Regulation of the aldosterone synthase gene- role in human hypertension and adrenal corticosteroid production

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Abstract

Despite many advances in technology and significant levels of funding, the genetic factors that regulate blood pressure in the population remain unclear. The public health implications of this lack of knowledge are conspicuous and a better understanding of the mechanisms behind hypertension would lead to better management of this important risk factor. The myriad of physiological systems involved in maintaining blood pressure can be variably dysregulated in patients with hypertension. Therefore, taking more account of hypertension as a heterogeneous condition may be a useful approach. Given the lack of success in dissecting the genetic factors to date, it seems reasonable to refocus attention on more carefully phenotyped groups of subjects, with the aim of identifying mechanisms specific to them.

Hypertension with inappropriate aldosterone level for salt and volume status, as measured by the use of the aldosterone to renin ratio, is one such sub-phenotype and is present in around 10% of hypertensive patients. This sub-phenotype has been associated with both altered adrenal corticosteroid production and genetic variation in the aldosterone synthase (CYP11B2) and 11ß-hydroxylase (CYP11B1) genes. However, the mechanisms behind this genotype-phenotype relationship are not apparent. It has been proposed that inefficient 11ß-hydroxylation leads to increased ACTH drive in order to maintain cortisol, and that this subtle increase over time leads to hyperplasia of the zona glomerulosa of the adrenal gland and an increased capacity for aldosterone production. Alternatively, as it is known that the aldosterone synthase and 11ß-hydroxylase genes are in a locus of high linkage disequilibrium. It may be that functional changes in the aldosterone synthase gene are co-inherited with functional changes in 11ß-hydroxylase and this digenic phenomenon leads to the phenotype observed. In order to test this second hypothesis, a number of investigations were carried out and are reported in this thesis.

Firstly, the pattern of linkage disequilibrium in the promoter region of the aldosterone synthase gene is examined in chapter 3. Polymorphic variation in this region has previously been reported but the degree to which these variations are inherited in a “haplotype block” was unclear. In this study, it is demonstrated that the -344 polymorphism in CYP11B2, which has been widely

investigated in the past and is associated with hypertension and an elevated aldosterone to renin ratio, can serve as a surrogate for at least 5 other single nucleotide polymorphisms within 2 kilo bases of the transcriptional start site of the aldosterone synthase gene. As such, any of these polymorphisms could be responsible for alteration in transcriptional activity of the gene.

In order to investigate the transcriptional activity of aldosterone synthase, a suitable *in vitro* model system is required. In chapter 4 the H295R cell line is assessed for this purpose. This cell line has been extensively used to explore a variety of aspects of adrenal function; it is the only human cell line capable of expressing the required steroidogenic enzymes and responding to trophins of steroid production. The investigations in chapter 4 confirm the presence of those characteristics of this cell line necessary for use as a model of transcriptional activity of the aldosterone synthase gene, as well as describing some of the limitations of this *in vitro* system. These include variation in steroidogenesis and enzyme expression over time in culture as well as the clear differences in terms of regulation of steroidogenesis from the *in vivo* system. In particular, the lack of zone-specific gene expression in the H295R cells in contrast to the normal adrenal gland is noted.

Having validated a model of aldosterone production, chapter 5 describes the use of this model for investigations of the functional effects of variation in the aldosterone synthase promoter region. A sequence of experiments is described in this chapter. Initially, a bioinformatic search was undertaken to prioritise further investigations. This confirmed the theoretical possibility that single base changes in this region may lead to altered transcription factor binding. Using this data, a single site of variation in CYP11B2 (-1651 rs13268025) was selected for further study. Differential transcriptional activity was confirmed using gene reporter assays. This demonstrated that the C allele at -1651 of the promoter region of CYP11B2 was associated with greater transcriptional activity that the T allele at this site. It was hypothesised that this was due to altered transcription factor binding. Therefore, the next investigation was an assessment of DNA: protein interaction in the presence of the T or C allele at -1651. An electromobility shift assay demonstrated that a larger (less mobile) complex of radiolabelled DNA and nuclear protein from H295R cells was formed in the presence of the T allele than the C allele. This indicated that a previously

unidentified protein that bound more avidly to this region of the gene in the presence of the T allele could act as a transcriptional repressor. In order to identify this protein, biotinylated probes bond in a protein: DNA complex were captured by streptavidin beads and, following trypsin digestion, analysed by mass spectroscopy. This analysis suggested that a transcription factor called APE1 was detectable in the presence of the T allele but not the C allele. In order to test whether APE1 could act as a transcriptional repressor, it was inhibited in a further reporter gene assay. This confirmed that when APE1 was inhibited, there was greater transcriptional activity of aldosterone synthase promoter and that this inhibition was greater in the presence of the T allele. In summary, the evidence presented in this chapter suggests that the polymorphic variation at position -1651 of CYP11B2 is associated with allelic-dependant variation in transcriptional activity mediated by APE1.

Finally, the effects of variation of the aldosterone synthase gene in human subjects were considered. Normal volunteers were studied under standardised conditions of posture and salt intake. Plasma responses to trophins of corticosteroid production were assessed as well as 24 hour urine collections for metabolites of corticosteroids. Data presented in Chapter 6 confirm that subjects homozygous for the T allele at -1651 have a lower excretion rate of aldosterone metabolites than subjects homozygous for the C allele. This is consistent with the notion that these individuals have less transcriptional activity of the aldosterone synthase gene. No allele-dependant difference was observed in response to salt intake or in response to trophins of aldosterone was observed, and some of the possible reasons for this are discussed.

In summary, this work confirms that the linkage disequilibrium across the aldosterone synthase gene promoter region is high, as is the linkage disequilibrium across the entire CYP11B1/CYP11B2 locus. It demonstrates that a number of polymorphisms are co-inherited with the -344 SNP in CYP11B2. Variation at -1651 of CYP11B2 is shown to be associated with allele-dependant variation in transcriptional activity of the aldosterone synthase promoter region. Data are presented to support the hypothesis that the transcription factor APE1 binds more avidly to the T allele at -1651 and acts as a transcriptional repressor. This is supported by the data from normal volunteers confirming that the T allele is associated with reduced aldosterone production. These findings

demonstrate how a common polymorphic variant might lead to a functional change in gene expression that translates into an important physiological phenotype. Further work is required to establish precisely how APE1 functions to alter transcriptional activity of this gene and whether this knowledge can be utilised to improve clinical care.

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Publications

Original Article

McManus F, Sands W, MacKenzie SM, Fraser R, Davies E, Connell JM. APEX1 regulation of aldosterone synthase gene transcription is disrupted by a common polymorphism in humans. *(Submitted)*

Review Article

McManus F, MacKenzie SM, Freel EM. Central mineralocorticoid receptors, sympathetic activity, and hypertension. Curr Hypertens Rep. 2009 Jun;11(3):224-30.

McManus F, McInnes GT, Connell JM. Drug Insight: eplerenone, a mineralocorticoid-receptor antagonist. Nat Clin Pract Endocrinol Metab. 2008 Jan;4(1):44-52

McManus F, Freel EM, Connell JM. Hypertension. Scott Med J. 2007 Nov;52(4):36-42.

Presentations

McManus F, Sands W, Fraser R, Davies E, Connell J. Polymorphic variation in the aldosterone synthase gene alters transcription via APEX1, a novel negative regulator of aldosterone synthase. 37th International Aldosterone Conference 2011, Boston, MA, USA. (Oral presentation). *Winner of Young Investigator Award*

McManus F, Sands W, Fraser R, Davies E, Connell J. Allele-Dependent Regulation of Aldosterone Synthase Is Mediated by APEX1: *In Vivo* and *In Vitro* Evidence. Endo 2011, Boston, MA, USA. (Poster presentation). *Winner of Outstanding Abstract Award* and *Presidential Poster Award*

McManus F, Sands W, Fraser R, Davies E, Connell J. APEX1, a novel, negative regulator of aldosterone synthase activity, differentially binds to a single nucleotide polymorphism in the aldosterone synthase gene and represses transcriptional activity both in vitro and in vivo. Society for Endocrinology 2011 Birmingham, UK. (Oral presentation).

McManus F, Sands W, Davies E, Connell J. Variation in the aldosterone synthase gene may alter gene transcription via altered transcription factor binding. 2010 Society for Endocrinology. Manchester, UK. (Poster presentation).

