorticoid receptor binding characteristics. Accordingly, the studies in this chapter were established to examine these factors. Specifically, analysis of concordance rates for glucocorticoid receptor binding characteristics in monozygotic and dizygotic twins would provide a measure of the genetic component of this variable, while the relationship between binding and cardiovascular risk factors could also be performed.

4.2 Effect of Age and Sex on Glucocorticoid Receptor Binding

4.2.1 Description of Cohort

A total of 150 twin pairs were recruited from west central Scotland for the Scottish Twin Study. (Inglis et al 1999; cohort recruited by L.Swan). Of these, 104 pairs had complete data collection and were therefore suitable for analysis in the study represented here. No selection was made on self-reported identity or non-identity and the cohort was therefore expected to represent both monozygotic and dizygotic twins. Subjects were, however, excluded if they had taken glucocorticoid medication within the previous year, if they were taking anti-hypertensive medication or if they were diabetic.

Microsatellite analysis was performed on each twin pair at 6 informative loci to establish zygosity (kindly performed by G.C. Inglis). Those twin pairs of different sex, or whose microsatellite analysis was discordant were excluded from further rounds of microsatellite analysis and were categorised as dizygotic. The distribution of zygosity and sex is shown for each individual in table 4.1.

Table 4.1 Sex and Twin Distribution of Subjects

Individuals in each twin-pair	Monozygotic	Dizygotic	Total
Female	84	72	156
Male	24	28	52
Total	108	100	208

No differences were found in sex distribution between monozygotic and dizygotic twins, or in twin type between males and females.

The age distribution for male and female subjects is shown in table 4.2.

Table 4.2 Age and Sex Distribution of Subjects

Age	Minimum	Mean	Standard Deviation	Maximum
Female	29	52.2	11.4	79
Male	29	52.4	14.6	80

No significant differences were found in the age distributions for males and females.

4.2.2 Measurements

Blood samples were taken for glucocorticoid receptor binding assay, plasma steroid measurement both before and 30 minutes after 250μg of synacthen injected intravenously, urea and electrolytes, liver function tests, calcium, albumin, phosphate, fasting glucose, fasting cholesterol, fasting triglycerides, and full blood count. Additional measurements were made on 24-hour collections of urine for urinary steroid metabolites, and dual X-ray absorption densitometry performed at femoral neck, and spine. A complete list of investigations is detailed in Appendix 3.

4.2.3 Glucocorticoid Receptor Binding Characteristics

Glucocorticoid receptor binding assays were performed as described in the chapter 2. The distribution of the binding characteristics is shown in table 4.3.

Table 4.3 Glucocorticoid Receptor Binding Characteristics for Whole Cohort

	Median	Interquartile Range
Kd	7.3	3.8
Sites per cell	7022	4668

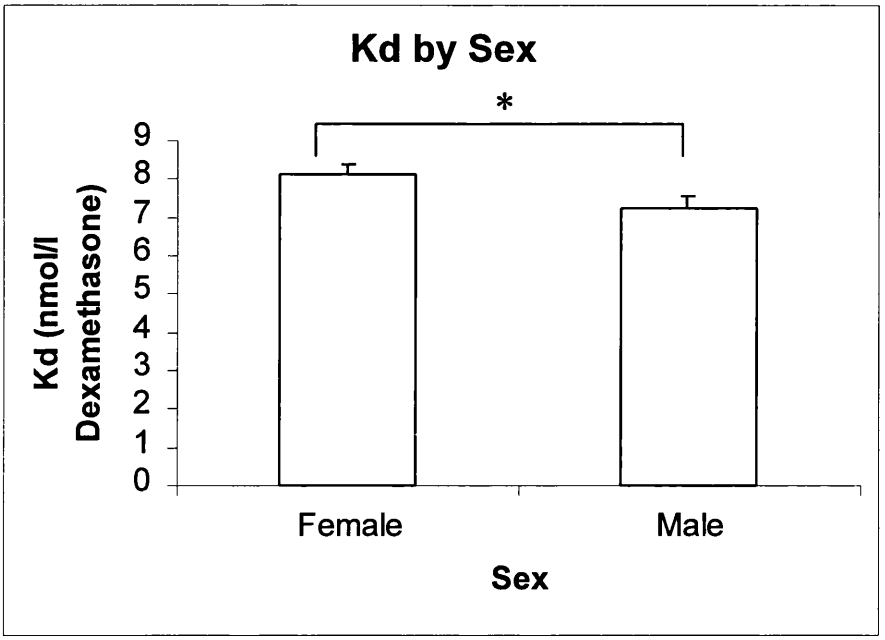
4.2.4 Sex Differences

Interaction with Kd

In order to examine the effect of sex on Kd, a 2-tailed non-paired homoscedastic t-test was performed. This showed a significant sex difference between men and women (T=2.09, p=0.039) with men having a lower Kd than women for dexamethasone binding (7.24 ± 2.42 versus 8.11 ± 3.09, mean ± standard deviation) and illustrated in figure 4.1.

Figure 4.1 Sex Difference in Glucocorticoid Receptor Kd

Kd is shown in nmol/l Dexamethasone. Error bars show standard error of the mean.



A biologically plausible mechanism to explain an influence of sex on Kd would be the effect of oestrogens and/or progestogens. If this were so, one would predict a difference between men and premenopausal women, but not between men and postmenopausal women. A summary of the subject characteristics by menopausal status is shown in table 4.4

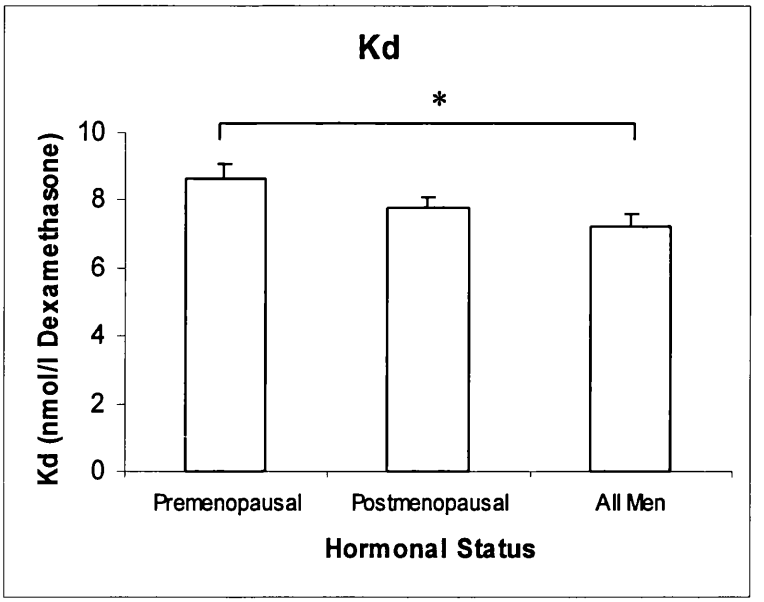
Table 4.4 Age and Receptor Binding Characteristics by Sex and Menopausal Status

	Number	Age		Kd		Sites perCell	
		Mean	*Std Dev*	*Mean*	*Std Dev*	*Mean*	*Std Dev*
Pre-menopausal ♀	62	43.39	8.7	8.647	3.214	7979	3908
Post-menopausal ♀	94	58.064	9.168	7.760	2.962	6904	3715
All ♂	52	52.38	14.62	7.242	2.422	7495	3177

No significant differences were found in age between men and premenopausal women or men and postmenopausal women, but a significant difference in age between pre- and postmenopausal women was shown ($T=-11.74$, $3p<0.001$). Comparison of pre-menopausal women with men, using a 2-tailed t-test, revealed a significant difference in Kd with men again having a lower Kd than pre-menopausal women for dexamethasone binding ($T=2.66$, $3p=0.027$, 7.24 ± 2.42 versus 8.65 ± 3.21, mean $\pm$ standard deviation, figure 4.2). There were no differences for Kd between pre-menopausal women and post-menopausal women, or between post-menopausal women and men. The possible confounding effect of age is addressed in the following section.

Figure 4.2 Difference in Glucocorticoid Receptor Kd by Sex Hormonal Status

A significant difference between men and premenopausal women only, is shown. No other significant differences between groups were found.



Interaction with sites per cell

The number of receptor sites per cell was compared between all men and all women, and between men and pre- or post-menopausal women. No significant differences were found.

4.2.5 Age Differences

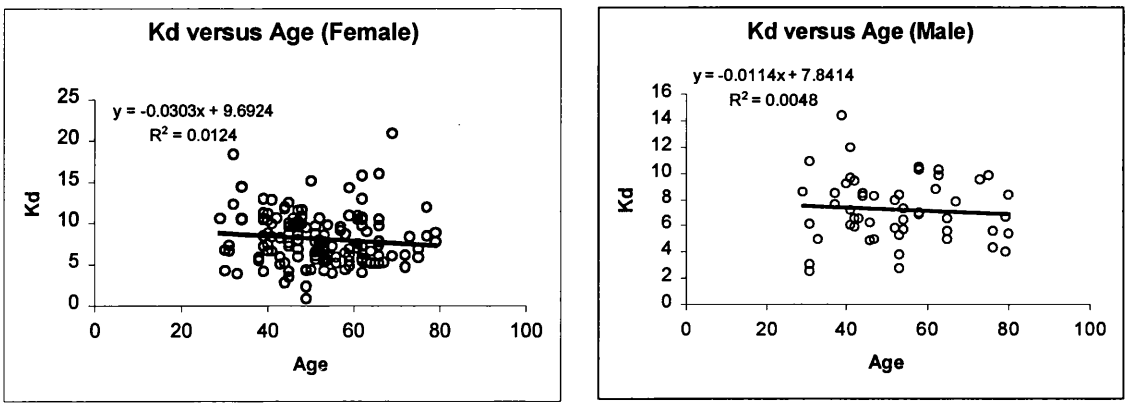
Interaction with Kd

To examine whether Kd altered with age linear regression was performed. This showed no significant interaction with r=0.1 and p=0.157.

However, as an interaction between sex and Kd was found, changes with age could occur independently for each sex that would be masked when both sexes were examined together. When linear regression was performed for Kd with age for females

or males separately, no association was found (females r=0.11, p=0.166:males r=0.07, p=0.626, figure 4.3). Further subgroup analysis by menopausal status also failed to show any significant interaction with age.

Figure 4.3 Receptor Kd by Age for Each Sex



Interaction with sites per cell

No association between age and sites/cell were found with r=0.09, p=0.167 for all subjects together, and r=0.13, p=0.113 for females, and r=0, p=0.917 for males.

4.2.6 Discussion

Kd showed an interaction with sex that was strongest between all men and premenopausal women suggesting oestrogen and progesterone either individually or together reduced affinity for glucocorticoid binding to the glucocorticoid receptor. The most likely explanation for this observation would be progestogen competing with glucocorticoid for binding at the receptor. Previous *in vitro* studies have shown that progesterone is able to interact with the glucocorticoid receptor at physiological concentrations (Sugino et al 1997) and that it reduces the affinity of the receptor for glucocorticoid, possibly by an allosteric mechanism (Svec et al 1980). No evidence exists for a similar effect of oestrogens on the receptor.

No effect on the number of receptor sites per cell was found suggesting sex steroids are not an important regulator of glucocorticoid receptor expression.

No effect of age on glucocorticoid receptor binding characteristics was found either when the cohort was analysed as a whole, or separately by sex.

4.3 Concordance

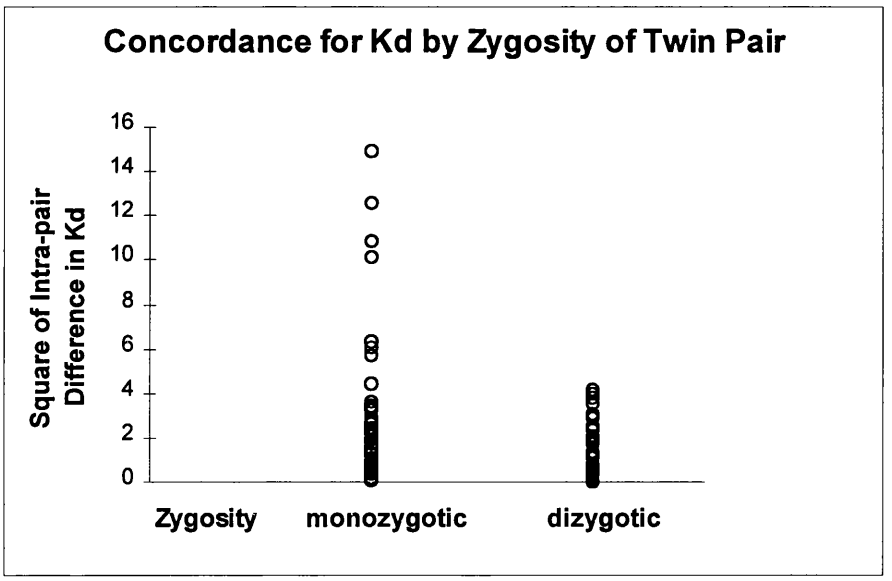
As receptor binding data were collected from twins on the same day under the same conditions, a measure of the inherited component of receptor binding can be assessed by comparing monozygotic twins with dizygotic twins. The square of the difference of Kd or sites per cell within each twin pair was taken and comparison between the two types of twins was done with a 2-tailed unpaired t-test.

4.3.1 Heritability of Receptor Binding Characteristics

Concordance for Kd

No difference was found between monozygotic and dizygotic twins for Kd (T=1.08, p=0.28) and illustrated in figure 4.4. This remained true when the sexes were analysed separately to allow for a sex-interaction (female T=1.00, p=0.32, male T=-0.05, p=0.96).

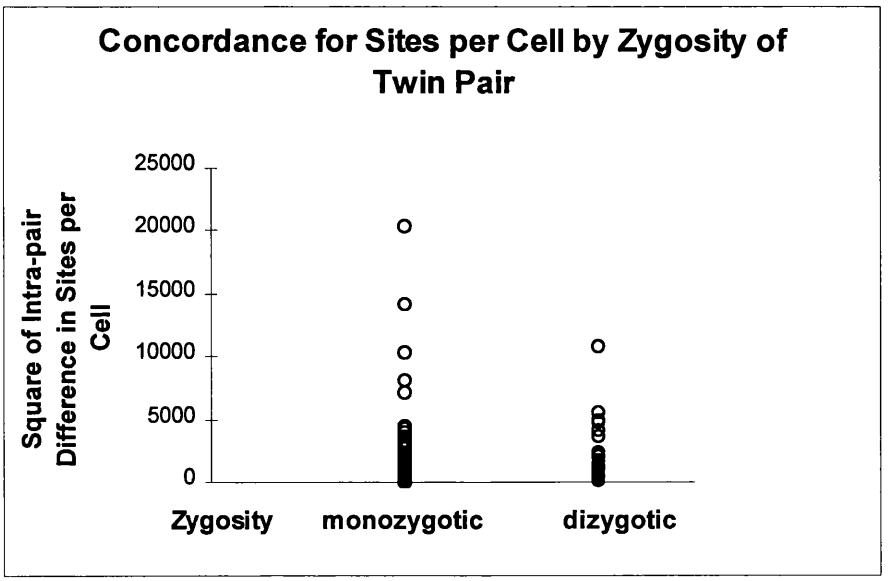
Figure 4.4 Concordance for Kd



Concordance for sites per cell

No differences were found for the number of receptor sites/cell (all twins T=0.79, p=0.43, female T=0.02, p=0.84, male T=0.97, p=0.35) as shown in figure 4.5.

Figure 4.5 Concordance for Sites per Cell



4.3.2 Twin-Twin Associations

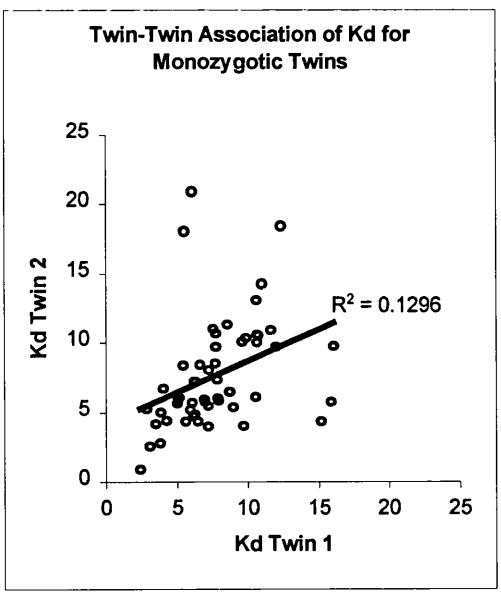
Comparing the concordance for Kd between monozygotic and dizygotic twins showed no significant evidence of a genetic component to either Kd or receptor sites per cell. If either of these binding characteristics were under environmental influence then twins from either monozygotic or dizygotic pairs would be expected to show similar values due to a shared environment.

Kd and sites per cell were analysed separately for monozygotic and dizygotic twin pairs plotting the value from one twin against the other twin: the choice of which twin was plotted on the *x* or *y* axis was random within each twin-pair.

Graphs for monozygotic and dizygotic twin-twin association are shown in figures 4.6, and twin-twin associations for sites per cell are shown in figure 4.7.

Figure 4.6 Twin-twin associations for Kd

Monzygotic twins are shown on the left and dizygotic twins on the right. R^2 values for linear regression within each twin cohort are shown in each graph.



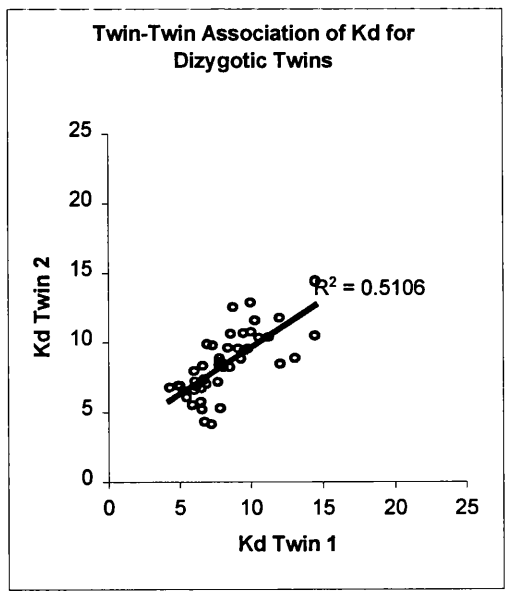
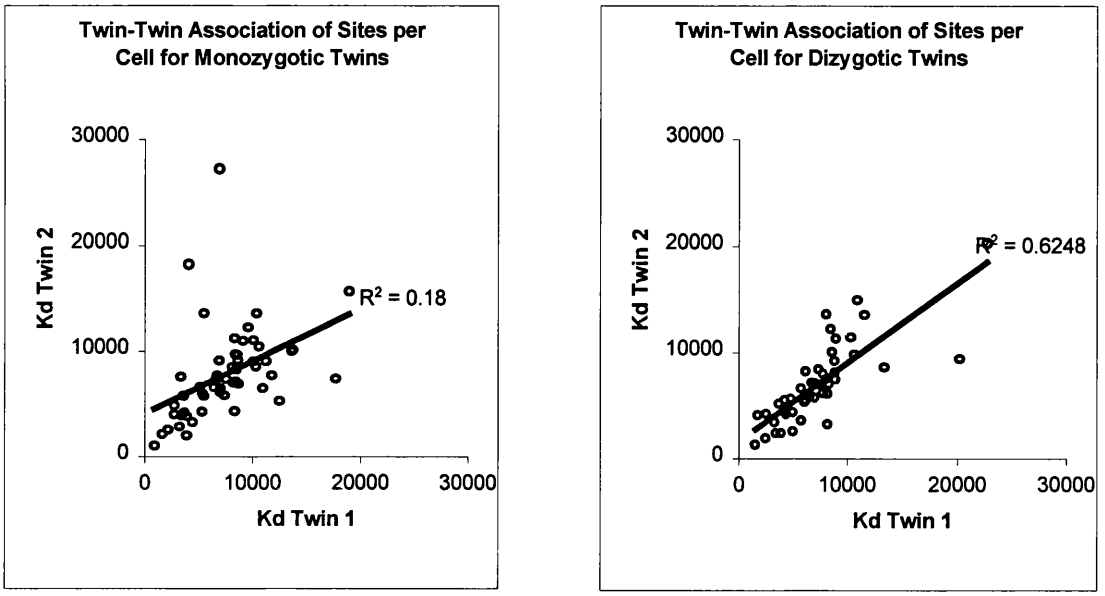


Figure 4.7 Twin-twin associations for Sites per Cell



Linear regression for each cohort of twins for Kd and for sites per cell was significant at the $p<0.005$ level.

4.3.3 Discussion

Comparing the concordance for Kd and sites per cell between monozygotic and dizygotic twins showed no evidence of increased concordance (as reduced variance) for monozygotic twins compared to dizygotic twins. This provides strong evidence that neither Kd nor sites per cell are under a significant genetic influence.

This would suggest that Kd and sites per cell may be under environmental control. To assess this, twin-twin associations were performed for each binding characteristic with the rationale that twins share environment from uterus through childhood and share many aspects of lifestyle into adulthood. This showed significant associations for Kd and sites per cell for monozygotic and dizygotic twins with R^2 values for Kd between

13% and 51% and for sites per cell between 18 % and 62%. That binding characteristics remain similar within a twin pair to adulthood in the absence of a genetic component is remarkable. Since twins would be expected to share most of the same environment in early life with lesser similarities in adulthood, this may suggest that early life programming of glucocorticoid sensitivity, as discussed in section 1.4, may be responsible for the effects seen. Alternatively, broader environmental influences such as water supply or climate which affect entire geographical regions, may exert an effect.

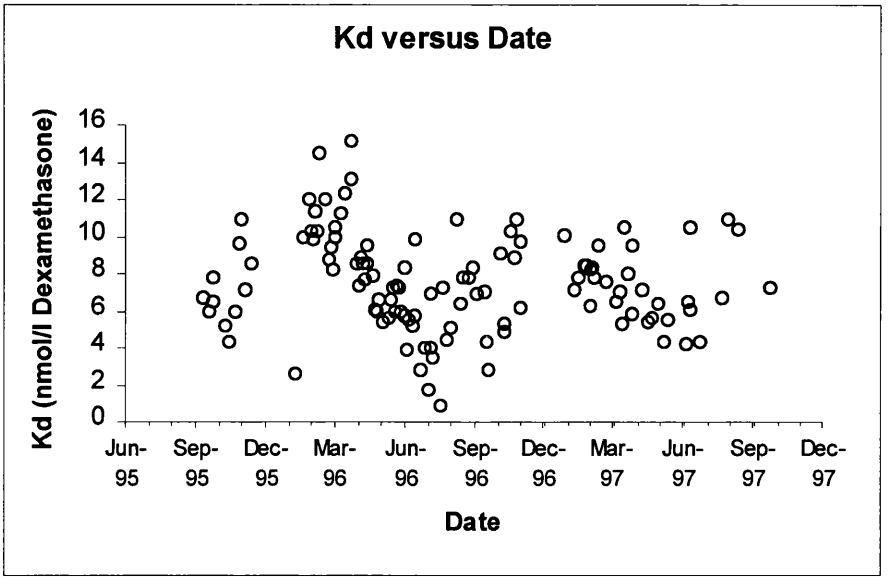
Finally, in view or the strong twin-twin associations found, subsequent analyses in this chapter are performed on a randomly chosen twin from each twin pair.

4.4 The Effect of Season

4.4.1 Interaction with Kd

As data was being collected it became clear that Kd appeared to vary predictably with time. When Kd was plotted against the date of sampling there appeared to be a sinusoidal pattern in Kd, figure 4.8.

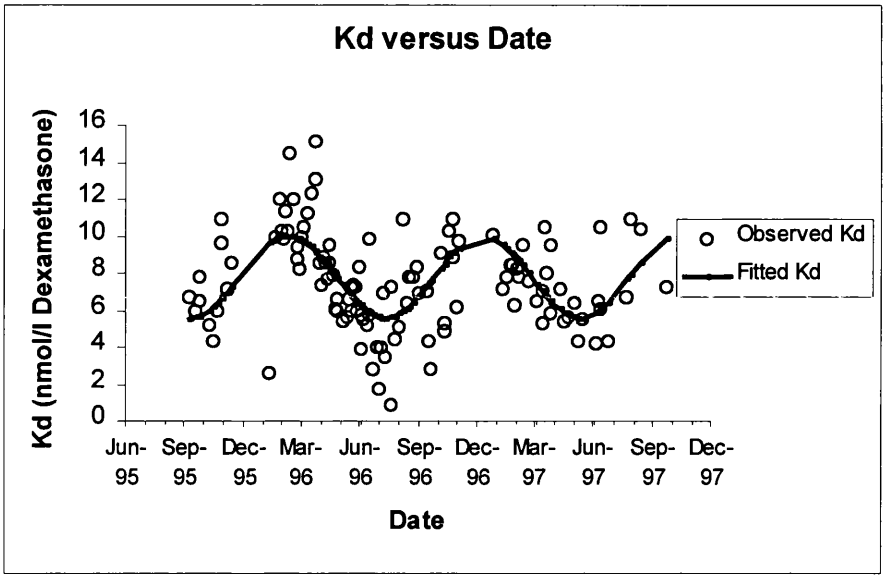
Figure 4.8 Variation of Kd with Time



Furthermore, the pattern appeared to vary with season with highest Kd in winter and lowest in summer and the highest Kd peak coincided with the coldest local winter on record with environmental temperatures of –20°C. Using a commercial curve-fitting program (CurveExpert – © Douglas Hyams, 911 Madden Bridge Road, Central, SC 29630, USA) able to employ a number of mathematical models to fit a data set by non-linear regression, a sinusoidal model fitted best ($r=0.5$, $p<0.0001$), Figure 4.9.

Figure 4.9 Kd by Day of Sampling with Fitted Sinusoid

The dates of sampling were converted to days since 1st January 1995, and fitted by non-linear regression. This provided a fit with equation y=7.76+2.21*cosine(0.021*day-2.37).



As a plausible seasonal effect could be mediated by climate, climatic data were collected from the local meteorological station (kindly supplied by the Meteorological Office). Linear regression for Kd with minimum environmental temperature, maximum environmental temperature and day length were all statistically significant as shown in table 4.5. Graphs of the linear regressions are shown in figures 4.10-4.12.

Table 4.5 Interaction between Kd and Climate

Interaction with Kd	*p*=	*r*=
Minimum Temperature	**<0.0001**	**-0.33**
Maximum Temperature	**<0.0001**	**-0.48**
Day Light	**<0.0001**	**-0.33**

Figure 4.10 Interaction of Kd with Minimum Temperature

Kd is in nmol/l dexamethasone. Minimum environmental temperature on the day of blood sampling is in °C.

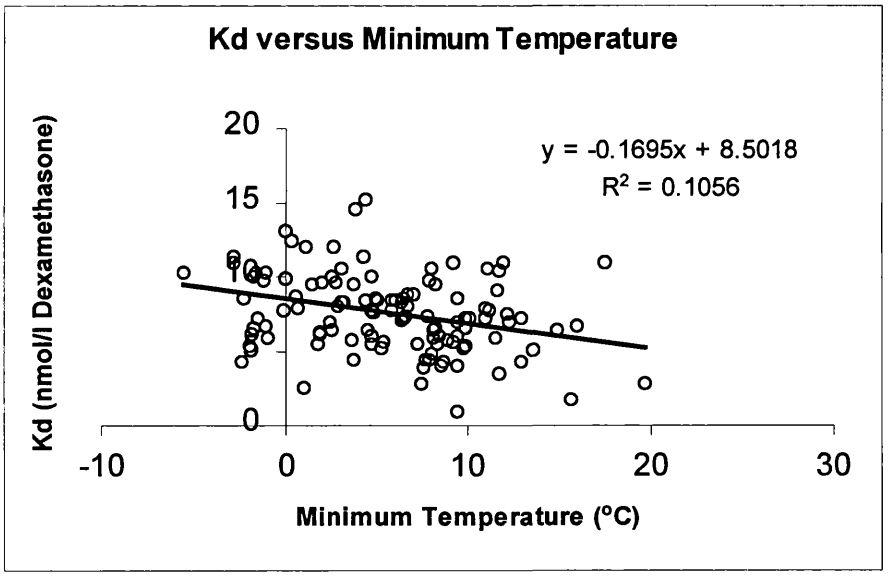


Figure 4.11 Interaction of Kd with Maximum Temperature

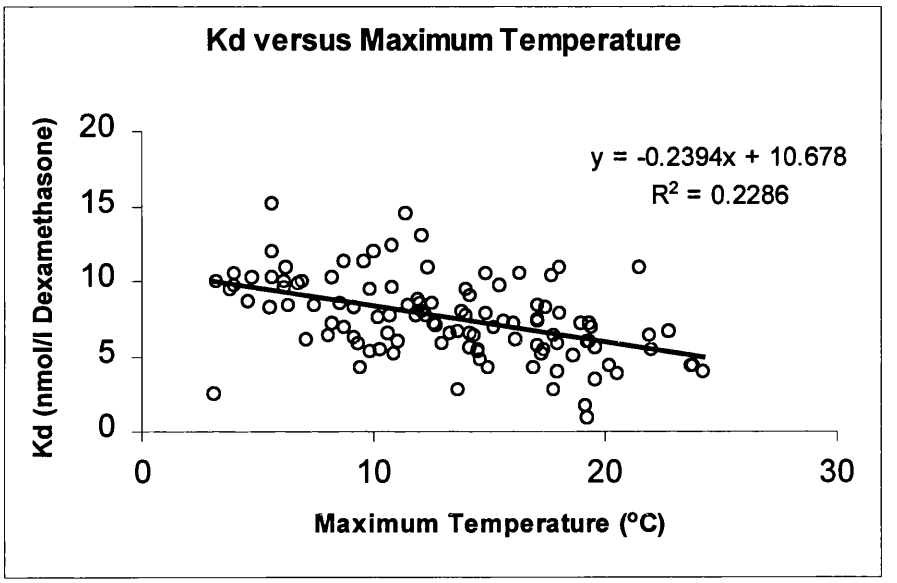
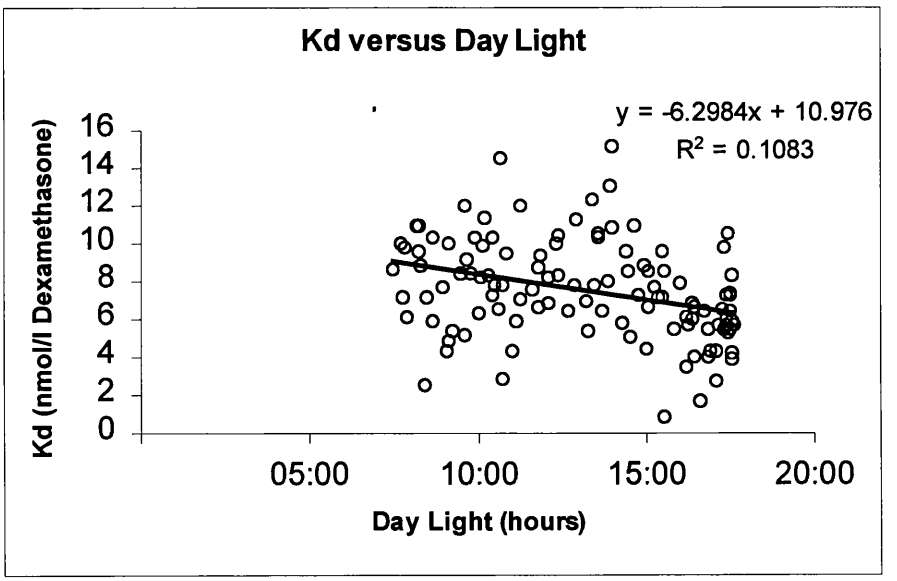


Figure 4.12 Interaction of Kd with Day Length

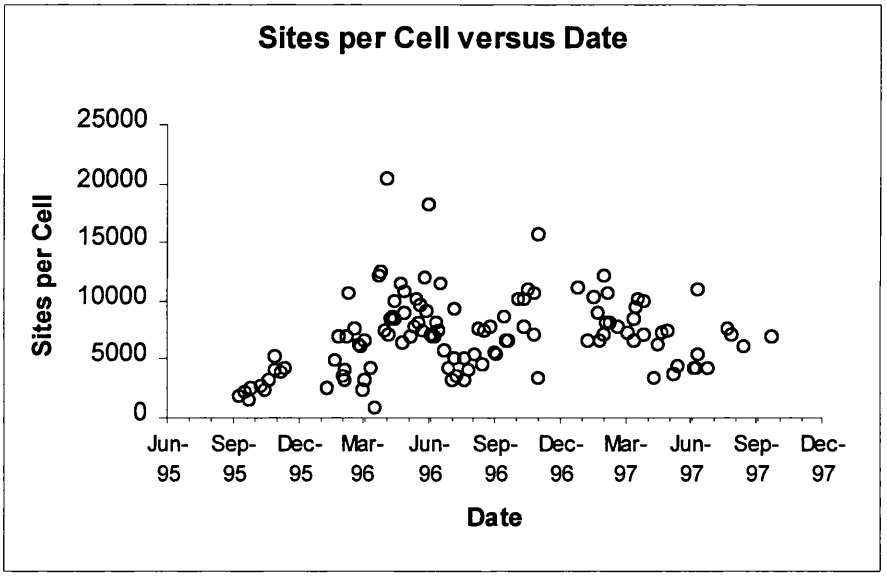


Using multiple regression with minimum temperature, maximum temperature and day length as explanatory variables for Kd, the total explained variance was unchanged compared to the univariate analysis for maximum temperature alone (r^2=0.229 $p<0.0001$ versus r^2=0.229 $p<0.0001$).

4.4.2 Interaction with Sites per Cell

When the number of receptor sites per cell was plotted against time there was less obvious seasonal variation in sites/cell, figure 4.13.

Figure 4.13 Receptor Sites per Cell by Time



When this data was modelled using the CurveExpert program there was no significant fit with a seasonal model, the best fit being provided by a simple linear model.

Furthermore, on linear regression analysis with sites per cell as the response variable and minimum temperature, maximum temperature or day light as the predictor variables, there were no significant associations as shown in table 4.6.

Table 4.6 Interaction between Sites per Cell and Climate

Interaction with Sites/cell	*p*=	*r*=
Minimum Temperature	0.96	0.0
Maximum Temperature	0.61	-0.04
Day Light	0.17	0.13

4.4.3 Discussion

Kd showed a striking seasonal pattern. Associations were found between Kd and minimum and maximum environmental temperature and for day length. The glucocorticoid receptor shows temperature sensitive binding characteristics with reduced affinity, but normal numbers of receptor sites, in hyperthermic subjects (Molijn et al 1995). This is in contrast to the effect found with season where higher affinity was found at higher environmental temperatures. We were careful to perform our binding assays at 25 °C for 20 hours in a thermostatically controlled environment to eliminate temperature-induced variation.

It seems unlikely, therefore, that environmental temperature directly affected receptor binding. A more plausible explanation would be that environmental temperature, and possibly day light, interacts *in vivo* with the glucocorticoid receptor by altering the production of an intermediate substance capable of modulating the receptor's affinity. This could be a simple molecule or a complex protein assembly. An obvious analogy for the simple molecule model is the effect of 2,3-bisphosphoglyerate that binds allosterically to haemoglobin increasing its affinity for oxygen (Baldwin and Clothia 1979). Any simple substance would have to be resistant to the repeated washing and

long incubations of the binding assays; cortisol itself is not a candidate molecule as it was undetectable after cell preparation. Large molecule interactions could include any of the components of the heat shock protein assembly (Pratt 1993).

In contrast, sites per cell showed no clear seasonal component, although variation throughout the year was nonetheless evident.

4.5 Biological Associations with Glucocorticoid Receptor Binding Characteristics

From discussion in section 1.7 glucocorticoid excess has many effects on body composition and metabolism, particularly affecting body mass, cardiovascular risk, electrolyte handling and bone metabolism. The glucocorticoid receptor is key in regulating the hypothalamo-pituitary-adrenal axis and to assess whether variation of glucocorticoid receptor binding characteristics affected steroid synthesis or metabolism we collected detailed steroid metabolite data from each subject. Anthropometric, biochemical and bone densitometry data were collected to assess the physiological impact of glucocorticoid activity.

4.5.1 Interaction with Steroid Metabolites

To examine the interaction of glucocorticoid receptor binding with steroid synthesis and metabolism, plasma and urinary steroid concentrations were measured using radioimmunoassay and gas chromatography mass spectrometry respectively (as described in Inglis et al 1999. RIA was kindly performed by Prof R Fraser, and GCMS by Miss M Ingram). The principal variables measured were basal plasma cortisol and aldosterone, cortisol and aldosterone responses 30 minutes after intravenous injection of 250μg of synacthen, and the unstimulated urinary metabolites of cortisol,

(tetrahydrocortisol [THF] and allo-tetrahydrocortisol [aTHF],) cortisone, (tetrahydrocortisone [THE]) and of aldosterone, (tetrahydroaldosterone [THaldo]). By taking the ratio of total cortisol metabolites (THF + aTHF) over total cortisone metabolites (THE), an index of overall 11β-hydroxysteroid dehydrogenase activity was derived, subsequently referred to here as the THF-ratio.

Interaction with Kd

Receptor Kd showed no interaction with plasma cortisol, aldosterone, or with any of the urinary metabolites THF, aTHF, THE or THaldo, as summarised in table 4.7.

Table 4.7 The Interaction of Kd with Corticosteroids and Metabolites

The *p* and *r* values are shown for the interaction between Kd and the variables listed using linear regression. Where the distribution of a variable deviated significantly from a normal distribution, the variable was log transformed. All log transformed variables had normal distributions. Residuals after each regression were normal. Statistically significant interactions are highlighted in bold text. Caveat - p values for regression were not adjusted for multiple comparisons on the basis that these were exploratory calculations: significance therefore has to be treated with caution.

Interaction with Kd	***p*=**	***r*=**
Log basal cortisol	0.21	-0.12
Log basal aldosterone	0.76	-0.03
Log stimulated cortisol	0.11	-0.15
Log stimulated aldosterone	0.27	-0.11
Log THF	0.12	-0.15
Log aTHF	0.52	-0.06
Log THE	0.14	-0.45
Log THF-ratio	0.78	0.03
Log THaldo	0.17	0.16

Interaction with sites per cell

Receptor sites per cell showed no interaction with plasma cortisol or aldosterone. Interactions were found between sites per cell and the urinary metabolites THF-ratio

and THaldo, but not with THF, aTHF or the THE. Results are summarised in table 4.8, with significant interactions shown in figures 4.12 and 4.13.

Table 4.8 Interaction of Sites per Cell with Corticosteroids and Metabolites

Interaction with sites per cell	*p*=	*r*=
Log basal cortisol	0.10	-0.07
Log basal aldosterone	0.95	0.0
Log stimulated cortisol	0.10	-0.16
Log stimulated aldosterone	0.37	0.08
Log THF	0.46	0.07
Log aTHF	0.82	0.0
Log THE	0.10	0.16
Log THF-ratio	**0.01**	**-0.24**
Log THaldo	**0.03**	**0.25**

Figure 4.14 Interaction of Sites per Cell with log THF-ratio

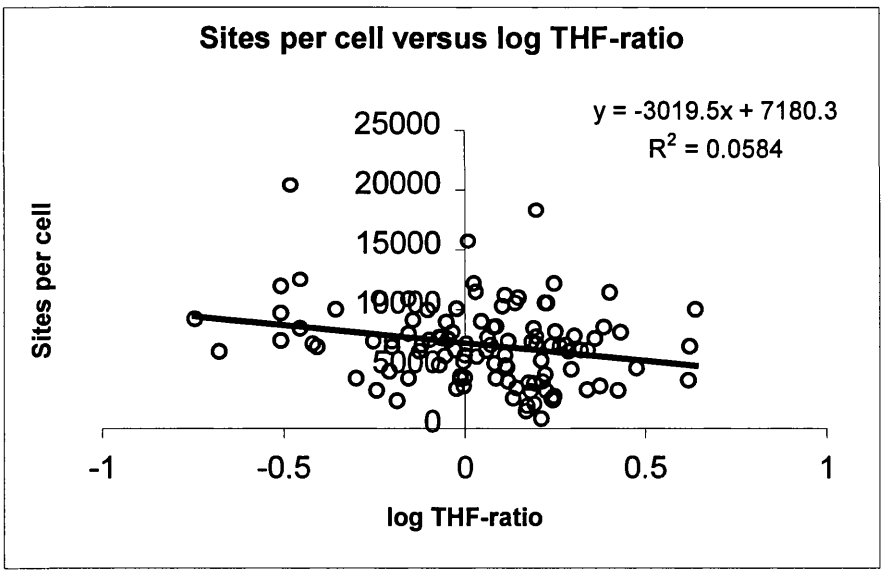
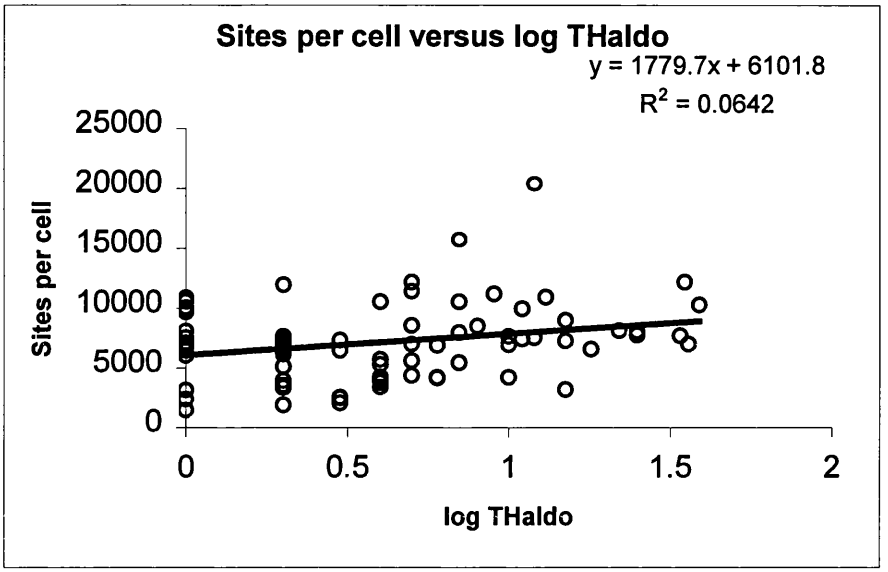


Figure 4.15 Interaction of Sites per Cell with THaldo



Discussion

No interaction between plasma cortisol or urinary cortisol metabolites and Kd or sites per cell was found suggesting the receptor assayed in leukocytes does not reflect central glucocorticoid receptor activity, and that physiological variation in cortisol synthesis does not affect leukocyte glucocorticoid receptor affinity.

The number of sites per cell did, however, show a negative correlation with the THF-ratio (an indirect measure of whole body 11β-hydroxysteroid dehydrogenase activity) and a positive correlation with THaldosterone urinary excretion. The mechanism for these changes, in the absence of any detectable change in plasma levels, remain unclear. Furthermore, the association between the number of sites per cell and THaldo excretion appears to be artefactual and caused by a number of low THaldo values skewing the regression. However, inhibition of 11β-hydroxysteroid dehydrogenase produces a rise in the THF-ratio and suppressed aldosterone production (Palermo et al

1996). What remains unknown is whether the glucocorticoid receptor increases 11β-hydroxysteroid dehydrogenase expression or activity.

4.5.2 Interaction with Anthropometric Data

Cortisol excess frequently produces a phenotype with increased central obesity with increased body mass index (BMI) and notably an increased waist:hip ratio (WHR) due to increased visceral fat deposition. In addition, cortisol exposure in utero has been proposed as a risk factor for intrauterine growth retardation and reduced birth weight. Data was therefore collected for height, weight, body mass index, waist hip ratio, and birth weight.

Interaction with Kd

No interaction was found between Kd and any of these variables. The results of linear regression are shown in table 4.9.

Table 4.9 Interaction of Kd with Anthropometric Measurements

Body mass index (BMI) was calculated as the weight in Kg divided by the square of the height in metres. Waist-hip ratio (WHR) was calculated as waist circumference divided by the hip circumference.

Interaction with Kd	***p*=**	***r*=**
Height	0.99	0.0
Weight	0.35	0.08
BMI	0.16	0.13
WHR	0.49	-0.07
Birth weight (by recall)	0.43	-0.08

Interaction with sites per cell

The interaction between sites per cell and anthropometric data was tested by linear regression. No significant interaction was found for any variable. Results of the regressions are shown in table 4.10.

Table 4.10 Interaction of Sites per Cell with Anthropometric Measurements

Interaction with sites per cell	***p*=**	***r*=**
Height	0.55	-0.05
Weight	0.49	0.06
BMI	0.21	0.12
WHR	0.42	0.08
Birth weight (by recall)	0.78	-0.03

Discussion

None of the variables weight, BMI or WHR were found to interact with Kd or sites per cell. This suggests that changes in Kd do not reflect peripheral tissue sensitivity to glucocorticoids. Conversely, states of relative obesity do not alter Kd or receptor sites per cell, in contrast to the effect of obesity on cortisol where plasma concentrations remain little changed, and urinary excretion greatly increases (Andrew et al 1998).

4.5.3 Interaction with Cardiovascular Risk Factors

The development of cardiovascular disease is associated with a number of risk factors that include hypertension, hyperglycaemia and dyslipidaemia. Cortisol excess is known to increase all these variables and may exert its adverse influence on cardiovascular disease through these mechanisms. To explore the interaction between altered glucocorticoid receptor signalling and a range of cardiovascular risk factors, blood pressure, fasting glucose and fasting lipids were collected from each subject.

Interaction with Kd

No significant interactions were found between Kd and the cardiovascular risk factors detailed above as shown in table 4.11.

Table 4.11 Interaction of Kd with Cardiovascular Variables

Interaction with Kd	*p*=	*r*=
Lying Systolic Blood Pressure	0.75	0.03
Lying Diastolic Blood Pressure	0.16	0.13
Lying Pulse	0.32	0.09
Standing Systolic Blood Pressure	0.57	0.05
Standing Diastolic Blood Pressure	0.37	0.08
Standing Pulse	0.46	0.07
Fasting Glucose	0.69	0.03
Log Fasting Triglycerides	0.29	0.10
Log Fasting VLDL	0.24	0.12
Fasting Cholesterol	0.16	0.13
Log LDL	0.09	-0.17
HDL	0.13	-0.15
HDL:LDL ratio	0.91	0.0

Interaction with sites per cell

No significant interactions were found between sites per cell and the cardiovascular risk factors detailed above as shown in table 4.12.

Table 4.12 The Interaction of Sites per Cell with Cardiovascular Variables

Interaction with sites per cell	*p*=	*r*=
Lying Systolic Blood Pressure	0.61	-0.04
Lying Diastolic Blood Pressure	0.79	-0.03
Lying Pulse	0.57	0.05
Standing Systolic Blood Pressure	0.97	0.0
Standing Diastolic Blood Pressure	0.81	0.0
Standing Pulse	0.51	0.06
Fasting Glucose	0.77	0.03
Log Fasting Triglycerides	0.26	0.10
Log Fasting VLDL	0.48	0.08
Fasting Cholesterol	0.31	-0.09
Log LDL	0.11	-0.16
HDL	0.45	-0.07
HDL:LDL ratio	0.91	0.0

Discussion

No association was found between Kd or sites per cell and blood pressure, lipid subfractions or glucose. This suggests that variation in leukocyte Kd does not reflect glucocorticoid activity in the key tissues of vasculature, liver and pancreas.

Previous studies have demonstrated that, in the leukocyte glucocorticoid receptor binding assay using dexamethasone as the radiolabel, hypertensive subjects have a lower affinity than normotensives when cortisol is used as the competing ligand, but not when dexamethasone competes (Mulatero et al 1997). Since the assay used in the present study used dexamethasone as the labelled and competing ligand these results are consistent. A previous report showed a positive correlation between receptor Kd and plasma cholesterol (Panarelli et al 1998). I did not replicate this finding. Finally, a lack of association between Kd and fasting glucose is, perhaps, unsurprising as subjects known to be diabetic were excluded from study, and this study lacked power to detect an effect within the closely regulated physiological concentrations of glucose.

4.5.4 Interaction with Plasma Electrolytes

Glucocorticoids bind to mineralocorticoid receptors with similar affinity to glucocorticoid receptors in vitro. In vivo, the distal tubules and collecting ductules are protected from the mineralocorticoid effects of glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase type 2. To examine the effects of glucocorticoids on renal electrolyte handling plasma electrolytes were collected.