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McManus F, Wood S, Ingram M, Davies E, Connell J, Fraser R. Quantitative Studies of Steroid Production in the H295R Cell by LC:MS- A Cell Line with Inefficient 11beta-hydroxylation? 35th International Aldosterone Conference, 2009 Washington DC, USA. (Poster presentation).

McManus F, Wood S, Ingram M, Davies E, Connell J, Fraser R. Steroid production in the adrenal cell line H295R varies with time in culture both qualitatively as well as quantitatively. Scottish Society for Experimental Medicine. 2009 Glasgow, UK. (Poster presentation).

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Author's Declaration

I declare that the work presented in this thesis was undertaken by myself and is my own work, unless specified otherwise in the text. It was carried out under the supervision of Professor John Connell and Professor Eleanor Davies in the Glasgow Cardiovascular Research Centre, Institute of Cardiovascular and Medical Sciences, University of Glasgow. This is a record of my research, composed by myself and has not been previously submitted for a higher degree.

Frances McManus

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Abbreviations

1,2 DAG	1,2 diacylglycerol
11βHDS1/2	11beta-hydroxysteroid dehydrogenase type 1 or 2 gene
11βHDS1/2	11beta-hydroxysteroid dehydrogenase type 1 or 2 enzyme
17OHP	17α-hydroxyprogesterone
17OH Preg	17α-hydroxypregnenalone
18OHB	18-hydroxycorticosterone
3βHSD	3beta-hydroxysteroid dehydrogenase gene
3βHSD	3beta-hydroxysteroid dehydrogenase enzyme
95% CI	95% confidence interval
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACTH	Adrenocorticotrophin Hormone
ADH	Antidiuretic hormone
AngII	Angiotensin II
AngIII	Angiotensin III
AngIV	Angiotensin IV
AP1	Activator protein (FOS and JUN)
APE1	APEX1, ref-1
ARR	Aldosterone to Renin Ratio
AT_1	Agiotensin type 1 receptor
AT_2	Angiotensin type 2 receptor
ATF-1	Activating trasncription factor 1
ATF-2	Activating trasncription factor 2
ATF3	Activating transcription factor 3
ATP2B1	Plasma membrane calcium-transporting ATPase 1 gene
AVP	Argenine vasopressin/ Anitdiuretic hormone
BER	Base excision repair
BRIGHT	British Genetics of Hypertension
BTG2	B-cell translocation gene 2
Bu_2cAMP	dibutyryl cAMP
c10orf107	chromosome 10 open reading frame 107 gene
Ca^{2+}	Calcium ions
CACNA1H	calcium channel, voltage-dependent, T type, alpha 1H subunit gene
CACNB2	calcium channel, voltage-dependent, beta 2 subunit gene
CAH	Congenital adrenal hyperplasia
CaMK	Calcium/ Calmodulin dependant protein kinases
cAMP	Cyclic adenosine monophosphate
CASZ1	castor zinc finger 1 gene
CDH13	Cadherin 13
CHARGE	Cohorts for Heart and Aging research in Genome Epidemiology
Cl-	Chloride ions
CLCN6	chloride channel 6
CLCNKA	Choride channel Ka gene
CLCNKB	Choride channel Kb gene
CLIP	Corticotrophin intermediate like peptide
Compound B	Corticosterone
Compound E	Cortisone
Compound F	Cortisol
Compound S	11-deoxycortisol
COUP-TF	Chicken ovalbumin upstream promoter- transcription factor
CRE	cAMP responsive element
CREB	cAMP responsive element binding
CREM	cAMP responsive element modulator

CRH	Corticotrophin releasing factor
Cry	Cryptochrome
CSK-ULK3	c-src tyrosine kinase/unc-51-like kinase 3 gene
Ct	Crossing threshold
CYP11A1	Cholesterol side chain cleavage enzyme
CYP11A1	Cholesterol side chain cleavage gene
CYP11B1	11beta-hydroxylase enzyme
CYP11B1	11beta-hydroxylase gene
CYP11B2	Aldosterone synthase enzyme
CYP11B2	Aldosterone synthase gene
CYP17A1	17α-hydroxylase gene
CYP17A1	17α-hydroxylase enzyme
CYP17A1	17alpha-hydroxylase enzyme
CYP17A1	17alpha-hydroxylase gene
CYP1A2	Cytochrome P450 1A2 gene
CYP21B	21-hydroxylase enzyme
CYP21B or *CYP21A2*	21-hydroxylase gene
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic blood pressure
DCT	Distal Convoluted tubule
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribose nucleic acid
DOC	11-deoxycorticosterone
EDTA	Ethylenediaminetetraacetic acid,
EMPHASIS-HF	Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure
EMSA	Electromobility shift assay
ENaC	Epithelial Sodium Channel
EPHESUS	Eplerenone Post–Acute Myocardial Infarction Heart Failure Efficacy and Survival Study
Erg	Early growth response
ERK	Extracellular-signal-regulated kinases
ESC	Embryonic stem cells
FDX1	Adrenodoxin/Ferrodoxin 1 gene
FDX2	Adrenodoxin/Ferrodoxin 2 gene
Fe/S	Iron Sulphur
GC:MS	Gas chromatography: tandem mass spectroscopy
GILZ1	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid receptor
GRA	Glucocorticoid Remedial Hyperaldosteronism
GRAPHIC	Genetic Regulation of Arterial pressure of Humans in the Community
GWAS	Genome Wide Association Study
HNRNPK	Heterogeneous Nuclear Ribonucleoprotein K
HPA	Hypothalamic pituitary adrenal axis
HPLC	High performance liquid chromatography
HWE	Hardy Weinberg equilibrium
IP_3	inositol trisphosphate
IPO7	Importin 7 gene
ITGA9	Integrin alpha 9
IUPAC	International Union of Pure and Applied Chemistry
JP	Joining Protein
K^+	Potassium ions
KCNJ1	Potassium inwardly rectifying channel subfamily J, member 1 gene
Ki-RAS	Kirsten Ras GTP-binding protein
LBD	Ligand binding domain
LC:MS	Liquid chromatography-tandem mass spectrometry

LD	Linkage disequilibrium
LOD	Logarithm of Odds
MAF	Minor allele frequency
Mb	Megabase
MCR2	Adrenocorticotrophin receptor 2
MEK	MAPK/ERK kinase
MI	Myocardial infarction
miRNA	Micro RNA
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
MSH	Melanocyte stimulating hormone
MTHFR	Methylenetetrahydrofolate reductase gene
Na^+	Sodium ions
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyl transferase
NBRE-1	NGFI-B response element
nCaRE	Negative calcium response elements
NED4-2	Neural precursor cell expressed developmentally down-regulated protein 4-2
NGF1B	Nerve Growth factor 1B
NGS	Next Generation Sequencing
NHR	Nuclear hormone receptor
NICE	National Institute for Clinical Excellence
NR4A1	Nerve Growth factor 1B
NR5A1	SF1, Steroidogenic factor 1
NURR1	nuclear receptor related 1
NURR77	Nuclear receptor related 77/ Nerve Growth factor 1B
NYHA	New York heart Association
Obs.Het	Observed heterozygosity
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2 enzyme
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2 gene
PAPY	Primary Aldosteronism Prevalence in Hypertension
PC1/2	Prohormone convertase 1/2
PCR	Polymerase chain reaction
PHAII	Pseudohypoaldosteronism type 2
PI3K	Phosphatidylinositol 3-kinases
PIAS1	protein inhibitor of activated STAT
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C
PLEKHA7	Pleckstrin homology domain containing, family A member 7 gene
PMS1	postmeiotic segregation increased 1 gene
Pol II	RNA polymerase II
POMC	Pro-opiomelanocortin
POR	P450 oxidoreductase enzyme
POR	P450 oxidoreductase gene
PRA	Plasma renin activity
PRC	Plasma renin concentration
PRDM8/FGF5	PR domain containing 8/ fibroblast growth family 5 gene
Pred.Het	Predicted heterozygosity
PTH	Parathyroid hormone
QTL	Quantative Trait Locus
RAAS	Renin-angiotensin-aldosterone system
Raf-1	RAF proto-oncogene serine/threonine-protein kinase
RALES	Randomised Aldactone Evaluation Study
REN	Renin gene