Interaction with Kd

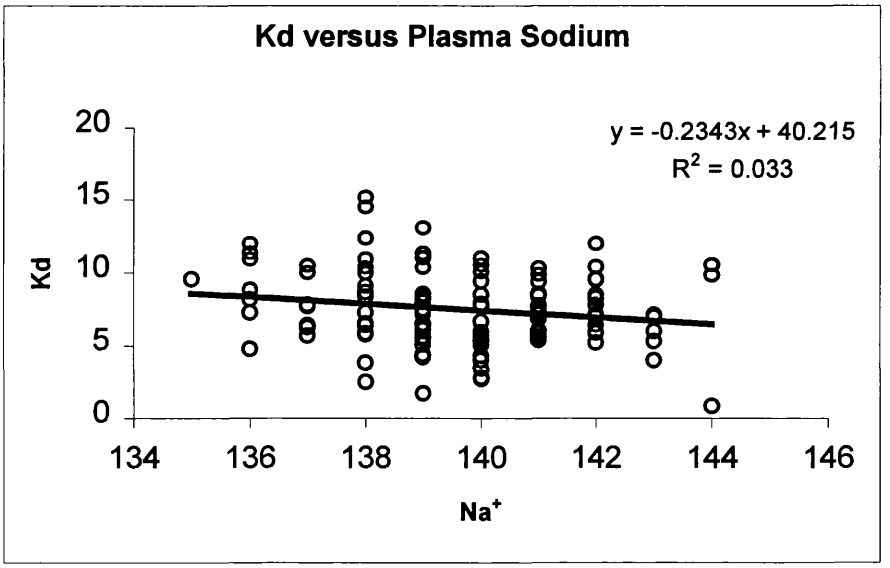
Linear regression showed a significant interaction between Kd and plasma sodium and creatinine as shown in table 4.13 and figure 4.16.

Table 4.13 Interaction of Kd with plasma electrolytes

Interaction of Kd with:	p=	r=
Sodium	**0.05**	**-0.18**
Potassium	0.87	0.0
Chloride	0.73	0.03
Carbon Dioxide	0.25	-0.11
Urea	0.63	-0.04
Creatinine	0.24	-0.11

Figure 4.16 Interaction of Kd with Plasma Sodium

Sodium is shown in mmol/l.



Interaction with sites per cell

No significant interaction between sites per cell and plasma electrolytes were found. Results of linear regression are shown in table 4.14.

Table 4.14 Interaction of Sites per Cell with Plasma Electrolytes

Interaction of Sites/cell with:	*p*=	*r*=
Sodium	0.46	-0.07
Potassium	0.12	-0.15
Chloride	0.49	-0.06
Carbon Dioxide	0.67	0.04
Urea	0.70	-0.03
Creatinine	0.10	0.15

Discussion

Kd showed a small interaction with plasma sodium concentration where a lower Kd was associated with a higher plasma sodium. This was a small effect, one that may have been due to confounding by a few outliers at the upper range of sodium concentrations, and should be interpreted with caution in the context of multiple significance tests. With those reservations however, glucocorticoid deficiency results in a characteristic hyponatraemia that is mainly due to reduced free water clearance (De Leacy et al 1991). If a higher Kd resulted in reduced glucocorticoid activity in the nephron, this may reduce free water clearance sufficiently to dilute the sodium concentration towards the lower end of the reference range. An alternative explanation that increased glucocorticoid activity in the nephron increases sodium reabsorption seems unlikely as glucocorticoid excess is not associated with increases in plasma sodium. The converse hypothesis that physiological changes in sodium concentration alter glucocorticoid receptor affinity does not apply in this system where the concentration of sodium in the incubation is strictly controlled.

No interaction between sites per cell and electrolytes was found. This is in contrast to a previous positive correlation between the number of receptor sites and plasma sodium (Panarelli et al 1998).

4.5.5 Interaction with Bone Metabolism

Long term exposure to relatively modest glucocorticoid excess is known to reduce bone mineral density. Part of our hypothesis was that altered glucocorticoid metabolism over time may manifest itself as reduced bone mineral density which might accelerate the rate of bone loss with increasing age. Each twin therefore had dual-X ray absorption densitometry measurements made at hip and spine for comparison with Kd and sites per cell.

Interaction with Kd

No significant interaction was found between Kd and bone mineral density by linear regression as shown in table 4.15.

Table 4.15 Interaction of Kd with Bone Mineral Density

Bone mineral density is shown as the T value. Similar results were found for Z values.

Interaction with Kd	***p*=**	***r*=**
Femoral Neck T	0.52	0.06
Trochanter T	0.33	0.09
Intertrochanter T	0.45	0.07
Ward's Area T	0.19	0.12
Femur T	0.45	0.07
L 1-4 T	0.35	0.09

Interaction with sites per cell

Significant interactions between sites per cell and bone mineral density were found at the greater trochanter, intertrochanteric region and total femur as shown in table 4.16 and figures 4.17-4.19.

Table 4.16 Interaction of Sites per Cell with Bone Mineral Density

Interaction of sites per cell with:	***p*=**	***r*=**
Femoral Neck T	0.23	0.01
Trochanter T	**0.01**	**0.24**
Intertrochanter T	**0.02**	**0.22**
Ward's triangle T	0.14	0.14
Femur T	**0.02**	**0.22**
L 1-4 T	0.88	0.0

Figure 4.17 Interaction of Sites per Cell with Trochanteric Bone Mineral Density

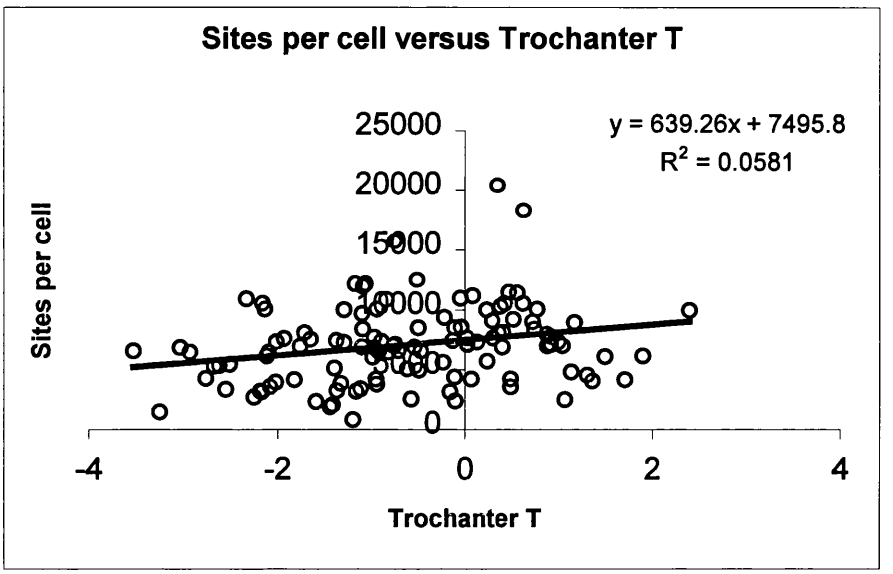


Figure 4.18 Interaction of Sites per Cell with Intertrochanteric Bone Mineral Density

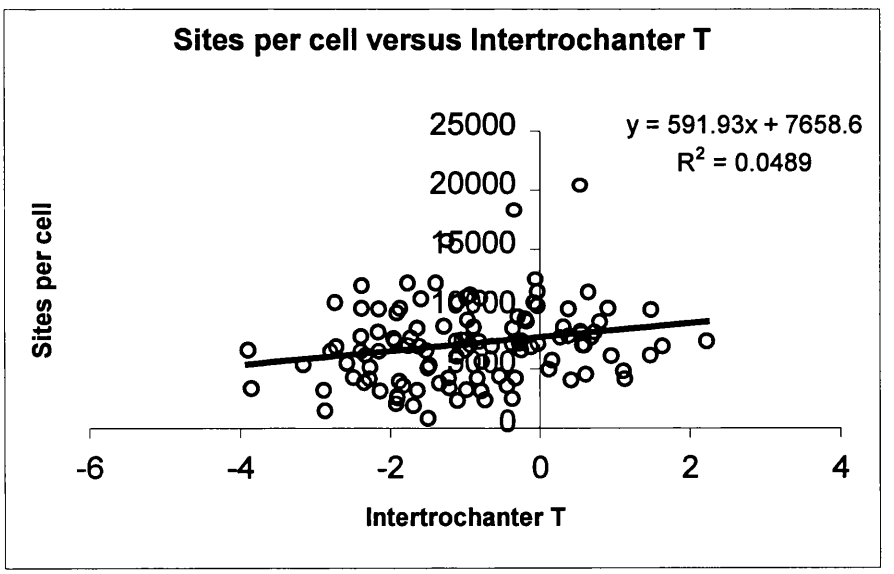
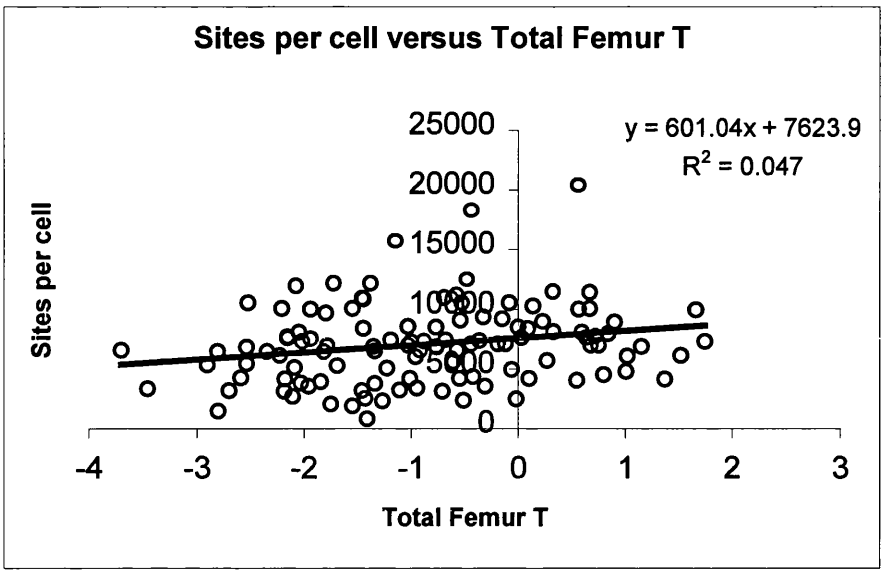


Figure 4.19 Interaction of Sites per Cell with Total Femoral Bone Mineral Density



Discussion

No association between glucocorticoid receptor Kd and bone mineralisation at femur or spine was found. However, strong associations were found between sites per cell and bone mineral density at the greater trochanter, intertrochanteric region and total femur. Previous studies have demonstrated pronounced bone mineral density loss caused by glucocorticoids that affects both spine and hip (Saag et al 1998). In this study we found a positive correlation between sites per cell and mineral density, a finding which would be contrary to the hypothesis that increased sensitivity to circulating glucocorticoids results in increased rates of bone loss. An alternative explanation, that both receptor number and mineral density are confounded by sex steroids is not borne out by subgroup analysis by sex or menopausal status.

4.6 Multivariate Analysis

A model was constructed to explain varitaion in receptor binding charactersistcs from the variables identified above. Using variables identified from the preceding univariate analyses that interacted with receptor binding characteristics, those variables with weak associations, those that may be confounded by outliers, and those for which no biological plausibility applied were excluded. This resulted in the exclusion of the interaction between sites and THaldo, Kd and sodium and sites and bone mineral density. A stepwise, forward selection approach was used to identify likely predictors from the pool of interacting univariate variables and these were then analysed using multiple regression. Where the introduction of a variable to the model failed to increase its predictive power significantly, the variable was rejected from the model.

Interactions with Kd

From the pool of putative predictor variables identified above, maximum environmental temperature alone predicted the highest variance in Kd of 22.9%. Adding sex, minimum temperature or day light failed to increase the predictive power of the model. The equation for Kd is :

Kd = 10.7 - 0.239*Max Temp

R^2=22.9%

$p<0.0001$

Interactions with sites per cell

Only Log THF-ratio showed a significant interaction for sites per cell, and the equation is given below.

Sites = 7180 - 3019*$\log_{10}$(THF-ratio)

R^2=5.8%

$p=0.01$

Chapter 5

Receptor Binding Characteristics in Normal Subjects

5.1 Introduction

In another cohort study, the Midspan study, the offspring of subjects characterised for cardiovascular risk factors in 1970 were recruited from west central Scotland. The original study, the Renfrew-Paisley study (Hawthorne et al 1974), was established between 1972 and 1976 to examine prevalent cardiovascular risk factors and subsequent cardiovascular and respiratory morbidity and mortality. In 1994-1996 the offspring of these original subjects were contacted and studied as part of a second generation family study of cardiovascular risk (Hawthorne et al 1995). Part of this study included measurements of anthropomorphic data (including weight, height, and waist-hip ratio and birth weight) cardiovascular risk factors (blood pressure, pulse, fasting glucose, cholesterol, triglycerides, LDL cholesterol and HDL cholesterol) and steroid metabolites (plasma cortisol and aldosterone). Taking the opportunity to measure glucocorticoid receptor characteristics in this cohort allowed me to explore whether there was any relationship between glucocorticoid receptor binding characteristics and cardiovascular risk factors.

5.2 Description of Cohort

A total of 180 subjects (106 females and 74 male) were recruited for whom a complete data set was available. The age distribution is shown in table 5.1.

Table 5.1 Age Distribution of Cohort

(Std Dev is standard deviation)

	Number	Minimum	Mean	Std Dev	Maximum
Female	106	21	44.4	5.2	54
Male	74	32	43.4	5.7	54

There were no significant differences between the ages of the females and males when compared by a 2-tailed t-test (T=1.16, p=0.25).

5.3 Measurements

Each subject had height and weight measured and body mass index calculated. Blood was taken for glucocorticoid receptor binding assay, basal cortisol, fasting glucose, fasting insulin, fasting triglycerides and fasting cholesterol. A 75g load of glucose was then given and blood taken at 2 hours for glucose and insulin measurement.

5.4 Glucocorticoid Receptor Binding Characteristics

Glucocorticoid receptor binding assays were performed as described in chapter 2. The distribution of receptor binding characteristics is shown in table 5.2.

Table 5.2 Glucocorticoid Receptor Binding Characteristics

	Minimum	Mean	Std Dev	Maximum
Kd	0.6	8.4	3.3	16.8
Sites per cell	1932	7956	3276	10015

5.5 Sex Differences

Interaction with Kd

Evidence of a sex interaction with Kd was tested with a 2-sample 2-tailed t-test. This showed no significant interaction with sex (T=0.12, p=0.91). This is in contrast with the age effect in Kd in the twin study. However, the present study was composed of a different population with correspondingly lower mean ages and smaller ranges in ages. As an interaction with age within the female group was found in the twin study, a smaller range in age in the present study would be expected to conceal any interaction with age and sex.

Interaction with sites per cell

No interaction with sex was found for sites per cell (T=-0.44, p=0.66).

5.6 Age Differences

Interaction with Kd

Using linear regression, no evidence of an interaction with was found (r=-0.08, p=0.16). There remained no interaction when females and males were analysed separately.

Interaction with sites per cell

No interaction between sites per cell and age were found by linear regression for females and males together (r=-0.05, p=0.43) or separately (r=-0.06, p=0.67).

5.7 Interaction with Season

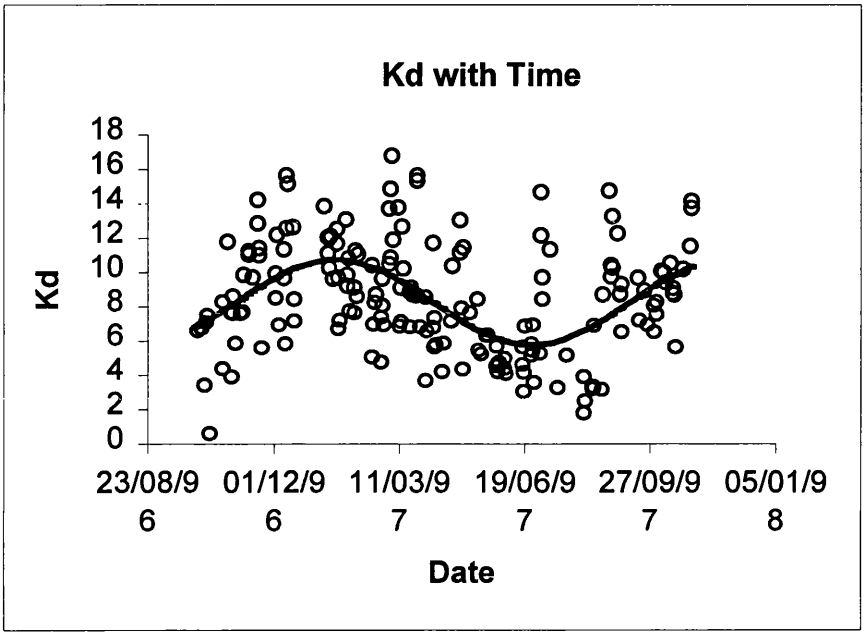
Interaction with Kd

As in the twin study, a clear seasonal effect on Kd was found. When Kd was plotted with time the most descriptive model using the CurveExpert package was a sinusoidal relationship ($p<0.0001$) as shown in figure 5.1.

Figure 5.1 Kd with Time

(Kd is shown in nmol/l dexamethasone).

The equation for the fitted sinusoid is 8.25+2.49*cosine(0.002*day-1.38) where the argument for cosine is in radians and the date is the number of days since the 1st of January 1996.

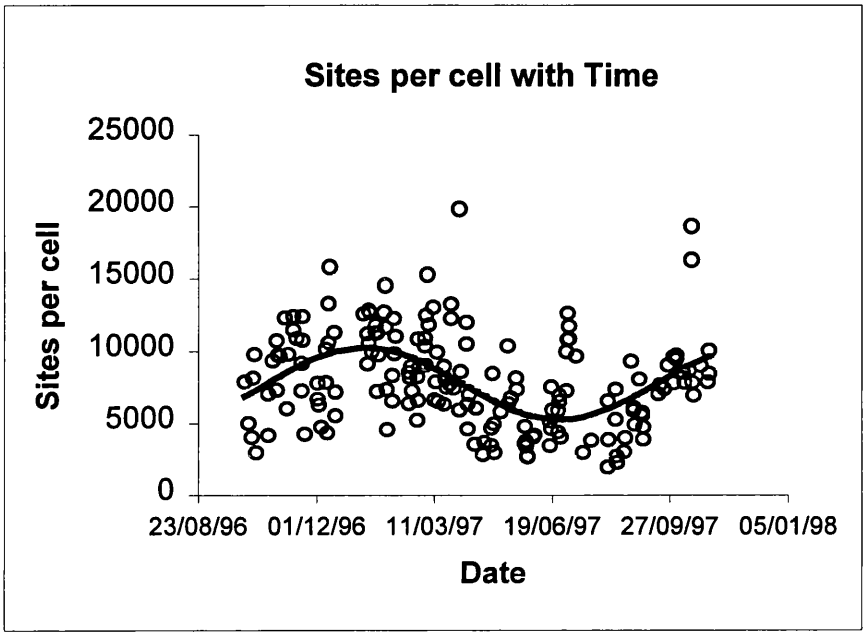


Interaction with sites per cell

Furthermore, the number of sites per cell showed variation with time and this showed a significant fit with a sinusoidal model ($p<0.0001$) as shown in figure 5.2.

Figure 5.2 Sites per Cell with Time

The equation of the fitted sinusoid is 7750+2480*cosine(0.019*date-0.85) where the argument for cosine is in radians and the date is the number of days since the 1st of January 1996.



Summary

Both Kd and sites per cell show a significant interaction with time which can be modelled by a sinusoid suggesting a seasonal interaction.

5.7.1 Effect of Climate

Interaction with Kd

To examine the effect of climate on Kd, meteorological data were collected and evidence for an interaction tested by linear regression using minimum environmental temperature, maximum temperature or day length to explain Kd. All showed significant interactions as shown in table 5.3 and in figures 5.3, 5.4 and 5.5.

Table 5.3 Interaction of Kd with Climate

Interaction of Kd with	*p*=	*r*=
Minimum temperature	**<0.0001**	**-0.25**
Maximum temperature	**<0.0001**	**-0.35**
Day length	**<0.0001**	**-0.45**

Figure 5.3 Interaction between Kd and Minimum Temperature

Kd is in nmol/l dexamethasone and minimum temperature is in °C.

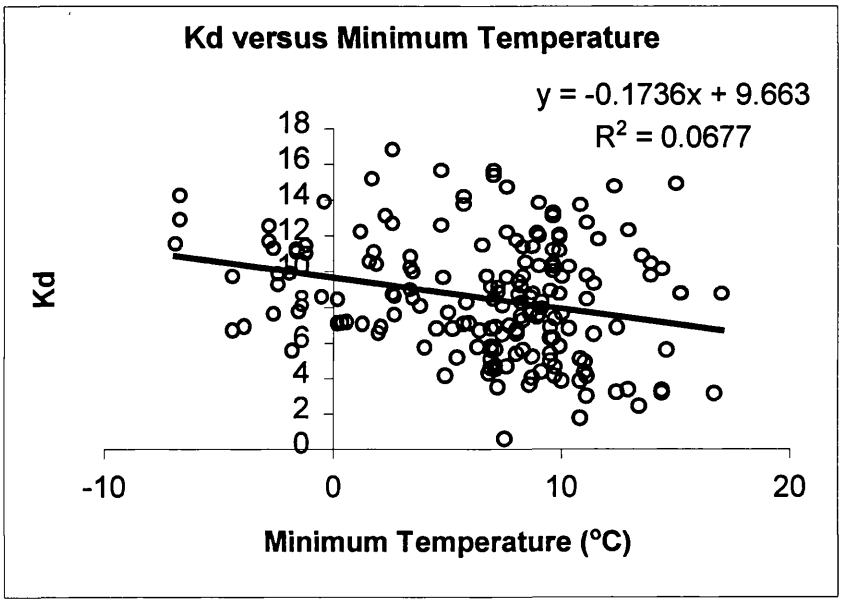


Figure 5.4 Interaction between Kd and Maximum Temperature

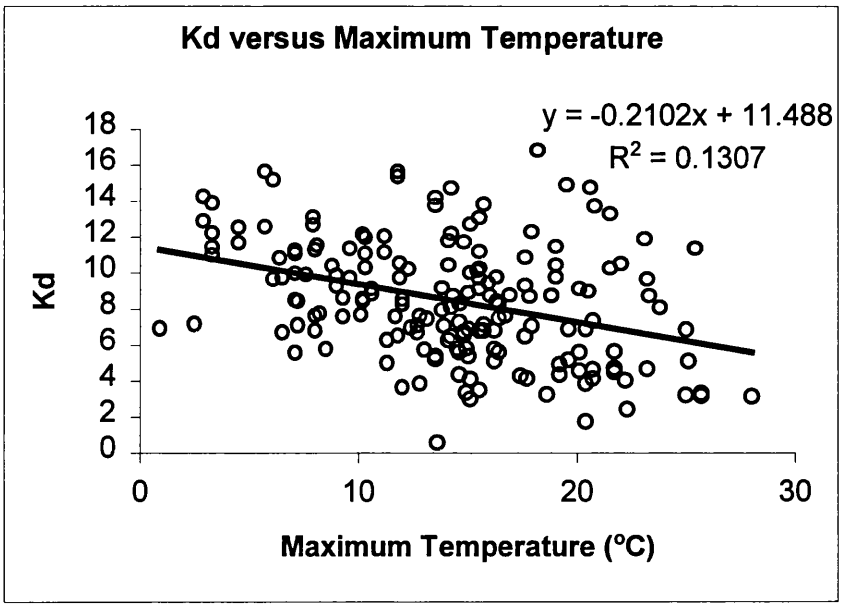
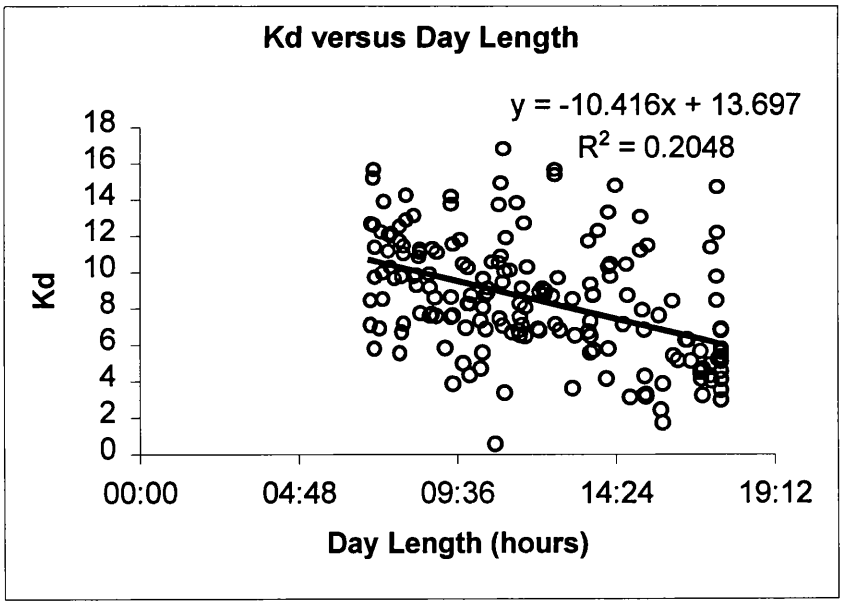


Figure 5.5 Interaction between Kd and Day Length



Interaction with sites per cell

Linear regression was performed to examine the relationship between climate and sites per cell. This showed significant interactions as shown in table 5.4 and in figures 5.6, 5.7 and 5.8.

Table 5.4 Interaction of Sites Per Cell with Climate

Interaction of sites per cell with	*p*=	*r*=
Minimum temperature	**0.001**	**-0.25**
Maximum temperature	**<0.0001**	**-0.41**
Day length	**<0.0001**	**-0.45**

Figure 5.6 Interaction of Sites Per Cell with Minimum Temperature

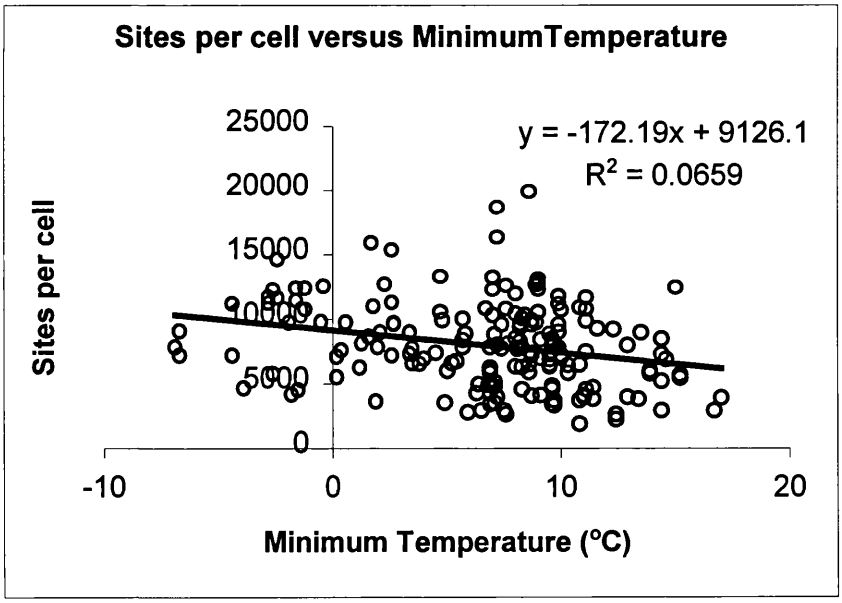


Figure 5.7 Interaction of Sites Per Cell with Maximum Temperature

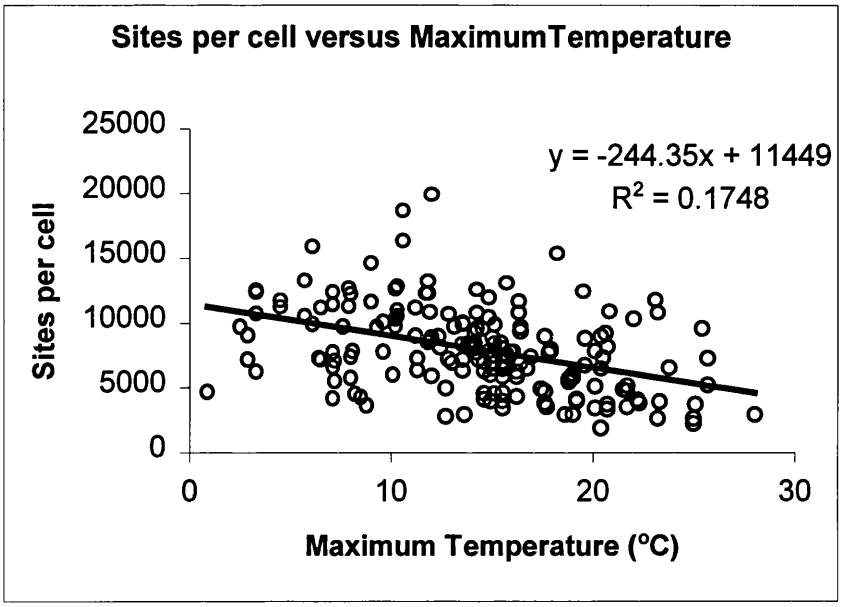
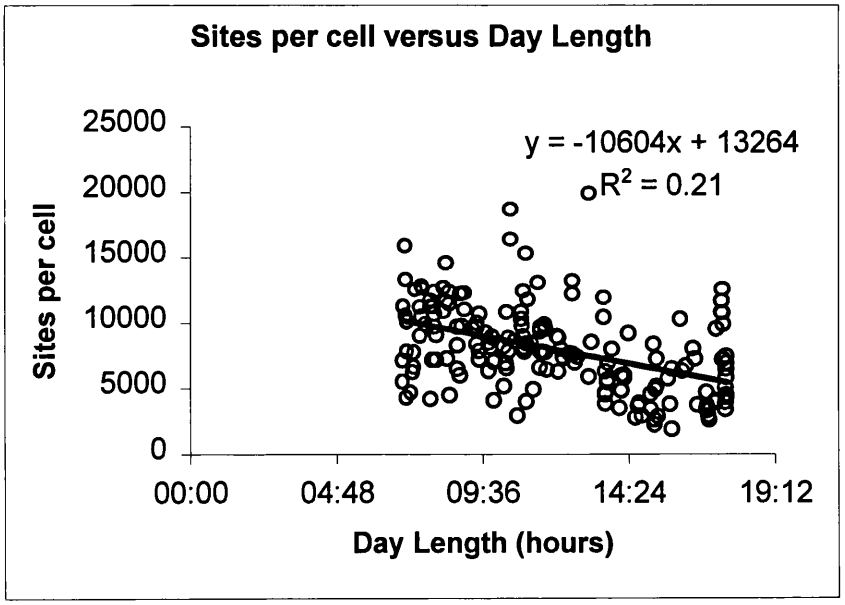


Figure 5.8 Interaction of Sites Per Cell with Day Length



Summary

Both Kd and sites per cell show significant association with minimum temperature, maximum temperature and day length.

5.8 Interaction with Cortisol

Basal cortisol was measured on an unstressed sample from each subject at 09:00 – 09:30am. No interaction between basal plasma cortisol and either Kd or sites per cell was found as shown in table 5.5

Table 5.5 Interaction between Plasma Cortisol and Kd or Sites Per Cell

Interaction with Plasma Cortisol	***p*=**	***r*=**
Kd	0.37	0.07
Sites per cell	0.21	0.09

5.9 Interaction with Anthropometric Data

Interaction with Kd

Subjects' height and weight were measured and from this the body mass index (BMI) calculated. There were no significant interactions with Kd and any of these variables as shown in table 5.6.

Table 5.6 Interaction between Kd and Anthropometric Measurements

Interaction with Kd	*p*=	*r*=
Height	0.53	0.0
Weight	0.51	0.0
BMI	0.37	0.07
WHR	0.20	-0.1
Birth weight	0.92	0.0

Interaction with sites per cell

No interactions between sites per cell and anthropometric measurements were found as shown in table 5.7.

Table 5.7 Interaction between Sites Per Cell and Anthropometric Measurements

Interaction with sites per cell	*p*=	*r*=
Height	0.37	-0.07
Weight	0.52	-0.02
BMI	0.31	-0.08
WHR	0.25	-0.09
Birth weight	0.76	0.0

5.10 Interaction with Cardiovascular Risk Factors

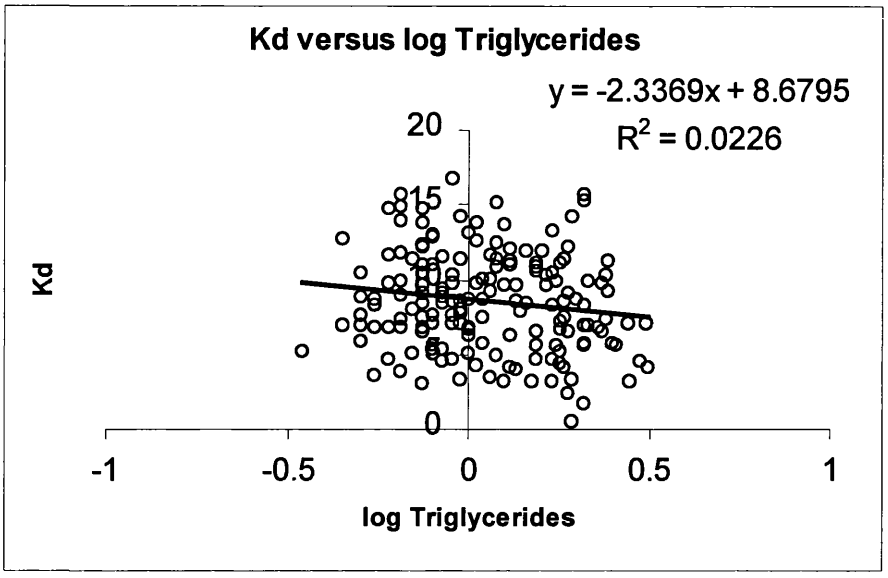
Interaction with Kd

A number of cardiovascular variables were measured on each subject including resting pulse, resting systolic blood pressure, resting diastolic blood pressure, fasting glucose, fasting insulin, fasting triglycerides, fasting cholesterol, and glucose and insulin 2 hours after a 75g glucose load. A significant interaction between Kd and log transformed fasting triglycerides was found, with the linear regression of the interaction shown in figure 5.9. The results of linear regression are shown in table 5.8. It should be noted that these were exploratory regressions and the p values are not adjusted for multiple tests. Values, therefore, need to be interpreted with caution.

Table 5.8 Interaction of Kd with Cardiovascular Variables

Interaction of Kd with	***p*=**	***r*=**
Resting pulse	0.89	0.0
Systolic blood pressure	0.86	0.0
Diastolic blood pressure	0.32	0.07
Fasting glucose	0.31	0.08
Log fasting insulin	0.47	0.05
Log Fasting triglycerides	**0.05**	**-0.15**
Fasting cholesterol	0.86	0.0
2 hour glucose	0.94	0.0
Log 2 hour insulin	0.77	0.03

Figure 5.9 Interaction of Kd with log Triglycerides



Interaction with sites per cell

No significant interaction was found between sites per cell and any of the cardiovascular variables listed above as shown in table 5.9.

Table 5.9 Interaction of Sites per Cell with Cardiovascular Variables

Interaction of sites per cell with	***p*=**	***r*=**
Resting pulse	0.32	0.08
Systolic blood pressure	0.98	0.0
Diastolic blood pressure	0.19	0.09
Fasting glucose	0.56	0.04
Log fasting insulin	0.87	0.0
Log Fasting triglycerides	0.09	-0.13
Fasting cholesterol	0.42	0.06
2 hour glucose	0.74	-0.03
Log 2 hour insulin	0.62	0.03

5.11 Interaction with Electrolytes

Interaction with Kd

No significant interaction between Kd and either plasma sodium or potassium was found as shown in table 5.10.

Table 5.10 Interaction of Kd with Electrolytes

Interaction of Kd with	*p*=	*r*=
Sodium	0.07	0.14
Potassium	0.79	0.0

Interaction with Sites per cell

No significant interaction between Kd and either plasma sodium or potassium was found as shown in table 5.11.

Table 5.11 Interaction of Sites per Cell with Electrolytes

Interaction of Sites per cell with	*p*=	*r*=
Sodium	0.06	0.15
Potassium	0.94	0.0

5.12 Multivariate Analysis

Interactions with Kd

Using the variables found to have significant interaction with Kd on univariate regression (i.e. minimum temperature, maximum temperature, day length and log triglycerides) a multivariate analysis was performed. From this, day length and log triglycerides explained more of the variance (23.8%) in Kd than the other variables.

Including plasma sodium, which had borderline significance, did not refine the predictive power of the model. The equation for Kd was:

Kd=14.1-10.9*day length – 2.68 $\log_{10}$ (triglycerides)

For sites per cell, day length also explained more variance in sites per cell than the other climatic variables (21.3%). Adding maximum temperature, but not minimum temperature, to the model significantly improved the predictive value explaining 23.8% of variance in sites per cell. Adding plasma sodium to the model did not increase its predictive power. The multiple regression equation is:

Sites per cell=13463-313 * day length-121 * maximum temperature

5.13 Conclusions

5.13.1 Age and Sex

The cohort was well matched for age and sex with no significant difference for age between female and male subjects. No interaction between Kd or sites per cell and age or sex was found.

5.13.2 Season

Kd showed a significant interaction with climate in keeping with findings in the twin cohort. On univariate regression, minimum temperature, maximum temperature and day length all showed significant interactions, but day length alone provided the best model, in contrast to the twin study where maximum temperature was most predictive. It should, however, be noted that all the measured climatic variables are highly correlated with one another and correlation in statistical analysis does not imply a causal mechanism. The finding of a seasonal effect in both studies is, therefore, entirely consistent with the earlier finding in the twin study.

For sites per cell all three climatic variables showed significant interaction individually, and when combined in multiple regression, day length remained the best explanatory variable with maximum temperature making a small, but significant, refinement to the model.

This is in contrast to the finding of no significant interaction between sites per cell and climate in the twin study. The Midspan study included a larger number of subjects that the Twin study (180 versus 104 subjects selected from each twin pair). This would be expected to increase the power of the study and may explain the apparent inconsistency between populations. A further confounding influence between the studies may be caused by the geographical situation of each population. In the Midspan study all subjects resided within the Renfrew or Paisley districts and climatic data were collected from the Glasgow airport meteorological station, a maximum distance of 11 kilometres from the subjects home. In contrast, subjects in the Twin study were recruited from the whole of Scotland and it was impossible to obtain accurate climatic data for the whole country. This discrepancy may explain part of the weaker or absent interactions seen between climate and receptor binding characteristics.

5.13.3 Steroid Metabolism

No interaction between Kd or sites per cell and plasma cortisol was found. It is plausible that the leukocyte receptor does not behave similarly to the receptor in the central nervous system, where glucocorticoid receptor activity is known to regulate cortisol secretion by the adrenal (Bornstein and Chrousos 1999). However, plasma cortisol has poor predictive power in assessing overall cortisol production. More powerful methods of measuring cortisol production include label dilution methods (Linder et al 1990) and 24 hour urinary metabolite excretion measurement (Yap et al 1992). The former method

uses radiolabelled or stable isotope labelled cortisol injected intravenously with the ratio of labelled to unlabelled (i.e. endogenous) cortisol measured at equilibrium giving an accurate measure of total circulating cortisol. The latter method measures steroid metabolites by their separation in a gas chromatography column and subsequent detection by mass and charge of molecular fragments in a mass spectrometer (gas chromatography mass spectrometry – GCMS). However, more detailed assessment of the cortisol phenotype was not possible in this study.

5.13.4 Anthropometry

No interaction between Kd or sites per cell and height, weight, BMI, WHR or birth weight was found. This finding is consistent with the twin study and suggests either that glucocorticoid receptor activity in leukocytes does not reflect glucocorticoid sensitivity in key tissues such as adipose tissue, or that glucocorticoids are not important in mediating growth and obesity.

5.13.5 Cardiovascular Risk Factors

No interaction between Kd or sites per cell and pulse, blood pressure, glucose or insulin (either fasting or 2 hours after a 75g glucose load) or fasting cholesterol were found. A weak interaction between Kd and fasting triglycerides was found. The role of triglycerides in cardiovascular disease remains controversial, however (Gotto 1998, Sattar et al 1998) and its effect on cardiovascular risk is not likely to be substantial.

5.13.6 Electrolytes

No significant interaction between Kd or sites per cell and plasma sodium or potassium was found although previous studies have demonstrated an interaction between plasma sodium and the number of sites per cell (Panarelli et al 1998).

Chapter 6

Effect of Season on Receptor Binding

6.1 Introduction

Assays of receptor binding characteristics in the large populations from the Twin study and the Fastcard study had both revealed a significant interaction between climatic variables and Kd, with variable effects on sites per cell. However, these were cross sectional studies and it is important to establish that the apparent seasonality is seen in individual subjects. In order to test the hypothesis that receptor binding characteristics varied within each individual in a seasonal pattern, subjects were recruited for serial receptor binding assays over 1 year. Nine healthy male subjects between 23 and 61 years of age were recruited: female subjects being excluded to avoid the risk of inducing iron deficiency anaemia from the repeated venepuncture, and to avoid the possible confounding effect of sex found in the Twin study (but not the Fastcard study). Blood was collected between 09:00 and 09:30 hours every 3 weeks and receptor binding assays performed as described previously. Plasma was separated for measurement of cortisol and tri-iodothyronine assays to test the hypothesis that these hormones affect glucocorticoid receptor activity.

6.2 Statistics

The statistical analysis of serial data is complex as data points are not necessarily independent of one another and cannot simply be analysed by standard regression methods. A model was developed using the Stata statistical package that allowed single and multiple regression to be performed making allowances for repeated measures and giving accurate estimates for probability.

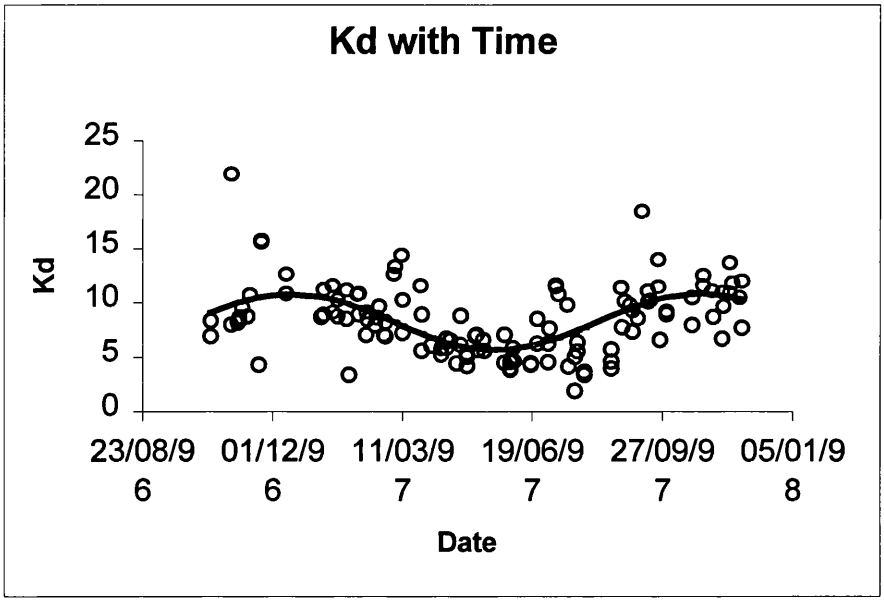
6.3 Interaction with Season

Interaction with Kd

For each subject Kd varied with season in a similar manner to that observed in the Twin and Fastcard populations with high Kd in the winter months and low Kd in summer. The best fit using the Curve Expert program was for a sinusoidal curve ($p<0.001$, F-test), figure 6.1.

Figure 6.1 Seasonal Variation in Kd

Kd is shown in nmol/L dexamethasone. The sinusoidal fit has the equation 8.24+2.57*cosine(0.02*(days since 1/1/96)+0.336) where the argument for cosine is in radians.



Univariate regression correcting for repeated measures was performed between Kd and the climatic variables minimum temperature, maximum temperature and day length. Ths showed significant interaction with Kd as shown in table 6.3 and in figures 6.2-6.4.

Table 6.1 Interaction between Kd and Climate

Interaction between Kd and	*p*	*r*
Minimum temperature	0.02	-0.22
Maximum temperature	0.01	-0.27
Day Length	0.001	-0.48

Figure 6.2 Interaction of Kd with Minimum Temperature

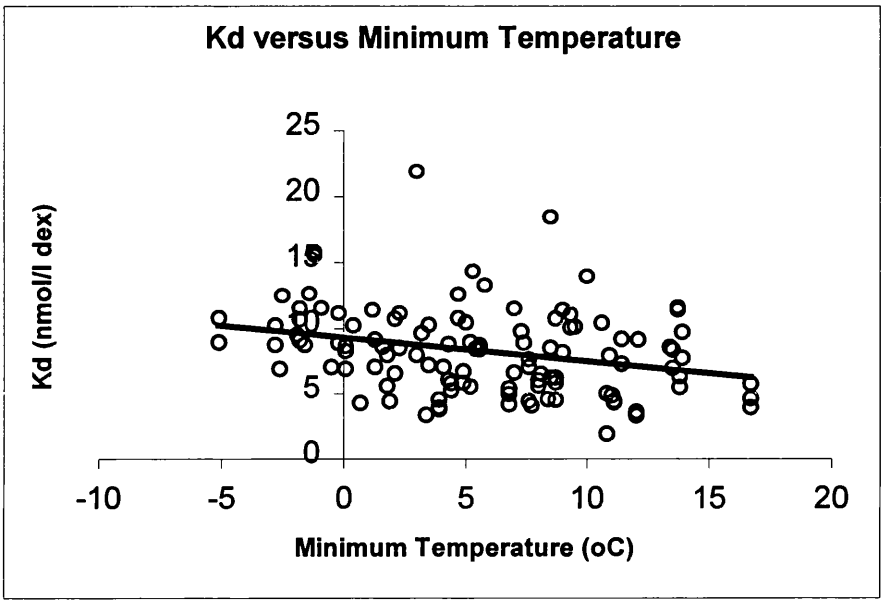


Figure 6.3 Interaction of Kd with Maximum Temperature

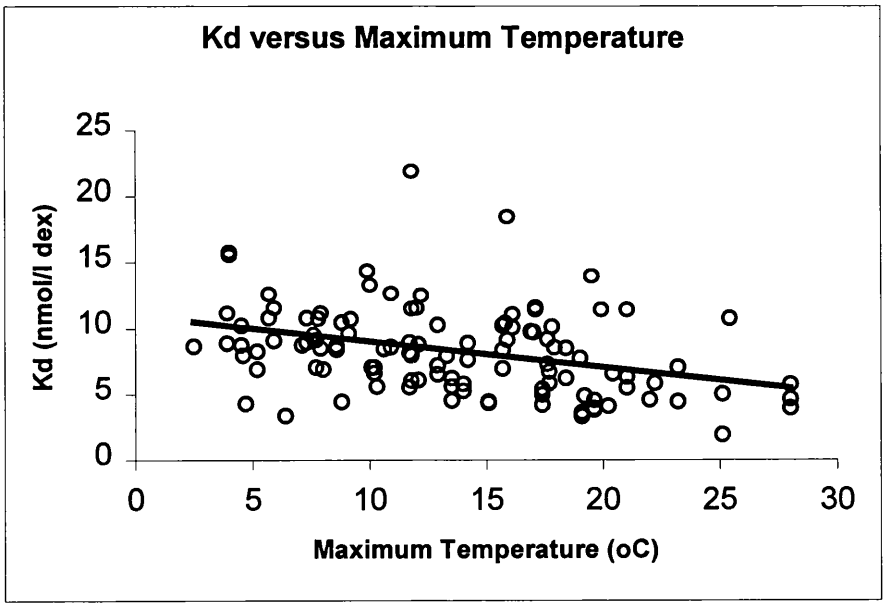
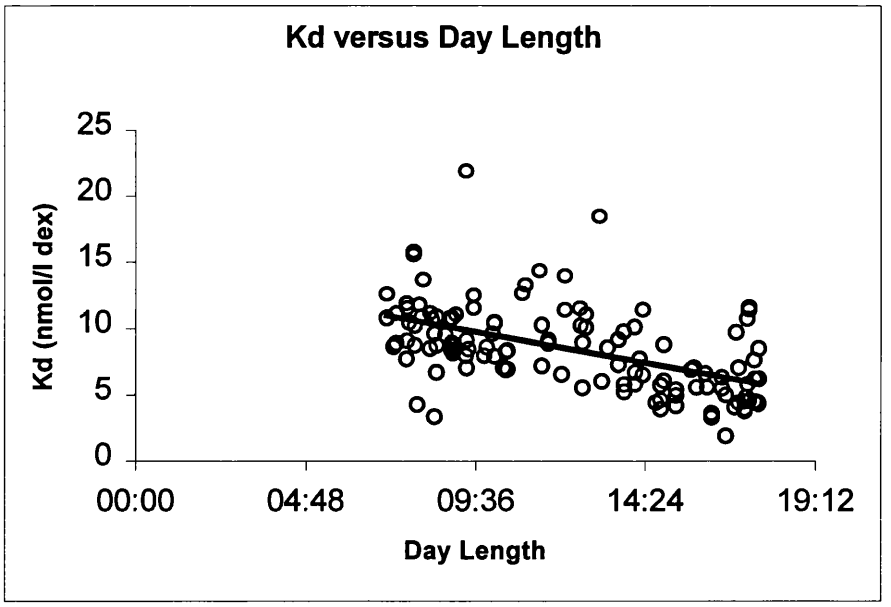


Figure 6.4 Interaction of Kd with Day Length

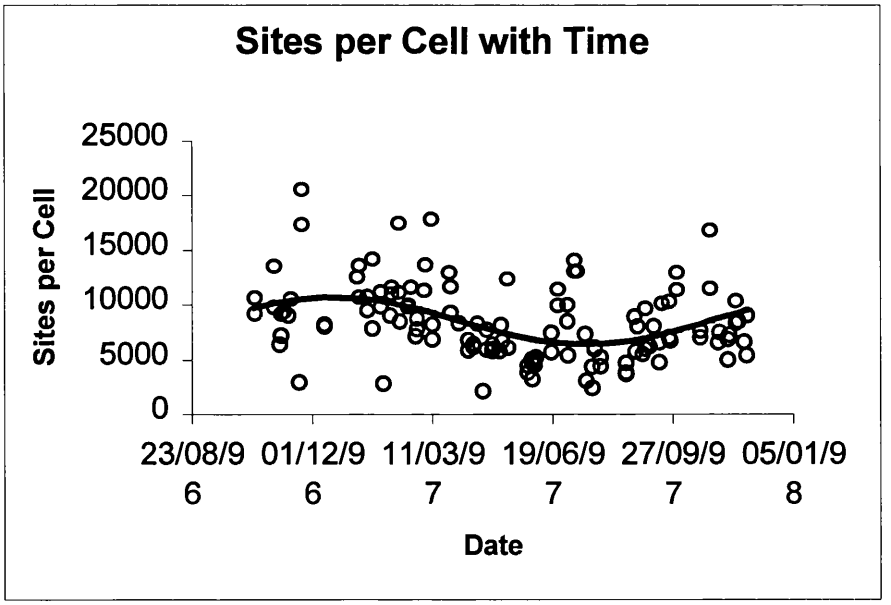


Interaction with sites per cell

Sites per cell also showed a significant seasonal variation, although this was less pronounced than for Kd. The best fit for this curve was also for a sinusoid ($p<0.001$, F-test), figure 6.5.