RESOLVD	Randomized evaluation of strategies for left ventricular dysfunction
RNA	Ribonucleic acid
RNAi	RNA interference
ROMK	Renal Outer Medullary Potassium channel gene
RT-PCR	Real-time polymerase chain reaction
SAVE	Survival and Ventricular Enlargement study
SBP	Systolic blood presure
SF-1	Steroidogenic factor 1
SGK1	Serine/threonine-protein kinase 1
SH2B3	SH2B adapter protein 3 gene
siRNA	Small interfering RNA
SLC12A1	Solute Carrier 12, member 1 gene
SLC12A3	Solute Carrier 12, member 3 gene
SLC24A4	Solute carrier family 24 member 4 gene
SNP	Single Nucleotide Polymorphisms
SPAK	Ste20-related proline-alanine−rich kinase protein
StAR	Steroidogenic acute regulatory hormone
STAT	signal-transducer and activator of transcription protein
StDev	Standard deviation
STK39	serine threonine kinase 39 gene
SULT2A1	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1
SUMO-1	small ubiquitin-like modifier-1
TAL	Thick Ascending Limb
TASK	Two-pore domain acid-sensitive potassium channel
TBX3/TBX5	T-box 3/ -box5 gene
THAldo	Tetrahydroaldosterone
Ubc9	Ubiquitin conjugating enzyme 9
ULK4	unc-51-like kinase 4 gene
WGHS	Women's Genome Health Study
WHO MONICA	World Health Organisation Monitoring Trends and determinants in cardiovascular disease
WNK	With no Lysine serene/threonine kinase
WT	Wild type
WTCC	Wellcome Trust Case Control Consortium
ZNF652	Zinc finger protein 652 gene
β-EP	β-endorphin
β-LPH	β-lipotrophin

Introduction

1. Introduction

1.1 Hypertension

1.1.1 Historical context

Hypertension remains the most significant modifiable risk factor for cardiovascular disease and makes a major contribution to the global burden of disease (World Health Organisation, 2002). Despite advances in understanding of the pathophysiology of hypertension as well as a significant expansion in therapies available, the effect of uncontrolled disease on morbidity and mortality continues to rise.

Historically, many of the consequences of high blood pressure have been recognised for hundreds of years, but it was only with the invention of accurate methods of measurement of blood pressure that the study of blood pressure advanced. Our current understanding of blood pressure began in the age of Enlightenment: this era inspired reasoned enquiry in the field of natural philosophy in general, and the study of cardiovascular physiology and blood pressure was not left behind. The spirit of the age was embodied by the Reverend Stephen Hale, who was the first to physically demonstrate blood pressure in 1733 (Hales, 1733). He inserted one end of a brass tube into the artery of a horse and attached a vertical glass tube, to the other. Blood pressure was demonstrated when the artery which had been tied off was released and blood rose in the tube to a height of eight feet three inches above the left ventricle of the heart. Despite this clear demonstration of physiology, it was not until 1896 that this was used in a practical sense when an Italian physician, Dr Scipione Riva-Rocci, developed an inflatable rubber cuff that occluded the artery of the upper arm (Riva-Rocci S., 1896). Systolic blood pressure corresponded to the pressure at which the brachial pulse could no longer be palpated and his device was strongly promoted by Dr Harvey Cushing the neurosurgeon and one of the founding fathers of endocrinology (Cushing H, 1903). However, the measurement of systolic and diastolic blood pressure did not become a common clinical measurement until 1905, when Dr Korotkoff described the systolic and diastolic sounds that can be heard with a stethoscope, just below the levels that Riva-Rocci palpated using his inflatable cuff (Korotkoff NC, 1905). Thus, having established a more accurate measurement technique,

the debate began regarding what was considered normal blood pressure, and was the stimulus to a rapid expansion in the investigation of the pathogenesis of the elevated blood pressure values. From a clinical perspective, it became possible for the first time to identify patients with elevated blood pressure prior to developing end organ damage as well as monitor treatment.

1.1.2 Hypertension: The clinical definition

Although the advances made by the pioneers of blood pressure measurement allowed physicians to objectively monitor blood pressure, blood pressure targets and the threshold for intervention has been a subject of controversy since that time. Hypertension could be defined as a "blood pressure level above which investigation and treatment do more good than harm"; a definition that was first used by Evans and Rose in 1971 (Evans and Rose, 1971) and this remains a useful tenet today. To that end, the concept of cardiovascular risk has gained prominence and an estimation of this can be calculated based on extensive epidemiological data gathered from the Framingham study (Anderson et al., 1991) or other sources (Woodward et al., 2007). This allows the synthesis of numerous risk factors (e.g. age, gender, cholesterol, blood pressure) in considering the prevention of cardiovascular disease. Nevertheless, cut off values are required, however arbitrary, and the British Hypertension Society gives clear guidelines regarding the thresholds for treatment as well as targets, in the context of overall cardiovascular risk (Williams et al., 2004) (see Table 1-1). Blood pressure targets are based on resting clinic blood pressure measurements, using instruments based on Riva-Rocci and Korotkoff's observations with standardised techniques in order to increase reproducibility and minimise error (Williams et al., 2004) (see Table 1-2).

CATEGORY	SYSTOLIC BLOOD PRESSURE (mmHG)	DIASTOLIC BLOOD PRESSURE (mmHG)
Optimal blood pressure	<120	<80
Normal blood pressure	<130	<85
High-normal blood pressure	130–139	85–89
Grade 1 Hypertension (mild)	140–159	90–99
Grade 2 Hypertension (moderate)	160–179	100–109
Grade 3 Hypertension (severe)	>180	>110
Isolated systolic hypertension (Grade 1)	140–159	<90
Isolated systolic hypertension (Grade 2)	>160	<90

Table 1-1 British Hypertension Society classification of blood pressure levels.

Report of the fourth working party of the British Hypertension Society, 2004—BHS IV (Williams et al., 2004).

GUIDELINES FOR BLOOD PRESSURE MEASUREMENT
Use a properly maintained, calibrated and validated device
Measure sitting blood pressure routinely: standing blood pressure should be recorded at the initial estimation in elderly and diabetic patients
Remove tight clothing, support arm at heart level, ensure hand relaxed and avoid talking during the measurement procedure
Use cuff of appropriate size
Lower mercury column slowly (2 mm/s)
Read blood pressure to the nearest 2 mmHg
Measure diastolic as disappearance of sounds (phase V)
Take the mean of at least two readings; more recordings are needed if marked differences between initial measurements are found.
Do not treat on the basis of an isolated reading

Table 1-2 Guidelines for blood pressure measurement.

Report of the fourth working party of the British Hypertension Society, 2004—BHS IV (Williams et al., 2004)

There is an awareness that blood pressure measurements as one-off, office assessments may not reflect the day-to-day blood pressure of patients and alternatives to this have been proposed including ambulatory monitoring, and home blood pressure monitoring (O'Brien et al., 2003; Stergiou and Bliziotis, 2011; Hodgkinson et al., 2011). It should be borne in mind that the thresholds for diagnosing hypertension are lower using these methods. There is mounting evidence to support both ambulatory and home monitoring as efficacious and cost effective in terms of diagnosis (Lovibond et al., 2011), and this has resulted in a recent change to the NICE guidelines (http://guidance.nice.org.uk/CG127) published in August 2011, to support the use of use of ambulatory blood pressure monitors to confirm the diagnosis of hypertension. However, there are limited data to inform clinicians regarding appropriate targets for treatment using this method of monitoring.

In addition, given the current and appropriate focus on blood pressure as a function of overall cardiovascular risk (British Cardiac Society, 2005), interest has been shown in measurements that may better reflect this. For example, pulse wave analysis as an indication of central pressure measurements has been proposed as a prognostic marker which could assist in cardiovascular assessment (Task Force Members: et al., 2007). Measurement of this index has been hampered by the invasive nature of the gold standard method of measuring central pulse pressure or by controversy over the relevance of surrogate markers. Williams et al have recently proposed a novel method of measurement and analysis of radial artery pulse wave velocity and recommend its clinical utility in assisting in identifying and monitoring disturbances of blood pressure (Williams et al., 2011). However, a lack of outcome data with all these measurements hinders their use in clinical practice and measurements seated in the clinician's consulting room, using the traditional brachial cuff instrument, developed over 100 years ago, remain the cornerstone of blood pressure management.

1.1.3 Aetiology of essential hypertension

Although distinct values have been determined for the diagnosis of hypertension, it is recognised that blood pressure is a continuous variable with a normal distribution and it has been many years since the continuous association

between increasing blood pressure values and increased cardiovascular risk was confirmed (Hamilton et al., 1964). In around 5% of patients with elevated blood pressure a clear pathophysiological mechanism can be identified but in the remainder, it is very likely that multiple genetic and environmental influences combine to determine blood pressure. This thesis will focus on the role of genetic variation in a candidate gene, aldosterone synthase, and its role in hypertension and relative aldosterone excess. However, I will first discuss some of the other contributing factors to the development of hypertension as well as a brief overview of investigations to date exploring relevant physiological and genetic factors leading to hypertension.

Lifestyle factors

The majority of this thesis will focus on genetic and pathophysiological mechanisms for the development of hypertension. However it is important to mention in brief the lifestyle mechanisms that have contributed to the significant increase in global burden of hypertension.

Weight gain is associated with a rise in blood pressure (Masuo et al., 2000) and strategies to lose weight have been shown to result in lower blood pressure. A meta-analysis of 25 randomised controlled trials included 4874 participants from different ethnic populations and showed a blood pressure reduction of 4.4/-3.6 mm Hg for around 5kg weight loss either by energy restriction, increased physical activity, or both (Neter et al., 2003). Exercise, even in the absence of weight loss improved blood pressure parameters. A further meta-analysis of 54 trials showed that blood pressure reduction of 3.8 mmHg SBP (95% CI 2.7 to 5.0 mm Hg, $p < 0.001$) and 2.6 mmHg DBP (95% CI 1.8 to 3.4 mm Hg, $p < 0.001$) with regular aerobic exercise. This effect was achieved in people regardless of their initial blood pressure, weight or ethnicity (Whelton et al., 2002).

Placebo-controlled studies have shown that reduced sodium intake lowers blood pressure and increases response to pharmacological therapy (Cappuccio et al., 1997; Elliott, 1989; Cutler and Stamler, 1997). However, until very recently, evidence of long term benefit was lacking. This has now been addressed in a recent major long term follow up study (Cook et al., 2007) where reduction in dietary sodium intake is associated with a reduced risk of cardiovascular

morbidity and mortality (relative risk 0.75, 95% CI 0.57 to 0.99, p=0.04 adjusted for trial, clinic, age, race, and sex). The interaction of potassium and sodium status has been proposed as an important factor in the regulation of blood pressure. Epidemiological data provide support for this view in that those societies with low sodium/ high potassium diets have lower blood pressure than industrialised societies (Adrogue and Madias, 2007). Potassium supplementation is associated with a reduction in blood pressure (Whelton et al., 1997) and the need for antihypertensive medication (Siani et al., 1991). In addition, the DASH trial (Appel et al., 1997) demonstrated that a fruit and vegetable rich diet containing a potassium content more than twice that of an average American diet, reduced blood pressure to a greater extent than controls with a similar level of sodium.

Genetic factors

Clearly environmental influences must be taken into account both by the patient, their physician, as well as public health organisations. However, environmental factors only account for part of blood pressure variation and genetic factors are estimated to account for 15-40%.

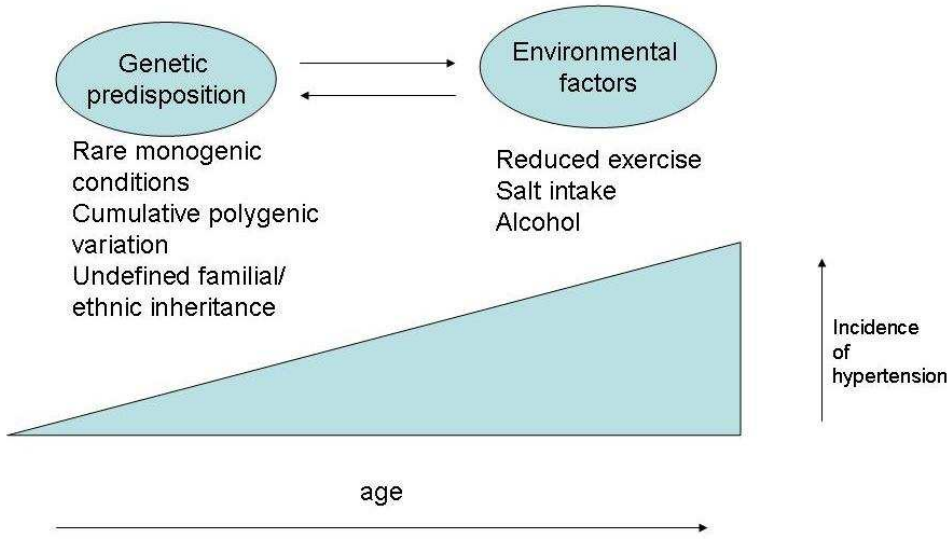


Figure 1-1 Interaction of genetic and environmental factors leading to elevated blood pressure.

The search for genes responsible for blood pressure variation remains ongoing. While some rare monogenic conditions have a large impact on an individual's blood pressure, more common genetic variations at multiple loci with smaller effects on blood pressure are likely to have a cumulative effect. Strategies for investigation of hypertension based on these approaches will be discussed in more detail in section 1.1.5 to 1.1.8.

As most investigation of culprit genes has focused on those involved in physiological systems known to regulate blood pressure, a brief overview of some of the homeostatic systems of blood pressure control will now follow.

1.1.4 Physiological regulation of blood pressure.

Normal control of blood pressure depends on a number of complementary neural, endocrine and paracrine mechanisms and the development of hypertension can result from perturbation of any or all of these. Blood pressure depends on cardiac output which is a function of heart rate and stroke volume multiplied by peripheral resistance. While systems that modulate all three of these variables will be discussed in the following section, the main focus will be on the renin- angiotensin- aldosterone system (RAAS) and in particular, aldosterone, which will be discussed at greater length in this work.

Renin-angiotensin- aldosterone system

The RAAS plays a crucial role in the regulation of blood volume. The classical mechanisms of homeostatic control mediated by the RAAS are demonstrated in Figure 1-2. Angiotensinogen, mainly synthesised in the liver, is cleaved by renin to form angiotensin I. Renin (encoded by the *REN* gene on chromosome 1) is released from the juxtaglomerular cells in the kidney in both an active and inactive form in response to signals from baroreceptors in the afferent arteriole, sympathetic neural activity and circulating catecholamines as well as chemoreceptor in the macula densa of the distal tubule. Prorenin (the inactive form) is cleaved to form active renin *in vivo* (Hsueh and Baxter, 1991) (this reaction requires recognition as cryoactivation of prorenin to renin can be a source of measurement errors unless samples are properly handled). Increased pressure to the juxtaglomerular cells inhibits renin release, thus forming a conventional negative feedback loop and maintaining homeostasis.

The angiotensin I peptide is the substrate for angiotensin converting enzyme (ACE), which cleaves angiotensin I to the biologically active angiotensin II (AngII). There are at least two subtypes of AngII receptors: the cardiovascular actions are effected mainly via the angiotensin type 1 (AT_1) receptor (de Gasparo et al., 2000), mediating numerous physiological actions including vasoconstriction and increased sodium and water retention both directly via the renal tubule cells and via aldosterone. These actions are described in Figure 1-2. The AT_2 receptor is thought to antagonise some of these effects of the AT_1 receptor and appears to have a mainly hypotensive action although the precise nature of this is still a subject of debate (de Gasparo et al., 2000).