Figure 6.5 Sites per Cell with Time

The equation of the fitted sinusoid is 8561+2144*cosine(0.015*(days since 1/1/1996)+0.199)

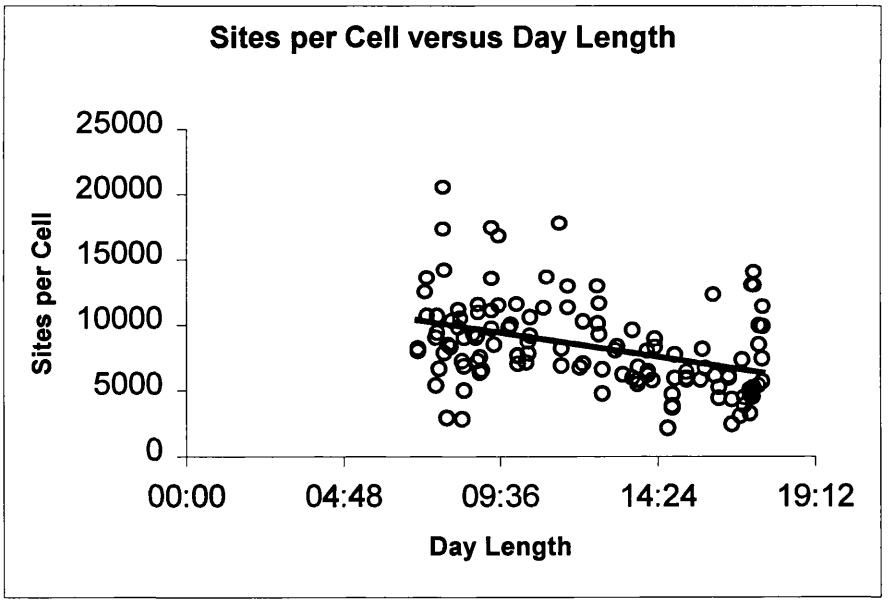


Univariate regression with correction for repeated measures was performed using sites per cell as the response variable and minimum temperature, maximum temperature and day length as explanatory variables. This showed a small significant interaction between day length and sites per cell only as shown in table 6.2 and figure 6.6.

Table 6.2 Interaction between Sites Per Cell and Climate

Interaction between sites per cell and	***p***	***r***
Minimum temperature	0.11	-0.10
Maximum temperature	0.07	-0.15
Day Length	**0.03**	**-0.20**

Figure 6.6 Interaction between Sites per Cell and Day Length



6.4 Interaction with Cortisol and Tri-iodothyronine

Interaction with Kd

Linear regression with correction for repeated measures was used to analyse for any interaction between Kd and cortisol or tri-iodothyronine. No significant interaction was found as shown in table 6.5.

Table 6.3 Interaction between Kd and Cortisol or Tri-iodothyronine

Interaction between Kd and	***p***	***r***
Cortisol	0.65	0.0
Tri-iodothyronine	0.67	0.0

Interaction with Sites per cell

No interaction between sites per cell and either cortisol or tri-iodothyronine was found using linear regression corrected for repeated measures, and shown in table 6.6.

Table 6.4 Interaction between Sites per Cell and Cortisol or Tri-iodothyronine

Interaction between sites per cell and	***P***	***r***
Cortisol	0.13	-0.04
Tri-iodothyronine	0.94	0.0

6.5 Summary

6.5.1 Season

In repeated observations of glucocorticoid receptor binding characteristics there was evidence of seasonal variation in Kd and sites per cell within individual subjects. For Kd, this showed an interaction with minimum temperature, maximum temperature and day length. Multiple regression using these candidate variables showed day length was the most predictive. For sites per cell, there was a small significant interaction with day length only.

These findings are consistent with the findings for Kd from the Twin and Fastcard studies. The seasonal interaction with sites per cell is consistent with the Fastcard study. All subjects studied lived within 20 kilometres of the meteorological station used to obtain the climate data and this may explain the greater strength of the association in the Fastcard study and weaker association in the Twin study.

6.5.2 Cortisol and Tri-iodothyronine

Neither Kd nor sites per cell showed any significant interaction with cortisol nor with tri-iodothyroinine suggesting neither hormone significantly regulates receptor binding characteristics.

6.6 Conclusions

In the twin study, there was no evidence for an inherited component to Kd or sites per cell suggesting the most important determinant of each would be environmental. Twin-twin associations were similar for both monozygotic and dizygotic twin pairs suggesting shared environment – either as climate, or indeed from shared *in utero* experience, was an important determinant of receptor binding characteristics.

Two large population based studies (the Twin and Fastcard studies) showed a significant effect of climate on glucocorticoid receptor Kd with an inconstant effect on sites per cell. These were cross-sectional studies and a prospective study with serial measurements of receptor binding characteristics was required to show the effect in physiological circumstances in individuals.

The finding of an effect of climate on leukocyte receptor binding is robust being resistant to several wash steps and long incubation times. This suggests climate alters a stable chemical signal within the cell, which may include small molecules such as lipophilic hormones or large molecular interactions such as protein expression. The present study suggested neither cortisol itself nor the active thyroid hormone, tri-iodothyronine, were likely to be responsible for seasonal interaction with receptor Kd and further work was designed to address this in greater detail (discussed in chapter 7).

The effect of season on the number of receptor sites was less consistent between studies, but where climatic data were local to the subject's home there was an interaction between day length and sites per cell with smaller interactions with maximum environmental temperature. Furthermore, an interaction between the number of receptor sites per cell and bone mineral density at the hip suggested local glucocorticoid action may play a role in bone metabolism. A candidate mediator that varies with season (and particularly day length) with an effect on bone mineral density is 1,25 dihydroxycholecalciferol and subsequent experiments were designed to study the effect of this hormone on binding (in chapter 7).

It is clear, however, that climate has an important effect on both the Kd and number of available receptor sites. Previous studies of glucocorticoid binding in leukocytes have not controlled for this effect and may be confounded by season. The net effect of a rise

in Kd and a rise in sites per cell in winter is difficult to predict, however, particularly as the leukocyte receptor does not appear to reflect activity in the central nervous system or important metabolic tissues such as adipose tissue or the pancreas.

Chapter 7

Candidate Modulators of Glucocorticoid Receptor Binding

7.1 Introduction

The preceding 3 chapters describe seasonal variation in glucocorticoid receptor binding principally affecting Kd and showing interaction with the climatic variables of maximum temperature, minimum temperature and day length. In hypothesising how these variables could affect glucocorticoid receptor binding the most likely mechanism would be one that either altered the structure of the receptor protein (affecting its ability to interact with ligand, heat shock proteins or its ability to translocate to nucleus) or that altered the accessory proteins in the receptor-heat shock complex. Since core body temperature, and therefore the temperature of lymphocytes, remains well controlled in most climatic circumstances any effect on receptor binding would be likely to be mediated by a chemical message.

Any chemical mediator would have to be intracellular or tightly adherant to the cell glycocalyx or membrane since washing cells thoroughly did not prevent its effects. Numerous lipid soluble hormones have been described to show seasonal variation and in the first series of experiments I performed glucocorticoid receptor binding assays in the presence and absence of each of these in physiological concentrations. Assays were performed at seasons when the endogenous concentration of the mediator or hormone would be expected to be at its lowest. Supplementing this concentration to high physiological levels might be expected to mimic the effects seen in different seasons.

7.2 Description of Candidate Mediators

In selecting candidate mediators I performed a literature search for all hormones known to shown seasonal or circannual variation. This identified cortisol (Wehr 1998, Maes et

al 1997), tri-iodothyronine (Maes et al 1997), melatonin (Asplund et al 1998), insulin (Behall et al 1984), retinol (Besu et al 1994), all-trans retinoic acid, and 1,25 dihydroxy cholecalciferol (Harris et al 1998). In addition to satisfying the seasonal pattern of variation, all these mediators are highly lipophilic, or demonstrate a high capacity for membrane binding.

Melatonin activates a specific receptor and, through activation of a G_i protein, inhibits adenylyl cyclase and cAMP generation (Morgan et al 1994). In amphibia increased melatonin secretion is accompanied by reduced pro-opiomelanocortin production and reduced cortisol secretion. Although less studied in man, seasonal variation in melatonin secretion also offers a plausible mechanism for modulation of glucocorticoid receptor binding.

The remainder of the mediators identified all act principally through nuclear receptors that are related to the glucocorticoid receptor. These receptors could modify glucocorticoid receptor binding through altered gene transcription, competition with accessory proteins, such as heat shock proteins or coactivators, or by forming heterodimers with monomeric glucocorticoid receptor protein. It is plausible that these mediators could have an influence on glucocorticoid receptor binding.

7.3 Binding Assays

Paired glucocorticoid receptor binding assays were performed on cells taken from the same subject on the same day. For one assay, all incubations of cells were co-incubated with vehicle alone (0.1% dimethylsulphoxide). In an identical assay, all incubations were co-incubated with physiological concentrations of each mediator as shown in table 7.1. Physiological concentrations of retinoids (Takeda et al 1994), 1,25 dihydoxycholecalciferol, trioiodthyronine, reverse tri-iodothyronine, insulin (Greenspan

et al 1997) and melatonin (van Reeth et al 1994) were obtained from the relevant literature and converted to S.I. units, where appropriate.

Table 7.1 Concentrations of Mediators Used in Incubations

Low physiological and high physiological concentrations of hormones are shown as low normal and high normal, and are derived from the references above.

Substance	Low Normal	High Normal	Incubation Conc.	Reference
All-*trans* retinol	1.5 µmol/l	2.0 µmol/l	1.75 µmol/l	Takeda 1994
All-*trans* retinoic acid	3.1 nmol/l	11.4 nmol/l	7.2 nmol/l	Takeda 1994
1,25-dihydroxycholecalciferol	48 pmol/l	184 pmol/l	116 pmol/l	Greenspan et al 1997
Tri-iodothyronine (T3)	1.5 nmol/l	2.9 nmol/l	2.2 nmol/l	Greenspan et al 1997
Melatonin	44 pmol/l	474 pmol/l	400 pmol/l	van Reeth et al 1994
Insulin	34 pmol/l	1.84 nmol/l	1.5 nmol/l	Greenspan et al 1997
Forskolin	-	-	10 µmol/l	

Retinoids were added to assays in the winter months, when the endogenous concentrations are at their lowest. Tri-iodothyronine, reverse T3, melatonin and insulin were added in summer months when concentrations are normally at their lowest. Forskolin was added in the winter months in an attempt to antagonise the inhibitory effects of melatonin (which is at its highest concentration in winter) has on adenylyl cyclase.

No detailed literature was found describing the metabolism of these mediators by leukocytes. The purpose of these experiments was to find whether any of these substances were capable of modifying glucocorticoid receptor binding and a positive result would be a supportive finding on its own merits. However the lack of any effect on receptor binding could not be taken as evidence of lack of effect.

7.4 Effect of Candidate Mediators

Receptor binding assays were performed as described above on at least 5 individuals each on separate days. The effect each mediator had on receptor Kd and the number of receptor sites per cell is shown in table 7.2.

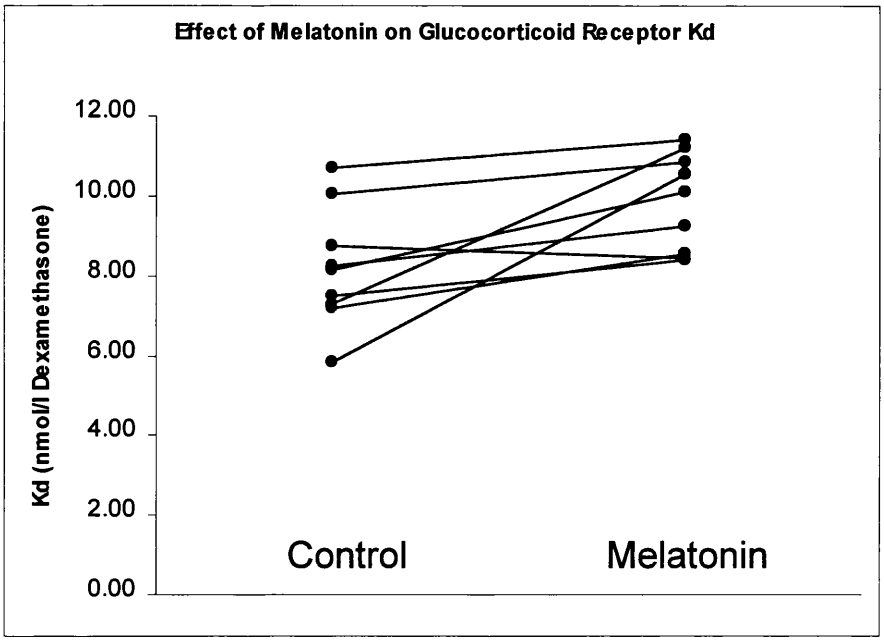
Table 7.2 The Effect of Various Substances on Glucocorticoid Receptor Binding

The abbreviations used are: t-retinoic acid=all *trans*-retinoic acid, c-retinoic acid=13-*cis* retinoic acid, 1,25 Vit D=1,25 dihydroxycholecalciferol, T_3=tri-iodothyronine, rT_3=3,3',5' tri-iodothyronine. Significance was tested using paired t-tests with p values corrected by the Bonferroni method: a level of 0.008 (i.e. α=0.05/6 tests) was considered significant.

	Mean Kd Control	Mean Kd Treatment	*p*	Mean Sites Control	Mean Sites Treatment	*p*
Retinol	11.5	12.4	0.430	8765	8878	0.882
***t*-Retinoic acid**	9.8	10.3	0.428	10774	11490	0.783
1,25 Vit D	11.3	11.2	0.697	8059	8195	0.141
T_3	9.4	8.6	0.184	6394	6176	0.543
Melatonin	8.2	9.9	**0.006**	9690	11113	0.080
Insulin	9.1	8.4	0.445	10312	8279	0.187

Melatonin was found to raise Kd significantly, with no significant effect on the number of receptor sites per cell as shown in figure 7.1.

Figure 7.1 Effect of Melatonin on Glucocorticoid Receptor Binding



To test whether the effect of melatonin was likely to be mediated by its effect on cAMP (Morgan et al 1994), a subsequent experiment using forskolin treatment was designed. This substance increases cAMP generation and would be expected to have opposite effects to metatonin that acts mainly by inhibiting cAMP generation. Forskolin lowered Kd significantly and caused a significant reduction in the number of receptor sites per cell as shown in table 7.3 and figure 7.2 and figure 7.3.

Table 7.3 The Effect of Forskolin on Glucocorticoid Receptor Binding

Significance was tested using a 2-tailed t-test with p at the 0.05 level considered significant.

	Mean Kd Control	Mean Kd Treatment	*p*	Mean Sites Control	Mean Sites Treatment	*p*
Forskolin	8.2	6.3	**0.03**	9250	7116	**0.02**

Figure 7.2 Effect of Forskolin on Glucocorticoid Receptor Kd

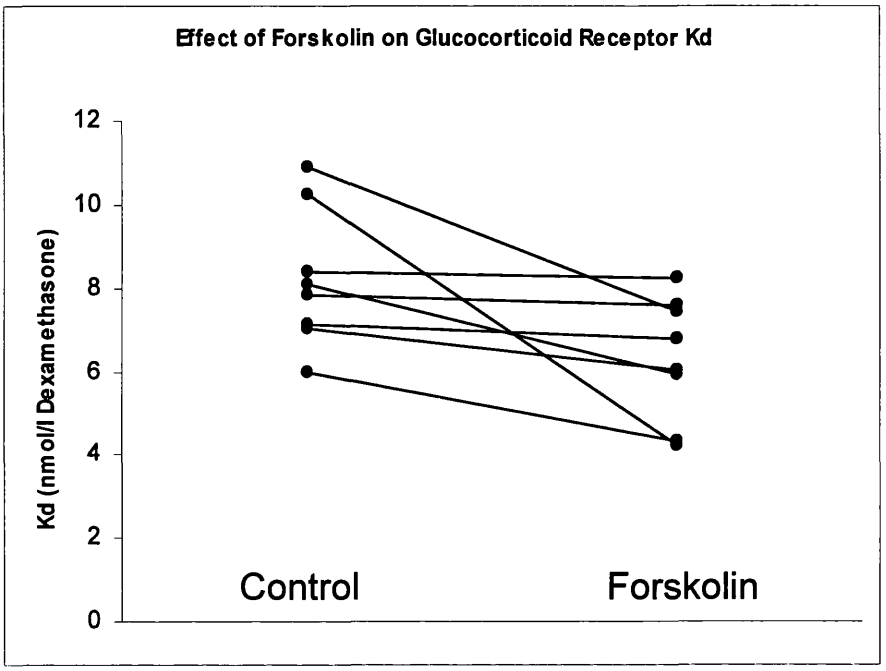
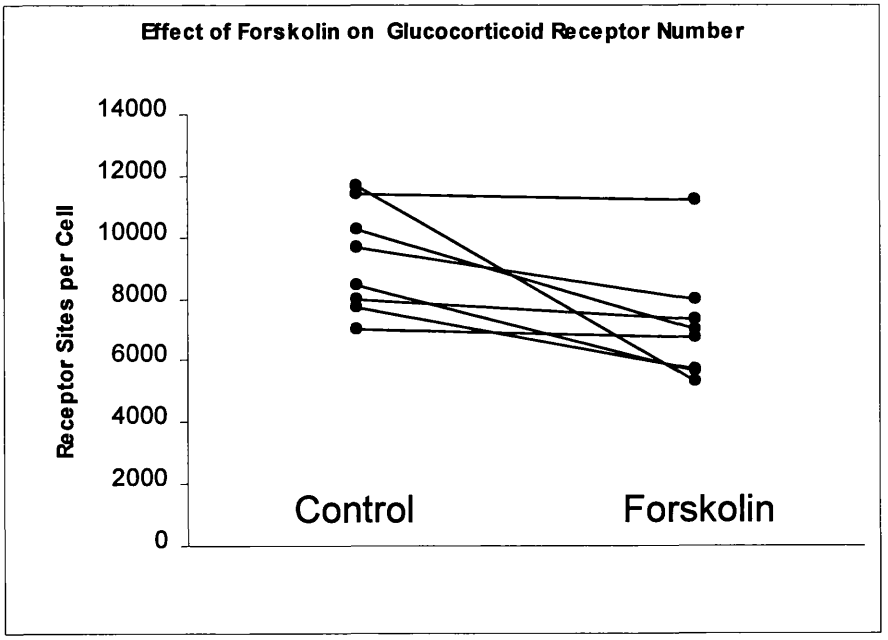


Figure 7.3 Effect of Forskolin on Glucocorticoid Receptor Number



7.5 Discussion

Of the substances tested, only melatonin and forskolin were found to alter glucocorticoid receptor binding. Melatonin added in the summer months produced a rise in receptor Kd but no significant change in receptor number. Forskolin treatment in the winter months produced a significant fall in receptor Kd and in receptor number.

Previous reports on the effects of melatonin on glucocorticoid receptor function are limited. One previous report also showed melatonin treatment increased Kd in receptors in rat brain (Marinova et al 1991) while an earlier report (Familiari et al 1988) showed no effect in receptor binding in rat thymocytes or on glucocorticoid-induced weight loss, adrenal atrophy or thymus atrophy.

The effects of forskolin on receptor binding remain controversial. Penuelas et al (1998) found forskolin increased promoter activity of the glucocorticoid receptor gene 2-fold in HeLa cells, although neither levels of receptor protein nor receptor binding were measured, this might be predicted to increase the number of receptors. In rat myocardial cells forskolin increased the affinity of the receptor for nuclear binding (Sato et al 1996) without increasing receptor protein levels. Receptor binding for ligand was, however, not measured. In a human breast carcinoma cell-line, forskolin treatment was found to increase glucocorticoid-mediated gene transcription without any detectable effect on receptor expression or receptor-ligand affinity (Moyer et al 1993). Using a rat hepatoma cell-line Dong et al (1989) found that forskolin increased receptor mRNA and saturation receptor binding capacity, although receptor affinity was not assayed. All of these experiments involved short term incubation with forskolin. The fall in Kd seen in the experiment here may, over a 20 hour incubation, increase the feedback on further

glucocorticoid receptor expression resulting in the fall in receptor number seen. Alternatively, the differences seen in the number of receptor sites may be a function of the different cell types used as many glucocorticoid-mediated effects show different responses in different tissue-types (e.g. the induction of phosphoenolpyruvate kinase in liver and the inhibition in adipose tissue).

Tri-iodothyronine is an extremely effective agent in inducing expression of the low-affinity glucocorticoid binding site in rat liver (Lopez-Guerra et al 1997). However, the identity of this binding site remains unknown and may represent a glucocorticoid and progesterone metabolising enzyme. Direct effects on the orthodox glucocorticoid receptor have been shown in the rat (Meaney et al 1987) where postnatal administration of tri-iodothyronine or thyroxine increased receptor concentrations in the hippocampus but not in hypothalamus or pituitary suggesting a tissue-specific effect. The only report in leukocytes is in the goat (Murakami et al 1980) where administration of thyroxine did not alter glucocorticoid receptor binding capacity, in keeping with the negative data from this study.

There are no reported data on the effect of insulin on glucocorticoid receptor binding or on receptor expression. In many tissues, the effects of these hormones are mutually antagonistic, although in some tissues their effects are synergistic (e.g. the induction of leptin in adipose tissue). This would tend to argue against a significant effect of insulin on glucocorticoid receptor binding: most of the reported interactions of these hormones have been reported to occur at a transcriptional level.

There are no reports of any effect of all-*trans* retinol, or *trans*-retinoic acid on glucocorticoid receptor binding although activation of the retinoic acid receptors RAR

and RXR are well known to cross-talk with glucocorticoid receptors at a transcriptional level in the expression of a number of genes.