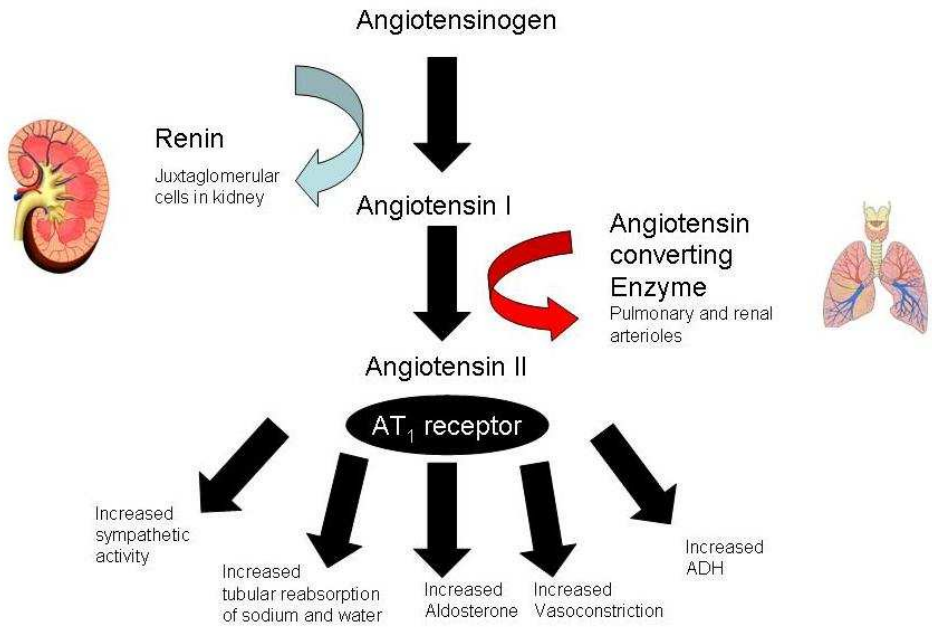


Figure 1-2 Schematic representation of the classical renin-angiotensin-aldosterone system.

Angiotensin II was until relatively recently thought to be the critical and central product of the RAAS, but current evidence suggests an important role of a number of other biologically active peptide products of the RAAS. Angiotensin II is swiftly degraded via the enzymatic action of aminopeptidase A, giving rise to angiotensin III (AngIII or Ang 2-8) and this can be further cleaved to form Angiotensin IV (AngIV or Ang 3-8), via aminopepdidase N. Ang III can bind to the AT_1 receptor and there is evidence that this peptide has a centrally acting pressor effect; intracerebroventricular injections cause blood pressure to rise

(Wright et al., 1985), and aminopeptidase A inhibitors that can cross the blood brain barrier have been shown to reduce blood pressure in experimental animals (Fournie-Zaluski et al., 2004).

Angiotensin I can also be converted to angiotensin 1-7 by neutral endopeptidase, and it can also be generated from angiotensin II by angiotensin converting enzyme 2 (ACE2) (Donoghue et al., 2000). Angiotensin 1-7 appears to act as a vasodilator and the discovery of the ACE2 enzyme and its product has refocused investigation of the RAAS. Much interest has been shown in understanding its mechanism of action as well as potentiating its effects (Ferrario et al., 2005) but the physiological implications of these novel peptides remain unclear, particularly given their very short half lives *in vivo*.

Bradykinin is a peptide with vasodilating properties and is part of the kallikrein-kinin cascade, an important system of modulation of the cardiovascular system in its own right. It is known to be an alternative substrate for angiotensin converting enzyme (ACE). Pharmacological inhibition of ACE leads to increased bradykinin (in clinical practice this manifests as the most common side effect of cough) and is has been proposed that this may contribute to the beneficial effects of these agents (Sharma, 2006). A summary of these novel products of the RAAS are demonstrated in Figure 1-3.

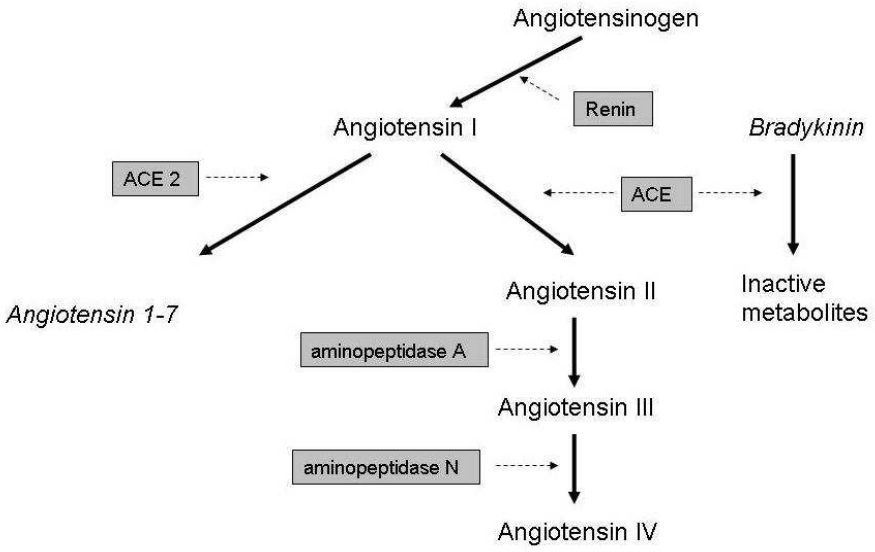


Figure 1-3 Schematic representation expanded renin angiotensin system.

Enzymes are shown in shaded boxes and their role in enzymatic reactions by dotted arrows. Peptides with vasodilating properties are in italics.

Thus, it is now apparent that the traditional concept of a simple, sequential pathway underestimates the complexity and sophistication of the RAAS. Further, it is clear that manipulation of the system as in the pharmacological treatment of hypertension can have multiple feedback consequences that were previously unrecognised. Of course, aldosterone is the end product of the RAAS and further discussion of its regulation and mechanism of action can be found in section 1.2 and 1.3.

Sympathetic nervous system

The sympathetic nervous system influences the control of blood pressure both by regulating heart rate directly by innervations of the cardiac muscle to increase heart rate (positive chronotropy), inotropy and conduction velocity (positive dromotropy), via beta-adrenoceptors, as well as activation of alpha adrenoreceptors in the adventitia, leading to vasoconstriction of the peripheral vasculature and increased peripheral resistance. In addition, it contributes in a more indirect manner via release of humoral factors into the blood stream. Renin release from the juxtaglomerular cells in the kidney is stimulated by sympathetic nerve activation, triggering the RAAS system discussed above (Gordon et al., 1967). Angiotensin II enhances the release of norepinephrine from sympathetic nerve endings and inhibits re-uptake, leading to enhanced sympathetic adrenergic function and producing a feedback loop (Wang et al., 1997). Catecholamine release from the adrenal medulla contributes to the maintenance of blood pressure. Indeed, as the medulla originates from neuroectoderm tissue, it can be viewed as a modified sympathetic ganglion. The crucial role of the sympathetic nervous system (and the kidney as a key modulator of a number of pathophysiological mechanisms) has recently been highlighted in a study demonstrating the efficacy of selective renal denervation in the management of treatment-resistant hypertension (The Simplicity HTN-2 Trial, 2010). The authors propose that selective denervation of the kidneys reduces renin release, renal blood flow and central sympathetic outflow and provide evidence that, in patients with hypertension resistant to pharmaceutical intervention, this technique is effective in lowering blood pressure by 32/12mmHg (St Dev 23/11). Although the role of the sympathetic nervous system in both the aetiology and treatment of hypertension is the subject of

ongoing investigation, it is beyond the scope of this current work and will not be discussed further.

Oxidative stress

The concept of oxidative stress as a key mechanism in the control of blood pressure and the development of hypertension has been the focus of much research. Increased generation or reduced clearance of reactive oxygen species leads to increased oxidative stress and this, it is suggested, leads to a myriad of alterations in cell signaling, ultimately leading to vascular injury, remodeling and hypertension (Briones and Touyz, 2010). While perturbations in oxidative stress have been shown to be associated with cardiovascular disease, it remains to be established whether this is a cause or a result of the underlying pathological mechanisms. For example, both animal models (Rocha et al., 1999) and human examples of aldosterone excess (Farquharson and Struthers, 2002) are associated with increased markers of oxidative stress.

Given these various factors, it is apparent that the mechanisms of maintaining blood pressure homeostasis are interdependent and interconnected, involving numerous humoral factors and organ systems but ultimately culminating in their major effect on salt and water homeostasis mediated via the kidneys. Data from both animal (Rettig et al., 1990) and human (Curtis et al., 1983) studies underline this, with the observation that renal transplantation can normalise blood pressure in previously hypertensive subjects. With this in mind, there will now follow a discussion of investigations of genetic factors that may contribute to perturbations of blood pressure regulation.

1.1.5 Genetic basis of hypertension

Hypertension has a strong genetic component, as clearly demonstrated by family and twin studies (Williams et al., 1991; Hunt et al., 1989; Havlik et al., 1979; Rose et al., 1979). While there are lifestyle factors, as discussed above (1.1.3), that influence blood pressure, it is a highly heritable trait and family history remains one of the strongest predictors of future risk of developing high blood pressure. It seems likely that these genetic factors will lie in genes involved in systems which are known to contribute to the control of blood pressure (as described above). However, despite much investigation focused on

these systems, a comprehensive understanding of the causative genetic factors has not yet been achieved.