The effects we see on glucocorticoid receptor binding are, therefore, consistent with previous reports for melatonin and forskolin. The antagonistic effects of melatonin and forskolin on receptor Kd may be accounted for by their effects on cAMP generation. Forskolin increases cAMP production by activating the enzyme adenylyl cyclase. Melatonin in contrast binds to a membrane-bound melatonin receptor: currently 3 melatonin receptors are characterised. The ML_{1A} receptor is mainly present in the suprachiasmatic nucleus and is involved with regulation of circadian rhythms (Reppert et al 1994). The ML_{1B} receptor is the product of a separate gene and is found in the retina and some areas of brain and is involved in retinal physiology (Reppert et al 1995). The ML_2 receptor has recently been cloned, although its physiological role remains to be determined (Ebisawa et al 1994). The ML_1 receptors both inhibit cAMP generation through a pertussis-sensitive G protein while ML_2 receptors increase inositol *tris*-phosphate breakdown (Popova et al 1995). A high-affinity binding site has recently been found on T-lymphocytes, but not B-lymphocytes, which has the binding characteristics of the ML_1 receptor and is present in greater amounts on CD4+ cells than CD8+ cells (Gonzalez-Haba et al 1995). This would suggest the main mechanism of action of melatonin in lymphocytes will be inhibition of cAMP. The opposite effects on glucocorticoid receptor Kd caused by incubation with either melatonin or forskolin could, therefore, be explained by their opposite effects on intracellular cAMP concentration.

How cAMP affects glucocorticoid receptor Kd is less well understood. An effect on receptor affinity would most probably be effected by altering the conformation of the

receptor either by covalent modification of the protein or by altering the properties of interacting proteins required to maintain normal receptor conformation. The most likely candidate mechanisms for an effect of cAMP would be altered protein phosphorylation by cAMP-dependent protein kinase-A mediated phosphorylase or dephosphorylase activity. Direct examination of receptor protein and Hsp90 protein (the major component of the heat-shock-receptor complex) shows striking increases in phosphorylation shortly after ligand binding (Orti et al 1992). However, neither measures which increase or decrease phosphorylation appear to alter receptor-ligand affinity (Bamberger et al 1996). The phosphorylation state of the receptor may instead be involved in subcellular localisation between the cytosolic and nuclear components (DeFranco et al 1991).

From discussion in sections 4.4.4, 5.7 and 6.4 Kd rises in winter and falls in summer. Melatonin, which is at its highest levels in winter when day length is at its minimum, produces similar effects on Kd increasing this above normal summer values. No effect was found after co-incubation of melatonin on receptor sites per cell, although a trend was found for an increase (adjusted $p=0.08$): a similar pattern found in winter months. The effect of forskolin on these effects was opposite producing a fall in Kd and a fall in sites per cell, broadly imitating the effects seen in summer. Melatonin acting through the M_{L1} receptor to alter cAMP levels is, therefore, a candidate mediator of the seasonal effect on glucocorticoid receptor binding.

Chapter 8

Screening the Glucocorticoid Receptor Gene for Mutations

8.1 Introduction

As discussed earlier in section 1.6, mutations of the glucocorticoid receptor gene that disrupt glucocorticoid receptor signalling often have a relatively subtle phenotype with hypertension, relative insulin resistance, hirsutism in women, hypercholesterolaemia (Arai et al 1994) and presumed increased cardiovascular risk. It is also noteworthy that the most striking features of glucocorticoid excess include central obesity, hypertension, hyperglycaemia, hyperlipidaemia, insulin resistance and increased cardiovascular risk (Ross et al 1982). These are also features of the metabolic syndrome X (Reaven 1988). Thus it is reasonable to ask whether some aspects of common cardiovascular phenotypes reflect variability in glucocorticoid action. There is indeed wide inter-subject variability in circulating cortisol (Huizenga et al 1998) and inter-individual sensitivity to exogenous glucocorticoids is also known to be widely variable (Hirano et al 1998). I hypothesised, therefore, that polymorphisms at the glucocorticoid receptor locus either altered glucocorticoid receptor protein through mutations in the coding sequence of the gene, or altered the expression of the gene by variation in the structure of the promoter. In considering the likely phenotypes of either of these types of mutation in the receptor gene, we acknowledged that mutations in the coding part of the gene may have relatively minor effects on receptor affinity or number. Furthermore, as such mutations would be expressed in all tissues with relative insensitivity centrally being compensated by increased cortisol concentrations circulating peripherally, a distinct phenotype may not be obvious.

Mutations in the promoter that affected receptor number to the same extent in all tissues would, for the same reason, be expected to produce little significant physiological disturbance. However, if tissue-specific components of the promoter contained polymorphisms then the possibility would exist for differential tissue regulation and for relative glucocorticoid imbalance in different tissues and cardiovascular disease.

As an illustration of this principal, the rare syndrome of thyroid hormone resistance (Chatterjee et al 1994) exists as generalised and as tissue-specific forms. In the generalised syndrome, the pituitary and peripheral tissues are partially resistant to the active thyroid hormone, tri-iodothyronine. As a result of reduced negative feedback at the pituitary, thyroid stimulating hormone secretion increases stimulating the secretion of thyroid hormones to a level that satisfies the pituitary requirement and to a variable extent peripheral tissue requirements.

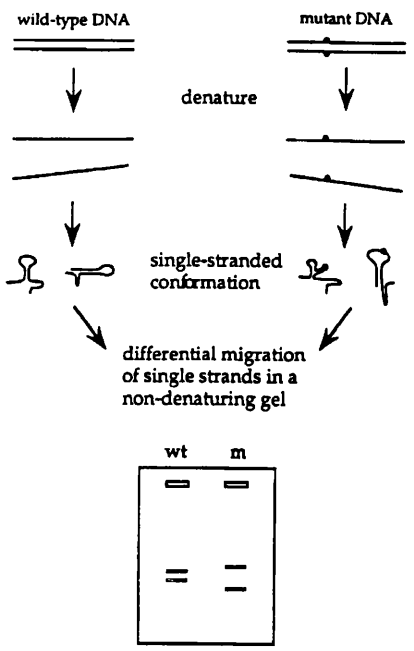
In the tissue specific forms the pituitary is relatively resistant to thyroid hormone. Reduced negative feedback at the pituitary increases the TSH drive to the thyroid and increases thyroid hormone secretion as before. However, as peripheral tissue sensitivity to thyroid hormone is normal this results in tissue hyperthyroidism with the usual symptoms and signs of thyroid hormone excess.

The glucocorticoid receptor is expressed from a single gene on chromosome 5q31. However, the start site of transcription from the promoter varies between tissues in the mouse (Strahle et al 1992), rat (Gearing et al 1993) and human (Chapman,K.L., personal communication). This raises the possibility that polymorphisms within the sequences of these start sites may influence the relative balance of glucocorticoid expression between different tissues.

8.2 Screening Strategy

The glucocorticoid receptor gene (see appendix 4 for sequence) was screened for mutations using single-stranded conformational polymorphism (SSCP) analysis (Orita et al 1989). Briefly, this method uses polymerase chain reaction (PCR) to amplify a region of interest and incorporates label (normally radioactive but fluorescent and digitonin labels have also been used) either at the 5' end of each strand or within the strand as it elongates. By denaturing the product and resolving it on a non-denaturing polyacrylamide gel, each single strand undergoes partial self-annealing within the gel that induces a secondary structure and affects its mobility in the gel. As a result each strand normally elutes at a slightly different rate within the gel allowing its detection by autoradiography. When a single point mutation exists within a product its self-annealing properties and elution rate are affected producing bands at differing positions on the final autoradiograph. Thus, in a heterozygote 4 principal bands will be seen in place of 2, and in a homozygote 2 principal bands will be found which differ in position when compared to product from a reference population (figure 8.1).

Figure 8.1 Single Strand Conformational Polymorphism



DNA from subjects homozygous for wild-type sequence or heterozygous for mutant sequence are shown.
Each of the single strands self-anneals to produce unique secondary structure with characteristic mobility on electrophoresis.
A point mutation results in both the sense and antisense strands adopting different conformations producing different bands on the final autoradiograph.
Modified from Grompe 1993

For the method to be optimally sensitive product size should be between 100 and 300 base pairs and 4 different running conditions (a glycerol containing gel run at 4°C and one at 25°C and a glycerol deficient gel run at 4°C and 25°C) should be used (Hayashi et al 1991).

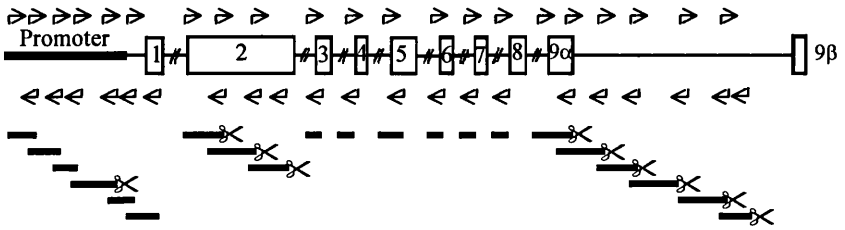
8.3 Primer Design

The glucocorticoid receptor gene is composed of a promoter, an untranslated exon 1, exons 2 to 8 and finally 2 alternatively spliced exons 9 (exon 9α and exon 9β). The β isoform of the receptor does not bind ligand and its function remains controversial. I therefore decided to screen only the sequences of the α isoform of the receptor for mutations including exon 9α and over 2000 bases of the 3' intron to include any enhancer sequence.

Primers were designed to generate fragments of suitable size for SSCP analysis (i.e. 100-300 base pairs, Hayashi et al 1991). This was done by generating PCR products of

appropriate size, or where exons were large, generating overlapping PCR fragments that were digested to the appropriate size by restriction endonuclease digestion. A diagram of the gene with ideograms of the primer positions is shown in figure 8.2. Using this method, primers were designed to amplify the promoter and exons of the glucocorticoid receptor gene using genomic DNA as template. As much of the intronic sequences were not known, primers were designed which spanned as much of the exonic sequence as possible.

Figure 8.2 Primers used for PCR-SSCP analysis



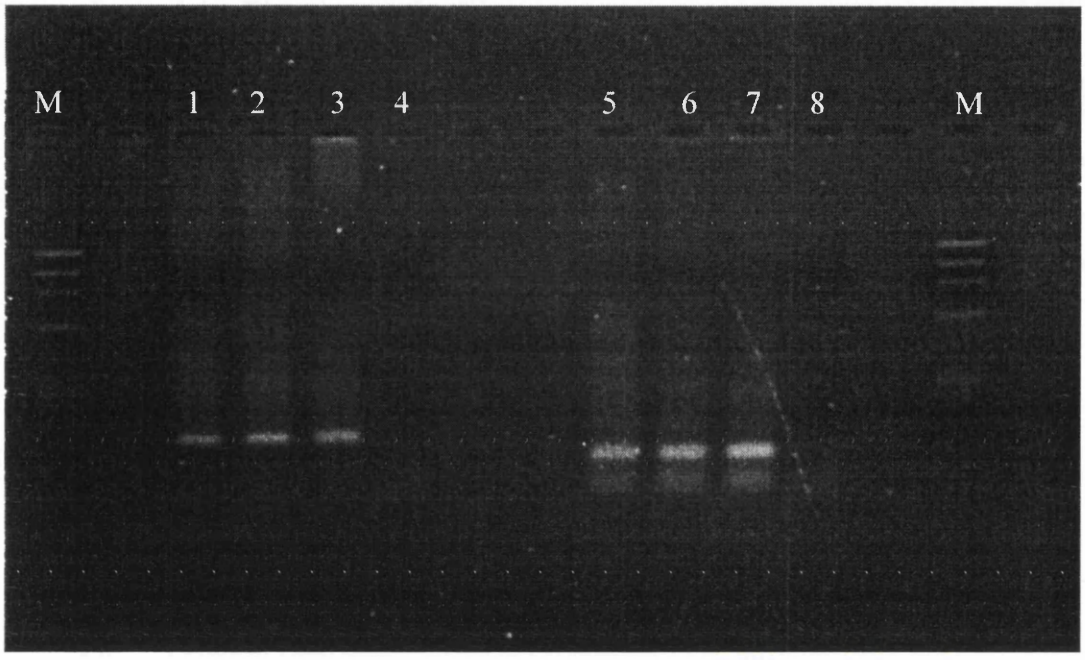
The structure of the glucocorticoid receptor gene is shown with numbered boxes denoting the exons of the gene, and the leading line representing the promoter.
The primer positions are shown as right-pointing arrows for the sense primers, and left pointing arrows for the antisense primers.
The PCR fragments are shown as lines below the gene and were arranged to produce overlapping fragments.
The PCR products that were subsequently digested with restriction enzymes are marked with scissors.
The position or each primer, fragment sizes and the enzymes used for digestion are detailed in appendix 5.

8.4 Optimisation of PCR

For each pair of primers, PCR of genomic DNA was performed to confirm the reaction produced satisfactory product, and where necessary to modify the reaction conditions.

Where PCR product was to be subsequently digested, restriction enzyme digest conditions were optimised. A sample PCR and restriction digest is shown in figure 8.3.

Figure 8.3 Optimisation of PCR and Restriction Digestion



Lanes 1-3 contain optimised PCR product from genomic DNA for exon 2 segment 3
Lane 4 contains PCR with no DNA
Lanes 5-7 contain PCR product digested with FokI
Lane 8 contains PCR product done with no template and digested with FokI
Lanes M contain ϕX174 bacteriophage/HaeIII digest marker DNA
Complete digestion of PCR product is seen in lanes 5-7 with no DNA contamination in lanes 4 or 8

Satisfactory product was not obtained for any of the promoter or exon 1 sequences. Initial PCR reactions produced multiple product bands suggesting multiple anneal sites or mis-annealing. Increasing the annealing temperature in 2°C steps up to a 10°C increment only reduced the amount of product produced without increasing specificity. A range of magnesium concentrations from 0.5mmol/l to 2.5mmol/l had no major

impact on PCR specficity. Increasing the initial denaturation time, prolonging annealing time or adding a final longer extension step of 5 minutes made no difference to reaction efficiency. Adding the detergent Tween, or the wetting agent Dimethylsulphoxide to the PCR reaction had no appreciable effect, although both have been described to increase reaction efficiency for difficult templates. Primers were redesigned using longer sequences and trying to avoid repetitive GC rich sequences. Unfortunately, none of these measures improved the specificity of the PCR reactions sufficiently to reproducibly amplify promoter sequence.

8.5 SSCP Reactions

PCR products were labelled either by "end-labelling" the primers or by incorporation labelling of the PCR products. End-labelling involves exchanging one of the phosphate groups at the 5' end of the primer with a [^{32}P] γ-phosphate group using the enzyme polynucleotide kinase purified from the T4 bacteriophage (Berkner, 1977). The reaction mixture for each primer was prepared as shown below and incubated for 1 hour at 37°C. This mixture produced sufficient primer for 50 SSCP reactions.

Primer	30μl	50μM
γ-[^{32}P] ATP	5μl	1.85MBq
Polynucleotide Kinase	1μl	5U
Buffer x 10	5μl	
H_2O	9μl	
Total	50μl	

In subsequent SSCP reactions, PCR products were labelled directly by incorporation of [α-^{32}P] CTP. This was achieved simply by adding 1μl (0.37 MBq) to the PCR master mix and adjusting the volume of H_2O added. No adjustment was made to the amount of unlabelled CTP added to the PCR mix as the concentration of α-[^{32}P] CTP compared to

unlabelled CTP was negligible (0.06μmol/l compared to 50μmol/l, a difference of 0.1%). Incorporation labelling had the advantage of increased sensitivity in detecting mutations in fragments digested by restriction enzymes. Digestion and denaturation of PCR product produces 4 single-stranded conformers. Where the product has been labelled by end labelling, only the 5' sense strand and the 3' antisense strand will be labelled, and therefore only 2 of the 4 conformers will be detected on autoradiography. Where incorporation labelling is used, all 4 single-stranded products will be detected with a theoretical increase in sensitivity.

For each SSCP reaction a master mix was prepared in quantities sufficient for 50 reactions using either labelled primers or the addition of labelled CTP as described above. The composition of a standard PCR mix is shown below. For some reactions the concentration of Mg^{2+} was optimised to reduce non-specific product formation.

	Final Concentration
Mg^{2+}	1.5 mmol/l
Taq Buffer	x 1
dATP	50 μmol/l
dCTP	50 μmol/l
dGTP	50 μmol/l
dTTP	50 μmol/l
Primer 1	1 μmol/l
Primer 2	1 μmol/l
Taq	50 U
H_2O	Added to total volume of 1.25 ml

Genomic DNA from each subject was aliquoted into each well of a 96 well PCR plate and air dried at room temperature to fix the DNA to the plastic well (Day et al, 1995). The PCR mix was then aliquoted onto each well in 25 μl volumes and overlaid with 2 drops of mineral oil. For each experiment a blank PCR reaction was included containing no added DNA to exclude contamination of the master mix. When any product was

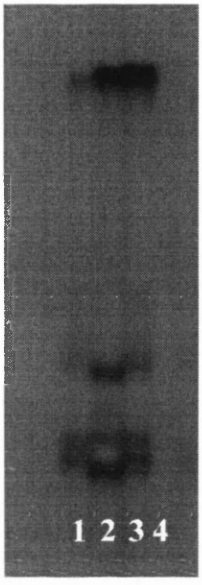
detected in the blank reaction, all samples were discarded and the experiment repeated. PCR cycling was performed at 3 temperatures: a denaturing 94°C step, an annealing step that depended on the base composition of the primers, and an extension step at 72°C. Where necessary, these steps and the time at each temperature were optimised. However, for the majority of PCR reactions this standard cycling programme was satisfactory:

Step	Temperature	Time
1.Initial denaturation	94°C	60 seconds
2.Denaturation	94°C	30 seconds
3.Annealing	55°C-65°C	30 seconds
4.Extension	72°C	30 seconds
Repeat steps 2-4 for 30 cycles		
5.End reaction	-4°C	10 minutes

8.6 Verification of SSCP

The SSCP method was tested for sensitivity using DNA containing a single point mutation. Plasmids containing cDNA for the human aldosterone synthase gene (Genbank sequence Gbmem:HUMCYPBB) ligated into a pGEM plasmid and containing either the wild-type sequence or a sequence with a $T_{3685} \rightarrow C$ point mutation were kindly provided by Miss A. Fisher. A segment of the insert was amplified using [^{32}P] end-labelled primers and a standard PCR reaction. The 225 base pair product was run on an SSCP gel with glycerol at 25°C at 30 watts for 4 hours. When developed, the autoradiograph showed an altered banding pattern, correctly identifying the point mutation (figure 8.4).

Figure 8.4 SSCP of point mutations of the aldosterone synthase gene



Lane 1 contains labelled PCR product from human genomic DNA
Lane 2 contains labelled PCR product from a plasmid with a site-directed point mutation of the CYP11B1 gene
Lane 3 contains labelled PCR product from a plasmid containing the wild type CYP11B1 gene
Lane 4 contains a labelled PCR control (with no DNA template)

The point mutation is clearly identified by altered mobility of 2 of the 4 principal bands

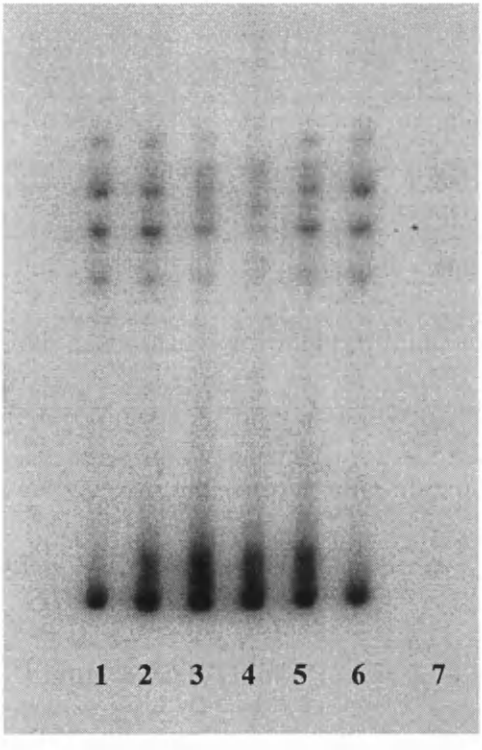
8.7 SSCP Screening of Subjects' DNA

Initially 40 healthy subjects from the MONICA IV study recruited from central Scotland (Davies et al 1999), were screened for mutations using a total of 22 primer pairs and run at 4 different conditions on polyacrylamide gels. The primer sequences for exonic PCR are shown in appendix 5. Although primers were designed against 1000 bp of the published promoter sequence of the glucocorticoid receptor gene, PCR and subsequent SSCP performed at a variety of optimising conditions, failed to produce product.

All exonic regions scanned produced successful reactions, if necessary after minor optimisation steps. In this initial run no polymorphisms were identified in any of the exonic regions.

As the frequency of polymorphisms in the normal population was unknown for this gene, it was decided to screen a further population of 40 healthy subjects recruited from the Monica IV study for whom detailed phenotypic data were available. The same strategy was used with the minor modification that PCR reactions labelled by incorporation of [α-^{32}P]-cytosine triphosphate. This provided the advantages of convenience, faster throughput and greater sensitivity in detecting mutations in restriction digested fragments. Using the same strategy, and the same primers, 4 subjects were found with an altered banding pattern for a region in exon 5 of the gene (figure 8.5).

Figure 8.5 SSCP of Exon 5



Lanes 1 and 6 show wild-type banding
Lanes 2 - 5 are products from the subjects with an altered banding pattern
Lane 7 is PCR control (with no DNA template)
Extra bands in lanes 2 to 5 are indicated by the white arrows.

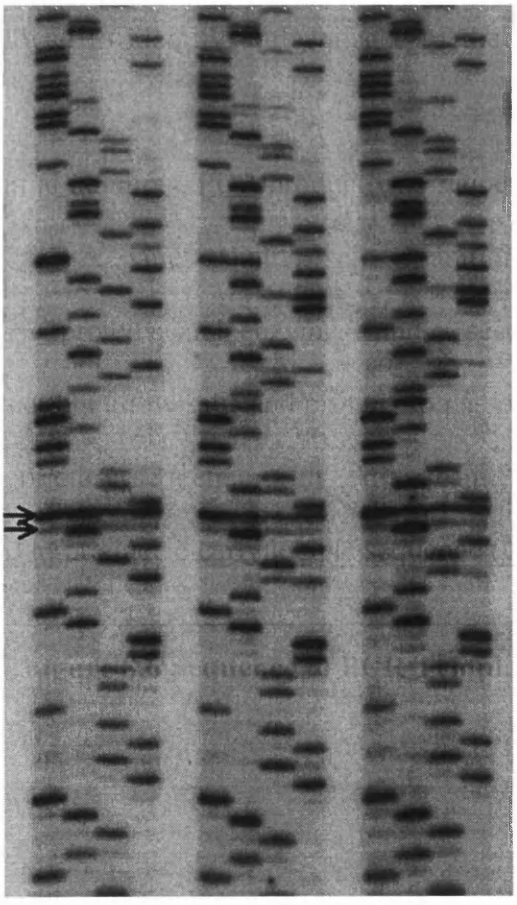
The PCR reactions for these subjects and 2 normal banding subjects were repeated and re-run on an SSCP gel to confirm the findings.

8.8 Sequencing

Several variations of the dideoxy termination sequencing method (Sanger, 1977b) were employed to identify the sequence of the polymorphic regions identified from SSCP screening.

In initial attempts, PCR product was sequenced directly, after separation from primers, salts and enzymes using a spin-separation column with a size filter of <10kDa (to remove proteins and enzymes) and a second filter of <100 base pairs to trap PCR product but allow salts and primers to pass unhindered (Amicon, USA). However, using a proprietary sequencing kit (Amersham, UK) and end labelled primers this method failed to produce clearly legible sequence (figure 8.6).