In recent years, two separate strategies have been proposed to investigate the genetic aetiology of hypertension. One focuses on comparing the prevalence of a specific candidate gene or genes identified from the systems previously described in groups of hypertensive or control subjects. The other is to seek to identify causative genetic regions by comparing the entire genome in groups of normotensive and hypertensive individuals. The following section will discuss some aspects of the progress made to date using these two approaches.

1.1.6 Candidate gene studies

This approach depends on a detailed knowledge of the disease, and of the systems that might be responsible, in order to make a "best guess" at genes that have a high probability of being involved. Many of the studies using this approach have examined genes known or suspected to be involved in monogenic disorders of blood pressure regulation, and postulating that "essential" hypertension is a *forme fruste* of these conditions. After selecting the candidate gene of interest, the hypothesis is that if variation in a particular gene is responsible for elevated blood pressure it will be over represented in a group of patients with hypertension compared with a matched group of controls. There are numerous examples of monogenic conditions causing blood pressure disturbance and subsequent investigations into the mechanisms underlying them has informed the study of population variation in blood pressure. The specific examples regarding monogenic disorders of sodium chloride transport in the renal tubule are given below. However, a fuller list of monogenic disorders of blood pressure regulation incorporating other mechanisms including adrenal steroidogenesis and action is given in Appendix 7.1.

Monogenic disorders of the distal convoluted tubule and the thick ascending limb

Monogenic disorders causing defects in the sodium chloride co-transporter of the apical (luminal) membrane in the distal convoluted tubule and thick ascending loop of Henle´ can lead to dysregulation of blood pressure and electrolytes- Gitelman, Bartter and Gordon syndromes are all examples of this.

Greater understanding of these disorders has resulted in the identification of a number of candidate genes for hypertension.

Gitelman's syndrome is characterised by renal salt wasting, resulting in hypokalaemia, hypomagnasaemia, hypocalciurea, metabolic alkalosis and low blood pressure. In addition, patients compensate for their salt wasting by activating the RAAS i.e. increasing serum renin and aldosterone levels. The molecular defect is known to be in the thiazide-sensitive sodium chloride cotransporter of the distal convoluted tubule (Figure 1-4). Mutations on solute carrier family 12 (sodium/chloride transporters), member 3 (SLC12A3) gene on chromosome 16, causes this condition (Simon et al., 1996) which is inherited in an autosomal recessive manner. This clinical syndrome resembles that seen in patients abusing thiazide diuretics.

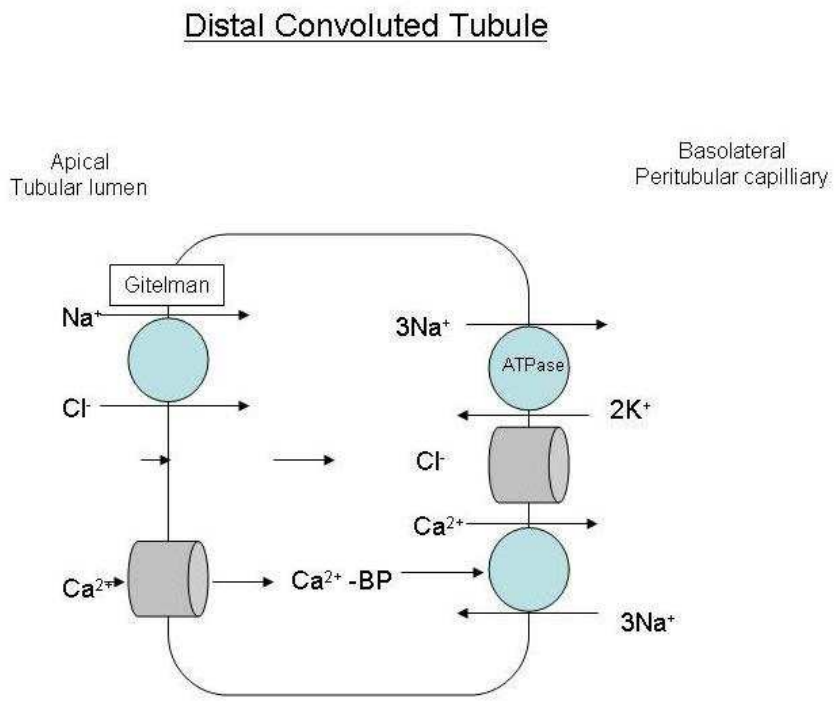


Figure 1-4 Schematic representation of salt losing tubulopathy Gitelman's syndrome.

In the distal convoluted tubule Na^+Cl^- moves from the tubular lumen into the cell along a concentration and electrical gradient. A Na^+-K^+ ATPase pump allows Na^+ to be reabsorbed at the basolateral membrane back into the circulation. Cl^- is reabsorbed via a Cl^- channel in the basolateral membrane. Calcium passes across the luminal membrane via a Ca^{2+} Channel and binds to vitamin D-induced binding protein (Ca-BP). It is then exchanged for Na^+ which follows the concentration and electrical gradient. The site of disruption of these processes in Gitelman's syndrome is shown in the apical membrane of the distal convoluted tubule.

The thiazide-sensitive sodium chloride transporter in the distal convoluted tubule can also be affected by a gain of function mutation causing high blood pressure. This causes a phenotype precisely opposite to Gitelman syndrome and is known as pseudohypoaldosteronism type 2 (PHAII) or Gordon syndrome. The clinical features of hypertension, hyperkalemia, metabolic acidosis, normal renal function, low or low-normal plasma renin activity and normal or elevated aldosterone concentrations are inherited in an autosomal dominant fashion. The primary physiological defect in PHAII is enhanced distal chloride reabsorption and it is interesting to note that the abnormalities can be corrected by a thiazide diuretic, which these patients are exquisitely sensitive to. However, in contrast to Gitelman's syndrome, there is no defect in the gene encoding the sodium chloride transporter in the DCT (SLC12A3) but rather mutations in genes located on chromosomes 12 and 17. These encode With No Lysine (WNK) serene/threonine kinases 1 and 4 respectively (Wilson et al., 2001). WNK1 and 4 localise to the DCT and cortical collecting duct and are involved in the regulation of the NaCl transporter. In the presence of WNK4, the transporter is down regulated and in the presence of some isoforms of WNK1, WNK4 itself is down regulated (Yang et al., 2003) The WNK enzymes act via a kinase cascade, phosphorylating the effector kinases SPAK (Ste20-related proline-alanine−rich kinase) and OSR1 (odd-skipped-related 1) shown in Figure 1-5.

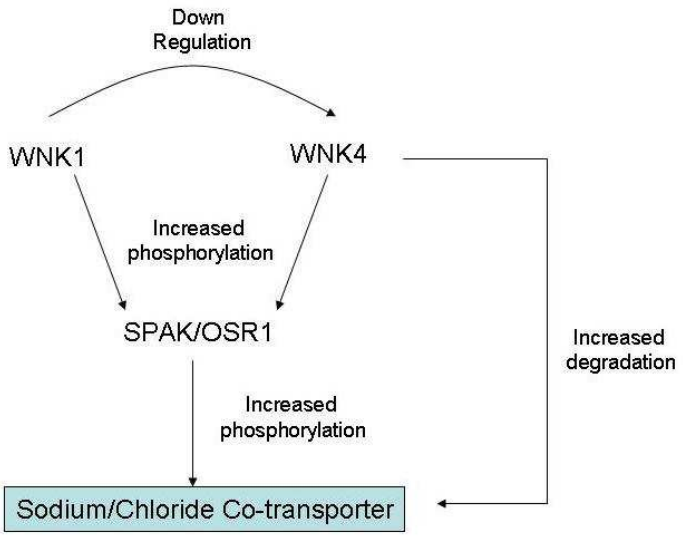


Figure 1-5 Kinase cascade regulating sodium/chloride transport.