Figure 8.6 Sequence of PCR Template



From left to right, sequences were loaded in the order G,A,T,C.
Sequence 1 is of a subject with a wild-type band for SSCP of exon 5.
Sequences 2 and 3 are from subjects with variant SSCP bands for exon 5.
Numerous ambiguous bands are seen in sequences 2 and 3 (labelled with white arrows).
Several ambiguous bands were seen in all four lanes of all three sequences (labelled with black arrows)

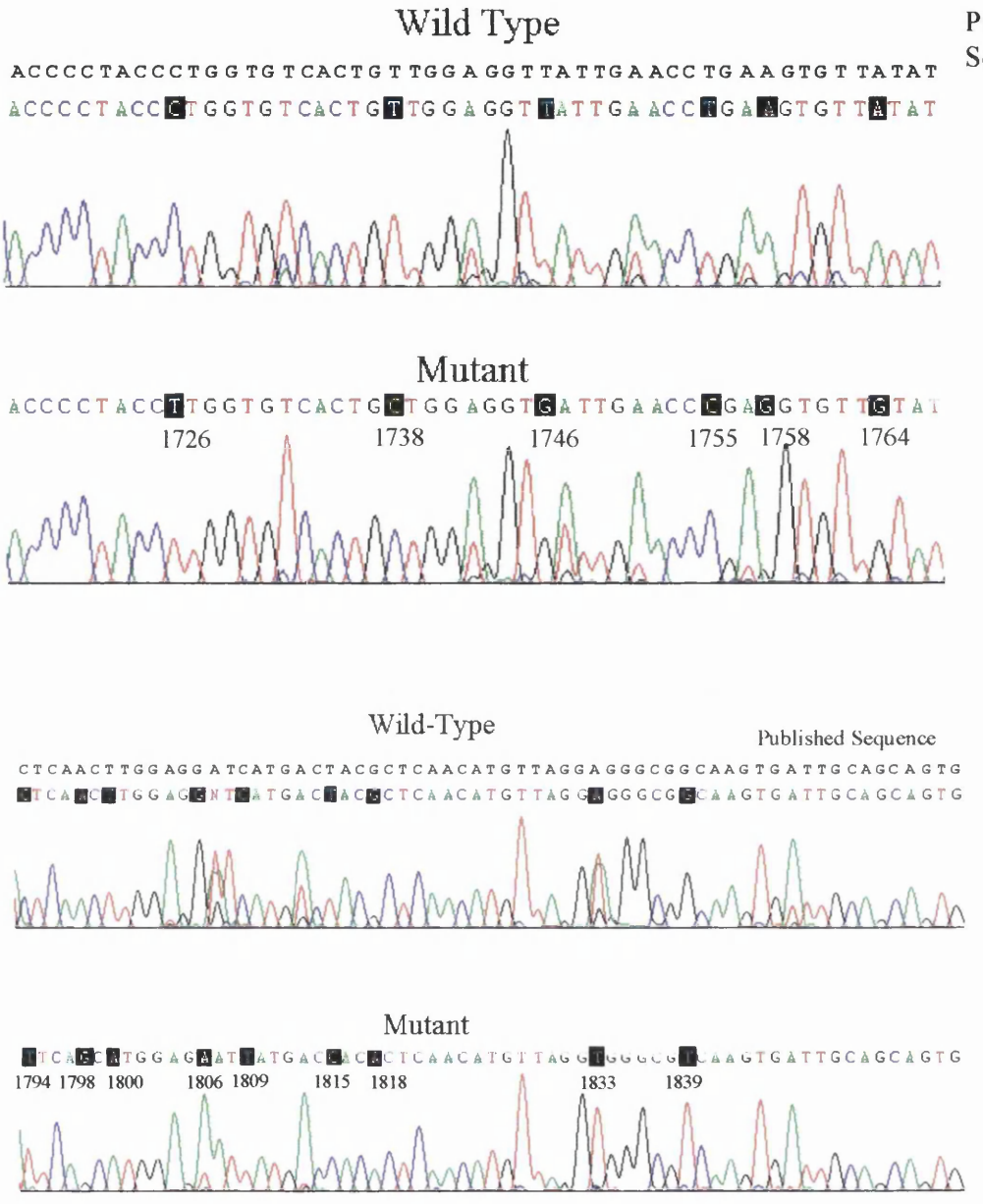
Bands in all four lanes ("BAFL") are normally a sequencing artefact caused by complex secondary DNA structure, residual primer in the template, or by high salt concentration interfering with the DNA polymerase activity. As there were numerous double bands in the sequences of the subjects with altered SSCP patterns, we felt these were also artefact bands. Repeating the PCR of exon 5 with repeated purification through centrifugation columns failed to yield better sequence data and many of the ambiguous bands remained. An alternative method of removing residual primers after PCR amplification using shrimp alkaline phosphatase to digest single stranded DNA and remaining nucleotides produced no better sequence data.

As an alternative method of sequencing, PCR products from each subject were subcloned into pGEM-T-easy vector and at least 6 subclones from each subject sequenced using the universal SP6 and T7 primers. The rationale for sequencing this number of subclones was to ensure that at least one clone from each allele was detected. Since the probability of detecting both alleles derives from binomial probability as p(both alleles)=$1-2^{(1-n)}$ where n is the number of subclones sequenced, using 6 subcones gave a probability of sequencing both alleles of 0.97. Using conventional radioactive sequencing methods we found what appeared to be several point mutations in the subjects suspected of having mutations in exon 5. However, some of these bands remained ambiguous.

In an attempt to improve sequence quality of these subjects, PCR products for exon 5 were freshly prepared and re-ligated into the pGEM-T-Easy sequencing vector. Using a commercial sequencing kit, sequence was generated with fluorochrome-labelled di-deoxynucleotides and resolved and read in an ABI 373 automated sequencer. Briefly, the method uses the same Sanger sequencing chemistry, but each dideoxy nucleotide is

labelled with a different fluourochrome that fluoresces at a different frequency of light. When the sequencing reaction is performed with all 4 dideoxynucleotides in the same reaction and resolved on a polyacrylamide gel, the identity of the base at each termination position is determined by its fluorescence and is read by a scanning laser-photodiode couple. Using this method, the ambiguous bands seen on manual sequencing were confirmed as mutations, "stop" artefacts were absent and several additional point mutations were identified in each subject. In total a complex of 15 point mutations in the exon was found ($C_{1726}T$, $T_{1738}C$, $T_{1746}G$, $T_{1755}C$, $A_{1758}G$, $A_{1764}G$, $C_{1794}T$, $A_{1798}G$, $T_{1800}A$, $G_{1806}A$, $C_{1809}T$, $T_{1815}C$, $G_{1818}A$, $A_{1833}T$, $G_{1839}T$). Each subject screened was a heterozygote for all 15 mutations. The automated sequences of wild-type and mutant sequences are shown below in figure 8.7.

Figure 8.7 Automated Sequences of Subcloned PCR Fragments of Exon 5



8.9 Discussion

SSCP is a well established method of triaging sequences that may contain mutations for subsequent sequencing. The sensitivity of the method has been estimated to be in excess of 99% for sequences of 100-300 base pairs when 4 different running conditions are used (Hayashi et al 1991), but falls dramatically to 90% for sequences in the range 300-450 base pairs.

Using SSCP with products in the size range 100-300 base pairs and running gels at the 4 recommended conditions, we confirmed the method worked for sequences with a known point mutation. Screening a population of 40 normal healthy subjects chosen at random I found no mutations in any exon. However, screening a different population of 40 normal subjects found 4 subjects with products with altered mobility, thus identifying them as possibly containing a mutation.

The failure to amplify any promoter sequences was disappointing. Several attempts were made to amplify the promoter from genomic DNA by PCR using different primers, altered cycling conditions, a range of magnesium concentrations and the addition of detergents and agents said to reduce secondary DNA structure. The failure of these measures and the multiple bands seen on PCR suggests this may have been due to multiple annealing sites. The glucocorticoid receptor promoter is extremely GC rich with a high annealing temperature and several repetitive motifs that could allow for primer misannealing. Promoters with high GC content are often difficult to PCR and sequence and detailed sequence analysis is often obtained by cloning the sequence in plasmid or cosmid vectors. Due to the limited time available for this project we had to abandon attempts to amplify the promoter sequence.

The PCR products with altered SSCP banding patterns were subsequently sequenced and found to have a complex pattern of 15 point mutations. First attempts at manual sequencing used PCR product as template. Multiple ambiguous bands in the sequencing ladder were interpreted as sequencing artefact. Attempts to further purify template did not resolve the extra bands, and in retrospect these represented heterozygote mutations. Subcloning PCR product had the advantage of producing clear bands with no heterozygote sequences as each allele is cloned and sequenced separately. However, several bands at the beginning or end of the sequence were not sufficiently clear to be sure of additional point mutations. Finally, using an automated sequencer which has greater resolution and base-calling sensitivity revealed clear evidence of all 15 point mutations. The use of these three separate methods with fresh template preparation on each occasion increases the confidence I have in this unusual mutation being genuine.

The effect of these point mutations on the expected protein sequence is surprisingly small. Two of the mutations ($A_{1798}G$ and $T_{1800}A$) change a codon from ACT (threonine) to GCA (alanine), but all the other mutations are conservative in the translated protein. The effect this has on receptor function remains unexplored in this study, but transient-transfection into a suitable cell-line would allow this to be explored.

The frequency of this haplotype in the population we screened was around 4 in 80 subjects (5%). Screening a large population to define the true frequency was not completed in this project and a more accurate estimate of prevalence is not available. However, phenotypic data collected from the 4 subjects we identified showed no significant difference from the reference population in glucocorticoid receptor binding characteristics, blood pressure, body mass index, fasting glucose or lipids. The lack of any difference may not be surprising given the small numbers involved and smaller

differences would require more subjects with these mutations to be identified. The mutations responsible for a codon usage change neither introduce nor abolish a restriction site. Rapid screening of a large population for this mutation would therefore have to use a dot-blot triage method followed by sequencing, or take a direct sequencing approach. The time allowed for this project was insufficient to extend work to these studies.

Chapter 9

Summary and Conclusions

9.1 Seasonal Variation Receptor Binding

In three separate populations I found seasonal variation in receptor binding with the peaks for both Kd and receptor sites per cell in winter and the troughs in summer. The physiological effect of combined changes in Kd and sites is difficult to predict. Although the response may be unchanged to a fixed concentration of ligand, the response to changing concentrations of ligand is grossly altered. When Kd falls, the gain on the system is increased, while a fall in sites reduces the maximum response achievable. This may be particularly important for hormones such as cortisol, which have a pronounced circadian concentration gradient.

How receptor binding characteristics are changed by climate requires some consideration. As discussed in section 6.6, Kd is likely to be altered by stable changes in the receptor itself such as covalent modification, or its interaction with other intracellular proteins such as the heat shock protein complex. Preliminary work, presented in Chapter 7, suggests this is due to an effect of melatonin, possibly by inhibiting adenylyl cyclase. Confirmation of this could be performed in cell culture, with a dose-response curve for melatonin-induced elevation in glucocorticoid receptor Kd. The effect of forskolin and cAMP concentrations could also be determined in this manner. If confirmed in this model, altered Kd could be due to changes in the receptor protein or accessory proteins. This could be tested by combining cell-free extracts enriched or depleted of glucocorticoid receptor (e.g. by passage through a dexamethasone-affinity column) from cells pre-treated with vehicle, melatonin or forskolin. A persistently elevated Kd after recombination of melatonin treated

glucocorticoid-enriched extract with vehicle treated glucocorticoid-depleted extract would suggest covalent alteration of glucocorticoid receptor protein.

9.2 Cardiovascular Risk

Although our original hypothesis that variation in glucocorticoid receptor Kd might predispose to cardiovascular risk, this proved not to be the case in the population studied. Consideration of a large number of established risk factors including blood pressure, cholesterol and body mass index showed no correlation with receptor binding variables. Furthermore, urinary steroid metabolites and plasma cortisol showed no correlation with receptor binding characteristics. However, it is possible that the receptor in lymphocytes is not regulated in the same way as the hypothalamic and pituitary receptors. Whether it reflects receptor binding in the key tissues involved in cardiovascular disease remains to be determined.

9.3 Glucocorticoid Receptor Gene Mutation Screening

After screening the gene for mutations by SSCP of 80 normal subjects, 4 subjects were found to possess a complex mutation. This had 15 substitutions in exon 5 only 2 of which affected the coded protein, substituting alanine for threonine, by affecting the same codon. This was a rare mutation in normal subjects, with no obvious phenotypic correlate. It would be of interest to examine a larger population for the mutation, particularly with reference to phenotypic changes of increased glucocorticoid sensitivity discussed in section 1.7.

The mutation described lies close to the secondary transactivation domain and the ligand binding domain of the receptor and could affect both of these activities. In vitro studies using site directed mutagenesis to generate the same mutation in a mammalian

expression vector could be used to examine both ligand binding and transactivation activity in more detail.

Appendix 1

Buffers for Molecular Biology

Cell Lysis Buffer

Sucrose	0.32 mol/l
TRIS	10 mmol/l
Magnesium Chloride	5 mmol/l
Triton X-100	1%
Adjusted to pH 7.5	

Nuclear Lysis Buffer

TRIS	10 mmol/l
Sodium Chloride	0.4 mol/l
EDTA	2 mmol/l
Adjusted to pH 2.8	

Phenol/Chloroform

Phenol (molecular biology grade)	500ml
Chloroform (molecular biology grade)	500ml

TE Buffer

TRIS	10 mmol/l
EDTA	10 mmol/l
Adjusted to pH 7.5	

TBE Buffer

Tris Base	54g
Boric Acid	27.5g
0.5M EDTA	20ml
Made up to 1000ml with dH_2O	
pH 8.0	

Formamide Loading Dye

Deionised Formamide	10ml
Bromophenol Blue	1mg
Xylene Cynol Green	1mg

Appendix 2

Non-Linear Regression Modelling of Receptor Binding.

Modelling Receptor Binding Characteristics

In saturation receptor binding assays the fractional occupancy (**r**) of a receptor population by ligand is defined in equation 1, where **Kd** is the dissociation constant of the receptor and **x** is the concentration of ligand.

$$r = \frac{x}{x + Kd} \quad \text{.....Equation 1}$$

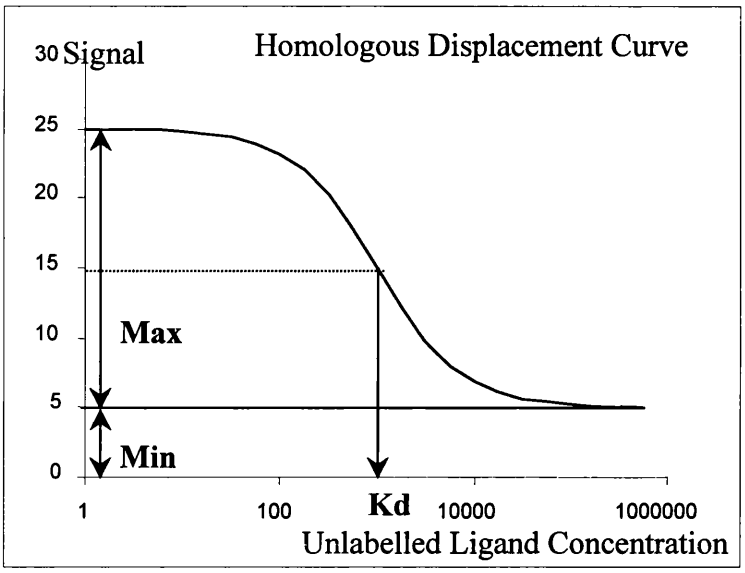
As ligand concentration increases receptor occupancy increases, reaching half-maximal occupancy when ligand concentration equals Kd.

In a homologous displacement receptor binding assay, the concentration of labelled radioligand is kept constant and the concentration of unlabelled, or "cold", ligand is increased. The signal measured from a scintillation counter at each concentration of "cold" ligand is the sum of specific binding of radioligand to receptor and non-specific binding to protein and lipid components of cells and the matrix used to handle receptor incubations and harvesting. The fractional occupancy due to radioligand binding will then be (1-r) and the total signal measured, **s**, will be the product of maximum specific binding, **Max**, plus the component due to non-specific binding, **Min**.

$$s = Max(1 - (\frac{x}{x + Kd})) + Min \quad \text{.....Equation 2}$$

This describes the curve seen in figure 1, where ligand concentration is shown in imaginary units on a logarithmic scale, the measured signal shown in imaginary liner units, and Kd, Min and Max are illustrated graphically

Figure 1 Ideal Homologous Displacement Curve



Development of Least-squares Non-linear regression Method

In order to extract values of Max, Min and Kd from experimental data with the least inaccuracy, a method of fitting equation 2 to observed data is required. Since error is proportional to variance, a least-squares method was used, and since equation 2 is non-linear (i.e. is not of the form y=mx+c) a non-linear fitting approach was taken.

The equation for the squared-residuals, $\underline{y}$, between expected (i.e. calculated data), $\underline{s}$, observed data, $\underline{d}$, will be:

$$y = (s - d)^2 \quad \text{.....Equation 3}$$

Expanding this equation using equation 2 gives:

$$y = \left(Max - \frac{Max.x}{x + Kd} + Min - d\right)^2 \quad \text{.....Equation 4}$$

By further expansion:

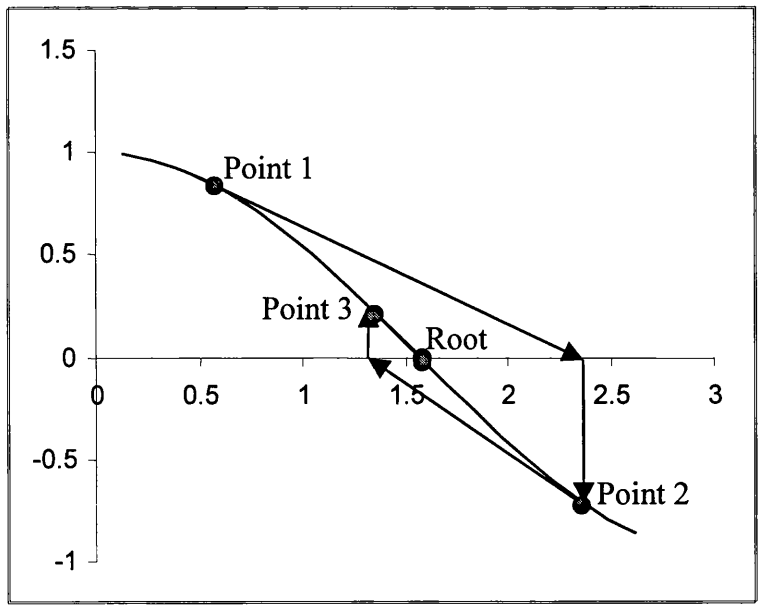
$$y = Max^2 + Min^2 + d^2 + 2.Max.Min - 2.d.Min - 2.d.Max - \frac{2.x.Min.Max}{x + Kd} - \frac{2.x.Max^2}{x + Kd} + \frac{2.x.d.Max}{x + Kd} + \frac{x^2.Max^2}{(x + Kd)^2}$$

.....Equation 5

The best fit of the calculated data, equation 2, to observed data occurs when the square of the residuals is minimised. This occurs when values are found for each of Max, Min and Kd that produce the minimum of equation 4.

For each variable Max, Min and Kd, the minimum of equation 4 is reached when the first derivative of equation 4 equals zero, i.e. at the root of the first derivative. To find the root of this first derivative, a recursive method, Newton-Raphson iteration, was used.

Briefly, for any function that crosses the x-axis, the nearest root can be found from a point on the function as follows. The gradient of the function at the point is found, a line extended to the x-axis along this gradient, and the new value of $\underline{x}$ applied to the function to find a new point on the function. By recursively applying this principle, the value of $\underline{x}$ is found such that f(x)=0.



The general gradient of a line is defined as:

$$m = \frac{y_2 - y_1}{x_2 - x_1} \quad \text{.....Equation 6}$$

where **m** is the gradient, and $\underline{y_2}$ and $\underline{y_1}$ are the y coordinates of the end and beginning of the line, and $\underline{x_2}$ and $\underline{x_1}$ are the x coordinates of the end and beginning of the line.

It follows that to find the new x-coordinate (from which to calculate the new point on the function at each step of the iteration), the increment in x on the previous x-coordinate ($\underline{x_1}$) will be (x_2-x_1). Since the new y-coordinate will be 0 as the gradient crosses the x-axis, this is calculated from equation 6 by rearrangement:

$$x_2 - x_1 = \frac{-y}{m} \quad \text{.....Equation 7}$$

Since the function for which we are trying to find a minimum is the first derivative of y, the gradient will be given by the second derivative. Thus the increment in x for $\underline{x_1}$ will be given by:

$$x_2 - x_1 = \frac{-\left(\frac{dy}{dx}\right)}{\left(\frac{d^2y}{dx^2}\right)} \quad \text{.....Equation 8}$$

This general principle is applied to find the value of Max which produces the minimum square residuals (i.e. minimum of **y** from equation 4), then applying this revised value of Max to calculate the value of Min with the minimum squared residuals and so on.

The equations for the first and second derivatives of y with respect to Max, Min and Kd are shown below:

$$\frac{dy}{dMax} = 2(Min - \frac{x.Min}{x+Kd} + Max - \frac{2.x.Max}{x+Kd} - d + \frac{x^2.Max}{(x+Kd)^2} + \frac{d.x}{x+Kd})$$

$$\frac{d^2y}{dMax^2} = 2(1 - \frac{2.x}{x+Kd} + \frac{x^2}{(x+Kd)^2})$$

$$\frac{dy}{dMin} = 2(Min + Max - \frac{x.Max}{x+Kd} - d)$$

$$\frac{d^2y}{dMin^2} = 2$$

$$\frac{dy}{dKd} = 2(\frac{x.Min.Max}{(x+Kd)^2} + \frac{x.Max^2}{(x+Kd)^2} - \frac{x^2.Max^2}{(x+Kd)^3} - \frac{d.x.Max}{(x+Kd)^2})$$

$$\frac{d^2y}{dKd^2} = 2(\frac{3.x^2.Max^2}{(x+Kd)^4} - \frac{2.x.Min.Max}{(x+Kd)^3} - \frac{2.x.Max^2}{(x+Kd)^3} + \frac{2.d.x.Max}{(X+Kd)^3})$$

Calculating Number of Receptor Sites per Cell

Three steps are involved in calculating the number of specific receptor sites per cell, each designed to reduce inter-assay variation.

Calculating the concentration of radiolabel

The specific activity for each batch of [1,2,4,6,7 ^{3}H] Dexamethasone came with the accompanying literature, and was generally around 3.07TBq per mmol. The Packard scintillation counter contained internal standards which allowed it to calibrate measured counts per minute with disintegrations per minute thus allowing direct calculation of radiolabel activity and concentration. The equation for dexamethasone concentration is given below, where **dpm** is disintegrations per minute, 3.07×10^{15} is the number of disintegrations per mole, and **vol** is the volume of sample in litres.

$$[Label] = \frac{dpm}{60 * 3.07 * 10^{15}.vol} \text{ mol/l}$$

Each batch of freshly prepared [1,2,4,6,7 ^{3}H] Dexamethasone was adjusted to a concentration of 2 nmol/l using this formula. To reduce pipetting errors and inadvertant errors in dilution, each receptor binding assay included quadruplicate 50µl samples for counting. From these, the concentration of label in each incubation (which represented a 4-fold dilution of label) was calculated as:

$$[Dex_{incubation}] = \frac{dpm * 20{,}000 * 1 * 10^{9}}{60 * 3.07 * 10^{15}} \text{ nmol/l}$$

Simplifies:

$$[Dex_{incubation}] = \frac{dpm}{36840} \text{ nmol/l}$$

Calculation of the Number of Receptor Sites Occupied by Radioligand

Since the specific activity per mole of radioligand was known, the number of receptor sites occupied by radioligand in the absence of "cold" ligand was simply calculated as:

$$R_b = \frac{dpm * 6.02 * 10^{23}}{60 * 3.07 * 10^{15}}$$

Calculation of Total Receptor Sites per Cell

At concentrations of radioligand used, only a fraction of the total receptor sites are occupied by ligand and the total number of receptor sites requires to be calculated. Since the fractional receptor occupancy is the ratio of receptors binding ligand to the total number of receptors available for receptor binding interactions, and since this is calculated simply from ligand concentration and receptor Kd, as seen in equation 1, RT can be calculated simply:

$$r = \frac{[R_b]}{[R_T]} = \frac{x}{x + Kd}$$

Rearrangement:

$$R_T = \frac{R_b.(x + Kd)}{x}$$

Since R_b, radioligand concentration and Kd are known with accuracy, the number of receptor sites, R_T, can be calculated.

Finally, to adjust to the number of receptor sites per cell, the number of cells per incubation are known from samples counted on an automated haemocytology counter.

Receptor sites/cell are calculated simply as:

$$R_c = \frac{R_T}{Cells}$$

Choice of Receptor Analysis

The mathematics involved in analysing the receptor binding data required some development. Two problems caused us to seek a custom solution. One was that no commercial software package was available at the time of beginning the project that was sufficiently robust or convenient to use for a large volume of data.

Most previously reported receptor binding studies have relied on Scatchard analysis. Although a useful graphical way to communicate binding data, Scatchard analysis has several mathematical and practical shortcomings. Firstly the method is prone to large errors when variation in binding data at either end of the binding curve (i.e. at high and low ligand concentrations) are evident. Furthermore, these errors are not linear, or symmetrically distributed and therefore are likely to give large errors when binding data deviate significantly from the expected curve. We therefore decided to use a non-linear regression method that was more robust and less prone to deviations due to variance in binding points.

We were careful to check the results we obtained for Kd, Max and Min with a commercially available package (Graphpad Prism, Graphpad Inc., California, USA) and found good agreement. For data with one point in the binding curve with high variance we found non-linear regression produced more reliable and less biased results than Scatchard analysis, as expected.