In PHAII mutant WNK4 leads to loss of inhibition of sodium chloride cotransporter and increased expression at the apical surface

With increased understanding of these monogenic disorders of blood pressure regulation, attempts have been made to implicate these in population blood pressure variation. Ji et al studied variation in the genes *SLC12A3* (Gitleman's syndrome), *KCNJ1* and *SLC12A1* (mutations here are known to be associated with Bartter's syndrome), in the Framingham Heart Study offspring cohort (Ji et al., 2008). 3125 individuals from this cohort had DNA available for analysis. They found that the carrier state for rare functional mutations in *SLC12A1*, *SLC12A3* and *KCNJ1* reduced blood pressure by on average 6.3mmHg systolic measurements and -3.4mmHg diastolic.

A further examination of polymorphic variants in these genes and association with hypertension was undertaken in the GRAPHIC (Genetic Regulation of Arterial pressure of Humans in the Community) cohort (Tobin et al., 2008). This family study recruited 2037 individuals from around 500 families. The investigators looked at variants with a frequency of >0.1 in genes known to be involved in monogenic forms of altered blood pressure regulation. The study demonstrated a significant association between 5 single nucleotide polymorphisms (SNP) in *KCNJ1* and mean 24 hour SBP and DBP. The effect size of the variants in this study was modest and in the order of -1.58mmHg for mean SBP and -0.95 for mean DBP. In this study, which was limited to examining common variants, no association was found in other genes involved in monogenic disorders, specifically those involved in other forms of Bartter's syndrome, Gitelman's syndrome, apparent mineralocorticoid excess, Liddle's syndrome and autosomal recessive pseudohypoaldosteronism.

The same investigators had previously interrogated this cohort to examine the effect of SNPs in the WNK1 (9 SNPs) and WNK4 genes (1 SNP) and hypertension (Tobin et al., 2005). 996 individuals from 250 families were studied and an association was found between 4 polymorphisms in the WNK1 gene and mean 24 hr SBP and 5 SNPs in WNK1 with mean DBP. The effect size was small, affecting both SBP and DBP by less than 1.5mmHg. There is little variability in the WNK4 gene in Caucasian population and in this study, there were no associations with variation in WNK4 (one polymorphism) and blood pressure. The British Genetics of Hypertension study (BRIGHT) (Newhouse et al., 2005) also confirmed an association between variations in the WNK1 gene and extremely hypertensive subjects. Importantly, the BRIGHT investigators extended and replicated these

findings in a large case control cohort composed of 6 sub-studies (Newhouse et al., 2009). Although the effect of WNK1 variation did not always reach statistical significance in the replication groups, there was consistency in the direction of effect, and in combination the statistical association was strong in the replication groups. These findings in a large study cohort, with replication in an independent sample, using careful and comprehensive genotyping and appropriate statistical analysis, provided convincing evidence of the involvement of WNK1 in the pathogenesis of hypertension. It is also interesting to note that the BRIGHT investigators also found a significant blood pressure effect of rare haplotypes, in line with the findings of Ji et al (Ji et al., 2008) and Tobin et al (Tobin et al., 2008) as discussed above.

1.1.7 Candidate Gene studies: Conclusions

Major advances in the understanding of pathways involved in blood pressure regulation, renal salt handling and monogenic disorders of blood pressure have increased dramatically and this has led to focused exploration of candidate genes. However, despite this expansion in knowledge, there has been limited success in extending this to our understanding of genetic causes of essential hypertension. There are significant limitations of the candidate gene approach as there remain areas of incomplete understanding and possible pathways as yet undiscovered. The candidate gene approach is distinctly biased towards known pathways and previously explored hypotheses and unexpected genes involved in novel pathways could be missed. In addition, as most candidate gene studies investigate only one gene or one pathway at a time, the impact of gene-gene interactions and pathway-pathway interactions is not taken into account. This may lead to false positive or false negative studies.

In addition, given current knowledge of the likely small effect size of each mutation, most candidate gene association studies were too small and, as a result, underpowered to detect an effect. Perhaps as a consequence, there have often been conflicting reports regarding whether the variant of interest is associated with disease, and there have often been as many negative studies associated with a gene of interest as positive. Many examples of these are described above. Further explanation for this is likely to lie in the different ethnicities of study populations; there is significant variation in the haplotypes

structures of different ethnic groups, as well as often widely varying allele frequencies. These population differences have a substantial influence on the outcome of case-control studies, particularly bearing in mind that positive studies only confirm association and not causation. Thus, given the well recognised phenomenon of linkage disequilibrium, the genotyped polymorphism may not be functional, but merely co-inherited with the functional variation. Further, differences in study population sizes, definitions of disease as well as altered and unquantified changes in environmental factors are all likely to contribute to the heterogeneity of the results of many candidate gene studies.

In response to some of the inherent flaws in the design of candidate gene studies, the alternative “genome wide” approach was proposed. This is discussed in the following section.

1.1.8 Linkage analysis and GWAS

Given that the inherent drawback of the candidate gene approach is the restrictive nature of the investigations in which results are limited to known pathways, alternatives have been proposed which do not suffer from this bias.

Linkage analysis

Linkage studies are a method whereby regions of a genome that contain genes which may be responsible for a trait can be identified. In order to understand linkage studies, two simple terms must be comprehended: linkage, and linkage disequilibrium. These terms have been used above but an understanding of the distinction between them is important. Two loci are linked if they are inherited together more often than expected if inheritance is random. Therefore, during meiosis, recombination occurs between the loci with a probability of less than 50%. However, linkage disequilibrium (LD) refers to the phenomenon observed in a population when the two loci are found together on the same haplotype more often than expected. When recombination events occur between the two loci, linkage disequilibrium is weakened. LD is preserved the closer the loci are to each other.

Linkage studies utilise family based cohorts and DNA is examined for areas that are shared by affected relatives but not by unaffected family members. Non-

identical siblings should share 50% of the genome, so for any individual marker, the chance of sib pairs carrying the same marker is 1:2. In sib pairs that are both affected by hypertension, the allele close to the locus causing hypertension should be should shared more often. This allows the identification of quantitative trait loci (QTL) within which the causative gene may be located but depends on the phenomenon of linkage disequilibrium between the marker used (either a SNP or microsatellite) and the causative gene. Linkage is assigned a logarithm of the odds (LOD) score; large positive scores are evidence for linkage, and negative scores are evidence against. A LOD score of 3 is regarded as significant evidence of linkage with genome wide significance and this is equivalent to a p value of 0·0001. The QTL may encompass a significant area of the genome and does not identify the gene responsible for the phenotype.

Numerous linkage studies for hypertension have been undertaken over the last 10 years. Many linkage peaks have been identified but few have achieved LOD scores that suggest genome wide significance and fewer still have been replicated. Studies that have successfully isolated statistically significant associations suggesting association with chromosome 17q (60-76cM) (Levy et al., 2000) from the Framingham cohort, 18q (80-94cM) in a genetically homogenous Icelandic cohort (Kristjansson et al., 2002), 4p (13-43cM) in a study of 18 Dutch families (Allayee et al., 2001) and 2p (26.5-27.1cM) in probands with severe hypertension from an isolated Sardinian village (Angius et al., 2002). In addition, the BRIGHT investigators identified a susceptibility locus on chromosome 6q in their cohort of severely hypertensive sib pairs (Caulfield et al., 2003). It is interesting that none of these results have been replicated within this group of studies, nor has there been any publication of replication with the same degree of statistical significance in the rest of the literature. The reason may be in the genetic heterogeneity of the different study populations with different genetic aetiologies underlying the causes of hypertension, altered effect size of the loci in different populations, and varying degrees of linkage disequilibrium in discrete ethic groups.

Genome wide association studies

With the advent of the Human Genome Project and the International HapMap project, a more complete knowledge of the reference sequence and the

common variations from this within the human genome, as well as a better understanding of the pattern of linkage disequilibrium was available. As a result, the linkage strategy was expanded and “tag SNPs” could be nominated to represent portions of the genome and give comprehensive coverage and the genome of cases and controls compared. High throughput genotyping using microarray chips enabled the practical application of this detailed understanding of the human genome to be interrogated further. This has allowed the genotyping of up to a million SNPs in thousands of cases and controls, and the conclusions of these studies were eagerly awaited and published in 2007. Initial results did not provide major insights into the origins of hypertension; the Wellcome Trust Case Control Consortium failed to identify any SNPs that were significant at a genome wide level (Wellcome Trust Case Control Consortium, 2007). However, proof of concept was achieved in the identification of association signals in bipolar disorder, coronary artery disease, Crohn’s disease, rheumatoid arthritis, Type 1 diabetes and Type 2 diabetes. It was suggested that the reason for a lack of association with any SNPs for hypertension may have been that the effect size for each SNP was too small and as such the study was underpowered to detect them. A potential confounding factor was the validity of controls in this study as there was a significant discrepancy in age (cases mean age 68, controls mean age 48). This may have led to misclassification of controls that may go on to develop hypertension over the next 10-20 years. In addition, it was suggested that greater coverage across the genome could be achieved and this was undertaken in subsequent studies using 1000K chips.