Appendix 3

List of Investigations in Twin Study

The following parameters were measured for each subject in the twin study:

Age
Sex
Receptor Kd
Receptor Sites per Cell
Zygosity
Lying systolic and diastolic blood pressure and pulse rate
Standing systolic and diastolic blood pressure and pulse rate
Weight
Height
Waist diameter
Hip diameter
Plasma sodium
Plasma potassium
Plasma chloride
Plasma carbon dioxide
Plasma urea
Plasma creatinine
Plasma calcium
Plasma phosphate
Plasma protein

Plasma albumin
Plasma bilirubin
Plasma alkaline phosphatase
Plasma gamma-glutamyl transpeptidase
Plasma aspartate aminotransferase
Plasma alanine aminotransferase
Plasma glucose
Plasma uric acid
Plasma triglycerides
Plasma cholesterol
Plasma very low density lipoproteins
Plasma low density lipoproteins
Plasma high density lipoprotein cholesterol
Plasma 25-Hydroxy cholecalciferol
Plasma C-reactive protein
White cell count
Neutrophil count
Lymphocyte count
Monocyte count
Eosinophil count
Basophil count
Red blood cell count
Haemoglobin concentration
Platelet count

Erythrocyte sedimentation rate
Femoral neck bone mineral density
Greater trochanter bone mineral density
Intertrochanteric bone mineral density
Ward's area bone mineral density
Lumbar vertebrae 1-4 combined bone mineral density
24 hour urinary tetrahydrocortisol
24 hour urinary *allo*-tetrahydrocortisol
24 hour urinary tetrahydrocortisone

In addition, blood was taken for cortisol, aldosterone, deoxycorticosterone, corticosterone and 11-deoxycortisol before and 30 minutes after 250μg of synacthen™ was given intravenously.

Appendix 4

Glucocorticoid Receptor cDNA Sequence

The following sequence was derived from Hollenberg et al 1985, and was obtained from GenBank (via the National Centre for Biotechnology Information at http://www.ncbi.nlm.nih.gov/).

```
LOCUS       HSGCRAR      4788 bp    RNA             PRI       12-SEP-
1993
DEFINITION  Human mRNA for alpha-glucocorticoid receptor (clone OB7).
ACCESSION   X03225 M10901
NID         g31679
KEYWORDS    glucocorticoid receptor.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae;
Homo.
REFERENCE   1  (bases 1 to 4788)
  AUTHORS   Hollenberg,S.M., Weinberger,C., Ong,E.S., Cerelli,G.,
Oro,A.,
            Lebo,R., Thompson,E.B., Rosenfeld,M.G. and Evans,R.M.
  TITLE     Primary structure and expression of a functional human
            glucocorticoid receptor cDNA
  JOURNAL   Nature 318 (6047), 635-641 (1985)
  MEDLINE   86092206
COMMENT     About 500 bp of the 5' region were derived from clone OB10
which
            represents the beta-glucocorticoid receptor (see X03348).
FEATURES             Location/Qualifiers
     source          1. .4788
                     /organism="Homo sapiens"
     CDS             133. .2466
                     /note="(aa 1-777)"
                     /codon_start=1
                     /product="alpha-glucocorticoid receptor"
                     /db_xref="PID:g31680"
                     /db_xref="SWISS-PROT:P04150"

/translation="MDSKESLTPGREENPSSVLAQERGDVMDFYKTLRGGATVKVSAS

SPSLAVASQSDSKQRRLLVDFPKGSVSNAQQPDLSKAVSLSMGLYMGETETKVMGNDL

GFPQQGQISLSSGETDLKLLEESIANLNRSTSVPENPKSSASTAVSAAPTEKEFPKTH

SDVSSEQQHLKGQTGTNGGNVKLYTTDQSTFDILQDLEFSSGSPGKETNESPWRSDLL

IDENCLLSPLAGEDDSFLLEGNSNEDCKPLILPDTKPKIKDNGDLVLSSPSNVTLPQV

KTEKEDFIELCTPGVIKQEKLGTVYCQASFPGANIIGNKMSAISVHGVSTSGGQMYHY

DMNTASLSQQQDQKPIFNVIPPIPVGSENWNRCQGSGDDNLTSLGTLNFPGRTVFSNG

YSSPSMRPDVSSPPSSSSTATTGPPPKLCLVCSDEASGCHYGVLTCGSCKVFFKRAVE

GQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGMNLEARKTKKKIKGIQQATTGVS

QETSENPGNKTIVPATLPQLTPTLVSLLEVIEPEVLYAGYDSSVPDSTWRIMTTLNML

GGRQVIAAVKWAKAIPGFRNLHLDDQMTLLQYSWMFLMAFALGWRSYRQSSANLLCFA

PDLIINEQRMTLPCMYDQCKHMLYVSSELHRLQVSYEEYLCMKTLLLLSSVPKDGLKS
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QELFDEIRMTYIKELGKAIVKREGNSSQNWQRFYQLTKLLDSMHEVVENLLNYCFQTF
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     misc_feature    3101. .3106
                     /note="pot. polyA signal"
     misc_feature    4679. .4684
                     /note="pot. polyA signal"
     misc_feature    4762. .4767
                     /note="pot. polyA signal"
     polyA_site      4788
BASE COUNT     1471 a    939 c    970 g   1408 t
ORIGIN
 HSGCRAR  Length: 4788  August 21, 1996 21:38  Type: N  Check: 5497  ..
       1  TTTTTAGAAA AAAAAAATAT ATTTCCCTCC TGCTCCTTCT GCGTTCACAA
      51  GCTAAGTTGT TTATCTCGGC TGCGGCGGGA ACTGCGGACG GTGGCGGGCG
     101  AGCGGCTCCT CTGCCAGAGT TGATATTCAC TGATGGACTC CAAAGAATCA
     151  TTAACTCCTG GTAGAGAAGA AAACCCCAGC AGTGTGCTTG CTCAGGAGAG
     201  GGGAGATGTG ATGGACTTCT ATAAAACCCT AAGAGGAGGA GCTACTGTGA
     251  AGGTTTCTGC GTCTTCACCC TCACTGGCTG TCGCTTCTCA ATCAGACTCC
     301  AAGCAGCGAA GACTTTTGGT TGATTTTCCA AAAGGCTCAG TAAGCAATGC
     351  GCAGCAGCCA GATCTGTCCA AAGCAGTTTC ACTCTCAATG GGACTGTATA
     401  TGGGAGAGAC AGAAACAAAA GTGATGGGAA ATGACCTGGG ATTCCCACAG
     451  CAGGGCCAAA TCAGCCTTTC CTCGGGGGAA ACAGACTTAA AGCTTTTGGA
     501  AGAAAGCATT GCAAACCTCA ATAGGTCGAC CAGTGTTCCA GAGAACCCCA
     551  AGAGTTCAGC ATCCACTGCT GTGTCTGCTG CCCCCACAGA GAAGGAGTTT
     601  CCAAAAACTC ACTCTGATGT ATCTTCAGAA CAGCAACATT TGAAGGGCCA
     651  GACTGGCACC AACGGTGGCA ATGTGAAATT GTATACCACA GACCAAAGCA
     701  CCTTTGACAT TTTGCAGGAT TTGGAGTTTT CTTCTGGGTC CCCAGGTAAA
     751  GAGACGAATG AGAGTCCTTG GAGATCAGAC CTGTTGATAG ATGAAAACTG
     801  TTTGCTTTCT CCTCTGGCGG GAGAAGACGA TTCATTCCTT TTGGAAGGAA
     851  ACTCGAATGA GGACTGCAAG CCTCTCATTT TACCGGACAC TAAACCCAAA
     901  ATTAAGGATA ATGGAGATCT GGTTTTGTCA AGCCCCAGTA ATGTAACACT
     951  GCCCCAAGTG AAAACAGAAA AAGAAGATTT CATCGAACTC TGCACCCCTG
    1001  GGGTAATTAA GCAAGAGAAA CTGGGCACAG TTTACTGTCA GGCAAGCTTT
    1051  CCTGGAGCAA ATATAATTGG TAATAAAATG TCTGCCATTT CTGTTCATGG
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    1201  ATTCCCGTTG GTTCCGAAAA TTGGAATAGG TGCCAAGGAT CTGGAGATGA
    1251  CAACTTGACT TCTCTGGGGA CTCTGAACTT CCCTGGTCGA ACAGTTTTTT
    1301  CTAATGGCTA TTCAAGCCCC AGCATGAGAC CAGATGTAAG CTCTCCTCCA
    1351  TCCAGCTCCT CAACAGCAAC AACAGGACCA CCTCCCAAAC TCTGCCTGGT
    1401  GTGCTCTGAT GAAGCTTCAG GATGTCATTA TGGAGTCTTA ACTTGTGGAA
    1451  GCTGTAAAGT TTTCTTCAAA AGAGCAGTGG AAGGACAGCA CAATTACCTA
    1501  TGTGCTGGAA GGAATGATTG CATCATCGAT AAAATTCGAA GAAAAAACTG
    1551  CCCAGCATGC CGCTATCGAA AATGTCTTCA GGCTGGAATG AACCTGGAAG
    1601  CTCGAAAAAC AAAGAAAAAA ATAAAAGGAA TTCAGCAGGC CACTACAGGA
    1651  GTCTCACAAG AAACCTCTGA AAATCCTGGT AACAAAACAA TAGTTCCTGC
    1701  AACGTTACCA CAACTCACCC CTACCCTGGT GTCACTGTTG GAGGTTATTG
    1751  AACCTGAAGT GTTATATGCA GGATATGATA GCTCTGTTCC AGACTCAACT
    1801  TGGAGGATCA TGACTACGCT CAACATGTTA GGAGGGCGGC AAGTGATTGC
    1851  AGCAGTGAAA TGGGCAAAGG CAATACCAGG TTTCAGGAAC TTACACCTGG
    1901  ATGACCAAAT GACCCTACTG CAGTACTCCT GGATGTTTCT TATGGCATTT
    1951  GCTCTGGGGT GGAGATCATA TAGACAATCA AGTGCAAACC TGCTGTGTTT
    2001  TGCTCCTGAT CTGATTATTA ATGAGCAGAG AATGACTCTA CCCTGCATGT
    2051  ACGACCAATG TAAACACATG CTGTATGTTT CCTCTGAGTT ACACAGGCTT
    2101  CAGGTATCTT ATGAAGAGTA TCTCTGTATG AAAACCTTAC TGCTTCTCTC
    2151  TTCAGTTCCT AAGGACGGTC TGAAGAGCCA AGAGCTATTT GATGAAATTA
    2201  GAATGACCTA CATCAAAGAG CTAGGAAAAG CCATTGTCAA GAGGGAAGGA
    2251  AACTCCAGCC AGAACTGGCA GCGGTTTTAT CAACTGACAA AACTCTTGGA
    2301  TTCTATGCAT GAAGTGGTTG AAAATCTCCT TAACTATTGC TTCCAAACAT
    2351  TTTTGGATAA GACCATGAGT ATTGAATTCC CCGAGATGTT AGCTGAAATC
    2401  ATCACCAATC AGATACCAAA ATATTCAAAT GGAAATATCA AAAAACTTCT
    2451  GTTTCATCAA AAGTGACTGC CTTAATAAGA ATGGTTGCCT TAAAGAAAGT
    2501  CGAATTAATA GCTTTTATTG TATAAACTAT CAGTTTGTCC TGTAGAGGTT
    2551  TTGTTGTTTT ATTTTTTATT GTTTTCATCT GTTGTTTTGT TTTAAATACG
    2601  CACTACATGT GGTTTATAGA GGGCCAAGAC TTGGCAACAG AAGCAGTTGA
    2651  GTCGTCATCA CTTTTCAGTG ATGGGAGAGT AGATGGTGAA ATTTATTAGT
    2701  TAATATATCC CAGAAATTAG AAACCTTAAT ATGTGGACGT AATCTCCACA
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2751  GTCAAAGAAG GATGGCACCT AAACCACCAG TGCCCAAAGT CTGTGTGATG
2801  AACTTTCTCT TCATACTTTT TTTCACAGTT GGCTGGATGA AATTTTCTAG
2851  ACTTTCTGTT GGTGTATCCC CCCCCTGTAT AGTTAGGATA GCATTTTTGA
2901  TTTATGCATG GAAACCTGAA AAAAAGTTTA CAAGTGTATA TCAGAAAAGG
2951  GAAGTTGTGC CTTTTATAGC TATTACTGTC TGGTTTTAAC AATTTCCTTT
3001  ATATTTAGTG AACTACGCTT GCTCATTTTT TCTTACATAA TTTTTTATTC
3051  AAGTTATTGT ACAGCTGTTT AAGATGGGCA GCTAGTTCGT AGCTTTCCCA
3101  AATAAACTCT AAACATTAAT CAATCATCTG TGTGAAAATG GGTTGGTGCT
3151  TCTAACCTGA TGGCACTTAG CTATCAGAAG ACCACAAAAA TTGACTCAAA
3201  TCTCCAGTAT TCTTGTCAAA AAAAAAAAAA AAAAAGCTCA TATTTTGTAT
3251  ATATCTGCTT CAGTGGAGAA TTATATAGGT TGTGCAAATT AACAGTCCTA
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3801  ATTATTTTAA ACAAAATAGA AGCTGTAGTA GCCCTTTCTG TGTGCACCTT
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3901  ATTTGATTTC TATTCAAGGT GGCCAAATTA TTTGTGTAAT AGAAAACTGA
3951  AAATCTAATA TTAAAAATAT GGAACTTCTA ATATATTTTT ATATTTAGTT
4001  ATAGTTTCAG ATATATATCA TATTGGTATT CACTAATCTG GGAAGGGAAG
4051  GGCTACTGCA GCTTTACATG CAATTTATTA AAATGATTGT AAAATAGCTT
4101  GTATAGTGTA AAATAAGAAT GATTTTTAGA TGAGATTGTT TTATCATGAC
4151  ATGTTATATA TTTTTTGTAG GGGTCAAAGA AATGCTGATG GATAACCTAT
4201  ATGATTTATA GTTTGTACAT GCATTCATAC AGGCAGCGAT GGTCTCAGAA
4251  ACCAAACAGT TTGCTCTAGG GGAAGAGGGA GATGGAGACT GGTCCTGTGT
4301  GCAGTGAAGG TTGCTGAGGC TCTGACCCAG TGAGATTACA GAGGAAGTTA
4351  TCCTCTGCCT CCCATTCTGA CCACCCTTCT CATTCCAACA GTGAGTCTGT
4401  CAGCGCAGGT TTAGTTTACT CAATCTCCCC TTGCACTAAA GTATGTAAAG
4451  TATGTAAACA GGAGACAGGA AGGTGGTGCT TACATCCTTA AAGGCACCAT
4501  CTAATAGCGG GTTACTTTCA CATACAGCCC TCCCCCAGCA GTTGAATGAC
4551  AACAGAAGCT TCAGAAGTTT GGCAATAGTT TGCATAGAGG TACCAGCAAT
4601  ATGTAAATAG TGCAGAATCT CATAGGTTGC CAATAATACA CTAATTCCTT
4651  TCTATCCTAC AACAAGAGTT TATTTCCAAA TAAAATGAGG ACATGTTTTT
4701  GTTTTCTTTG AATGCTTTTT GAATGTTATT TGTTATTTTC AGTATTTTGG
4751  AGAAATTATT TAATAAAAAA ACAATCATTT GCTTTTTG
```

Appendix 5

Primer Sequences

```
Promoter/Exon 1 Segment 1
forward primer (18-mer): -1001 CGCTATCCCGTCCCTTCC -984
reverse primer (21-mer): -864 CCGAGTTGCGTGAAGTGTGTC -884
product length: 138
optimal annealing temperature: 62.8 degrees Celsius
```

```
Promoter/Exon 1 Segment 2
forward primer (19-mer): -916 TTTCCGTGCAACCCCGTAG -898
reverse primer (19-mer): -651 CGTTAAGAGGGCCACCGAG -669
product length: 266
optimal annealing temperature: 64.6 degrees Celsius
```

```
Promoter/Exon 1 Segment 3
forward primer (22-mer): -703 GGGAAGGAGGTAGCGAGAAAAG -682
reverse primer (18-mer): -595 CAGGAAAAAGGGTGGCGG -612
product length: 109
optimal annealing temperature: 60.1 degrees Celsius
```

```
Promoter/Exon 1 Segment 4
forward primer (18-mer): -650 CCGCCCCAGAGAGACCAG -633
reverse primer (19-mer): -224 GCTCGCAAAATGGAGGAGG -242
product length: 427
PstI restriction site at -466 -> product lengths 184,243
optimal annealing temperature: 68.0 degrees Celsius
```

```
Promoter/Exon 1 Segment 5
forward primer (18-mer): -271 GTGTCCGCGCTCTCTTCC -254
reverse primer (18-mer): -165 ACCCACAGAATCCGTCCC -182
product length: 107
optimal annealing temperature: 60.6 degrees Celsius
```

```
Promoter/Exon 1 Segment 6
forward primer (18-mer): -241 CTCCTCCATTTTGCGAGC -224
reverse primer (25-mer): -16 AGAGCCCCTATTTAAGAAAGTCTCC -40
product length: 226
optimal annealing temperature: 63.5 degrees Celsius
```

```
Exon 2 Segment 1
forward primer (22-mer):    128 CACTGATGGACTCCAAAGAATC 149
reverse primer (20-mer):    579 AGCAGACACAGCAGTGGATG   560
product length:452
BglII restriction site at 360 -> product lengths 232,219
optimal annealing temperature: 57.8 degrees Celsius
```

```
Exon 2 Segment 2
forward primer (18-mer):    542 AGAACCCCAAGAGTTCAG 559
reverse primer (18-mer):   1015 CTTGCTTAATTACCCCAG 998
product length:474
Sty I restriction site at 767 -> product lengths 225,249
optimal annealing temperature: 54.6 degrees Celsius
```

```
Exon 2 Segment 3
forward primer (18-mer):    950 TGCCCCAAGTGAAAACAG  967
reverse primer (19-mer):   1291 TTCGACCAGGGAAGTTCAG 1273
product length:342
FokI restriction site at 1134 -> product lengths 184,157
optimal annealing temperature: 56.4 degrees Celsius
```

Exon 3
forward primer (19-mer): 1317 ***(tttag)***CCCCAGCATGAGAC 1330
reverse primer (22-mer): 1480 CCACTGCTCTTTTGAAGAAAAC 1459
product length: 169
optimal annealing temperature: 56.1 degrees Celsius

Exon 4
forward primer (20-mer): 1484 ***(tatag)***GACAGCACAATTACC 1498
reverse primer (18-mer): 1603 TACCTTCCAGGTTCATTC 1586
product length: 125
optimal annealing temperature: 50.8 degrees Celsius

Exon 5
forward primer (20-mer): 1702 ACGTTACCACAACTCACCCC 1721
reverse primer (18-mer): 1874 ATTGCCTTTGCCCATTTC 1857
product length: 173
optimal annealing temperature: 56.1 degrees Celsius

Exon 6
forward primer (20-mer): 1886 GGAACTTACACCTGGATGAC 1905
reverse primer (19-mer): 2015 ATCAGATCAGGAGCAAAAC 1997
product length: 130
optimal annealing temperature: 52.3 degrees Celsius

Exon 7
forward primer (19-mer): 2025 GCAGAGAATGACTCTACCC 2043
reverse primer (20-mer): 2154 TGAAGAGAGAAGCAGTAAGG 2135
product length: 130
optimal annealing temperature: 51.1 degrees Celsius

Exon 8
forward primer (18-mer): 2156 ***(tttag)***TTCCTAAGGACGG 2168
reverse primer (19-mer): 2311 CATGCATAGAATCCAAGAG 2293
product length: 161
optimal annealing temperature: 51.4 degrees Celsius

Exon 9 Segment 1
forward primer (20-mer): 2361 GACCATGAGTATTGAATTCC 2380
reverse primer (22-mer): 2812 GAAGAGAAAGTTCATCACACAG 2791
product length: 452
HaeIII restriction site at 2625 -> product lengths 263,189
optimal annealing temperature: 52.0 degrees Celsius

Exon 9 Segment 2
forward primer (20-mer): 2741 AATCTCCACAGTCAAAGAAG 2760
reverse primer (18-mer): 3166 GTGCCATCAGGTTAGAAG 3149
product length: 426
NlaIII restriction site at 2912 -> product lengths 170,256
optimal annealing temperature: 52.1 degrees Celsius

Exon 9 Segment 3
forward primer (22-mer): 3121 CAATCATCTGTGTGAAAATGGG 3142
reverse primer (22-mer): 3520 GCTGGTTATCTGGAATCACAAC 3499
product length: 400
FokI restriction site at 3360 -> product lengths 238,162
optimal annealing temperature: 54.5 degrees Celsius

Exon 9 Segment 4
forward primer (19-mer): 3461 TCAGGACCTTTTGAAGTAG 3479
reverse primer (19-mer): 3923 GCCACCTTGAATAGAAATC 3905
product length: 463
NlaIII restriction site at 3728 -> product lengths 266,197
optimal annealing temperature: 51.5 degrees Celsius

Exon 9 Segment 5
forward primer (19-mer): 3905 GATTTCTATTCAAGGTGGC 3923
reverse primer (21-mer): 4348 ACTTCCTCTGTAATCTCACTG 4328
product length: 444
PstI restriction site at 4062 -> product lengths 156,288
optimal annealing temperature: 51.2 degrees Celsius

Exon 9 Segment 6
forward primer (18-mer): 4301 GCAGTGAAGGTTGCTGAG 4318
reverse primer (20-mer): 4706 GAAAACAAAAACATGTCCTC 4687
product length: 406
FokI restriction site at 4475 -> product lengths 169,237
optimal annealing temperature: 54.1 degrees Celsius

Notes
Sequences shown in ***bold italics*** refer to sequence in the preceding intron. Nucleotide numbers then refer to the first nucleotide in upper case, and coincide with the published cDNA sequence from Hollenberg et al 1985 (Genbank GEMAM:HUMGCRA)

Primers Exon 9 segment 1 cover the major part of exon 9 while Primers Exon 9 segments 2-6 cover over 2 kilobases of the 3'untranslated region of the gene.

Appendix 6

Informed Consent

The form used to obtain the informed consent for subjects to have venepuncture and urine collections take is copied below.

[redacted]NFREW - PAISLEY FAMILY HEALTH STUDY CONSENT FORM

ırther Information Is Available From:

· Mark Upton - Principal Investigator
'partment of General Practice, Glasgow University,
)odside Health Centre, Barr Street, Glasgow G20 7LR
eephone: 0800 413 772

If you would like further information about the study from someone who is not part of the research team, please contact:

Ms Lisa Schwartz - Ethics Consultant
Department of General Practice, Glasgow University,
Woodside Health Centre, Barr Street, Glasgow G20 7LR
Telephone: 0141 332 8118

ID Label

ULL NAME .....(BLOCK LETTERS)

Is this the name your GP knows you by? If not, the name is .....)

DDRESS .....

.....

ELEPHONE ..... **DATE OF BIRTH** ...../...../.....

PARTICIPATION
I agree to participate/to the subject participating* in this study.
I have read this consent form and Subject Information Sheet and had the opportunity to ask questions about them.
I understand that I am/the subject is* under no obligation to take part in this study, and has the right to withdraw at any time.

Signature of subject/guardian* .....

GENERAL PRACTITIONER NOTIFICATION ABOUT PARTICIPATION
I agree for notice to be sent to my/the subject's* General Practitioner about my/their* participation in this study.

Signature of subject/guardian* .....

GENERAL PRACTITIONER NOTIFICATION ABOUT RESULTS
I agree for my/the subject's* General Practitioner to be notified about my/the subject's* blood pressure, height, weight, ECG, cholesterol level and any other clinically significant information.

Signature of subject/guardian* .....

VENEPUNCTURE
I agree to a blood sample being taken from me/the subject*, and for this to be analysed routinely for biochemistry including glucose, cholesterol, blood clotting and rheology, vitamins and cotinine (a marker of cigarette smoke exposure).

Signature of subject/guardian* .....

DNA (GENETIC MATERIAL)
I give permission for tests to be carried out on genetic material from the white blood cells in the blood sample that I have/the subject has* given. I understand that all results will be used anonymously.

Signature of subject/guardian* .....

STORED PLASMA AND SERUM
I give permission for some of the blood sample that I have/the subject has* given to be stored so that further tests can be carried out in the future. I understand that all results will be used anonymously.

Signature of subject/guardian* .....

MEDICAL RECORDS
I give permission for my/the subject's* progress to be followed through medical records.

Signature of subject/guardian* .....

GNATURE OF INVESTIGATOR .....

ATE .....

**delete as appropriate*

White copy: to be retained by Investigator
Yellow copy: to be retained by Subject.
Blue copy: to be sent to Subject's GP, *if consent given*

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