In recognition that larger populations were needed, researchers collaborated to pool resources internationally. Two “mega-analyses” were constructed by collecting numerous biobanks of data. This resulted in the publication in 2009 of the Global BP-Gen consortium (Newton-Cheh et al., 2009) and the Cohorts for Heart and Ageing Research in Genome Epidemiology (CHARGE) BP consortium (Levy et al., 2009). These studies included 34,433 individuals from 17 studies in the Global BP-Gen and 29,136 individuals from 6 cohorts in the CHARGE study. Both undertook a comparison with data from the other study and were published simultaneously in Nature Genetics in 2009. The CHARGE study identified 13 SNPs for SBP, 20 for DBP and 10 for hypertension at $P < 4 \times 10\text{-}7$ while the Global BP-Gen study identified 3 SNPs for SBP and 5 for DPB. However, due to differences

in analysis, the results from each investigator were not identical and only *CYP17A1* and SH2B2 achieved genome wide significance in both analyses for phenotypes of blood pressure (See Table 1-3). The magnitude of the effect on blood pressure varied between 0.5-1.5mmHg.

Chromosome	Gene	Possible Pathway	Phenotype	Study
Ch10	*CYP17A1*	Steroidogenesis	SBP HTN	CHARGE BP-Gen
Ch12	*SH2B3*	Negative regulator of cytokine signalling	DBP	CHARGE BP-Gen
Ch 1	*MTHFR*	Homocystine metabolism	SBP DBP	BP-Gen
Ch 17	*PLCD3*	Phospholipase C, catalyses formation of second messengers (DAG, IP3)	SBP	BP-Gen
Ch 4	*PRDM8/ FGF5*	Fibroblast growth factor family	DBP HTN	BP-Gen
Ch 15	*CYP1A2*	Drug metabolism	DBP	BP-Gen
Ch 10	*c10orf107*	Uncharacterised protein	DBP	BP-Gen
Ch 17	*ZNF652*	Zinc finger protein, transcriptional repressor	DBP	BP-Gen
Ch 11	*PLEKHA7*	Cellular structural protein	SBP	CHARGE
Ch 12	*ATP2B1*	Plasma membrane calcium pump	SBP DBP HTN	CHARGE
Ch 10	*CACNB2*	Voltage dependant calcium channel protein	DBP	CHARGE
Ch 15	*CSK-ULK3*	Sereine/threonine protein kinase	DBP	CHARGE
Ch 12	*TBX3-TBX5*	Transcriptions factors involved in embryological development	DBP	CHARGE
Ch 3	*ULK4*	Sereine/threonine protein kinase	DBP	CHARGE

Table 1-3 Results from the Global BP-Gen and CHARGE meta-analyses

SNPs which achieved genome wide significance $p<5x10^{-7}$. Possible pathways (summarised from NCBI Gene http://www.ncbi.nlm.nih.gov/gene) are given but it should be borne in mind that the SNPs examined are associated with hypertension but there is currently no proof of causation. The phenotype associated with SNP and the study which contributed evidence is displayed.

Attempts have been made to replicate these findings in other, independent populations. The Women's Genome Health Study (WGHS) was examined in which 28 345 female health professionals from North America were genotyped for nominated SNPs (Ho et al., 2011). Many of the findings of the Global BP-Gen/CHARGE studies were reproduced. *SH2B3, ATP2B1, MTHFR, CYP17A1* and *PLEKHA7* were significantly associated with SBP. Eight SNPs were associated with DBP in the WGHS: *CACNB2, ATP2B1, CYP1A2, c10orf107*, *SH2B3, ZNF652*, were significant. The significance level was set at $P < 1.2 \times 10^{-3}$ (this was arrived at after correction for multiple testing and is less stringent than required for genome wide significance). In addition, a new locus was identified, *CASZ1,* which did not reach significance in the Global BP-Gen or CHARGE studies. Reanalysis of the GRAPHIC study in a "pathway-omic" manner (Tomaszewski et al., 2010) (ie high throughput analysis of common variant in genes involved in nominated candidate pathways) concurred with the finding that SNPs in *MTHFR* (encompassing the *CLCN6* gene) are implicated.

Two further studies have successfully identified SNPs that are significant at a genome wide level. First, Wang et al examined a group of Amish subjects and identified *STK39* as independently associated with blood pressure increase of increases of 3.3/1.3 mm Hg in SBP/DBP (Wang et al., 2009). The investigators replicated their findings in 4 other Caucasian populations and found a consistent, albeit weaker BP effect. The *STK39* gene encodes the SPAK protein, which interacts with WNK kinases (discussed in section 1.1.6) and thus a possible functional explanation exists as to why variation in this gene may influence blood pressure. Further work has supported this hypothesis and a polymorphism in *STK39* has been associated with hypertension in a large group of middle aged Swedes (Fava et al., 2011) but not in a cohort of British subjects (Cunnington et al., 2009) and the *STK39* gene has not been found to associate with hypertension in either of the two larges genome wide studies described above. Second, Org et al identified variation in *CDH13* gene as being associated with blood pressure and hypertension (Org et al., 2009) although the association was not consistent in all cohorts examined. *CDH13* encodes for a calcium-dependent cell–cell adhesion glycoprotein T-cadherin and is also an attractive susceptibility gene as it interacts in vascular endothelial and smooth muscle cells and may regulate

vascular wall functions. However, to date, there have been no further studies directly linking this gene to hypertension.

GWAS from other ethnic populations have also been published within the last year. A large (25 826 subjects) Japanese study (Takeuchi et al., 2010), concluded that *MTHFR, FGF5, CYP17A1*, *ATP2B1*, and *CSK-ULK3* were important loci in a non-European cohort as well as the Caucasian populations studied in CHARGE and Global BP-Gen. In addition, two further loci (*CASZ1, ITGA9*) which had been implicated but not confirmed in CHARGE and Global BP-Gen achieved statistically significant associations in this Asian cohort. A study of African-American participants identified variation in *PMS1, SLC24A4, YWHA7, IPO7*, and *CACANA1H* as associated with hypertension (Adeyemo et al., 2009). Genes that were identified by CHARGE and Global BP-gen were associated with hypertension in this cohort but with less statistical significance. These results suggest that, while there may be similarities between ethnic groups, there are likely to be different genes involved in blood pressure disturbance and this should provoke caution when investigators are attempting to replicate their results in other populations.

1.1.9 Linkage analysis and GWAS: Conclusions

The advent of techniques to analyse the genome in its entirety gave great promise for the understanding of heritable disease. However, disappointingly, despite the very large numbers of subjects studied and significant economic cost, results have not provided clear and incontrovertible candidate genes, nor have they yet suggested novel treatable pathways.

Although there is disparity between results in different replication studies and different ethnic populations, there have been some consistent messages from GWAS. However, it is important to remember that SNPs identified within these genes are “Tag SNPs” (as listed in Table 1-3) and that these represent many other SNPs that they are in linkage disequilibrium with, sometimes spanning significant portions of the genome. The causative SNP could lie a large distance away or even within another gene.

However, the major message from the GWAS era has been the small effect size of many of the identified genes which leaves a large proportion of the heritable

component of blood pressure unexplained. This “missing heritability” has been the subject of much controversy. It has been proposed that hypertension is even more polygenic than previously thought, with many genes each having a very small effect, and consequently that studies have been underpowered to detect them. “Mega-analyses” sought to address this problem of loss of power but there remain significant missing data to account for the heritable nature of blood pressure variation. It has been proposed that another possible source of the “missing heritability” is in rare alleles. GWAS studies were set up to capture allele frequencies of greater than or equal to 5%. However it has been suggested that multiple rare alleles (rather than a few common alleles) may be the explanation for blood pressure heritability. Even the combined resources of the “mega-analysis” GWAS trials were not adequately powered to detect these rare variations. Further, the hypothesis-free approach of these studies results in multiple comparisons and consequently, there is a need to increase the stringency required to achieve statistical significance. This may have resulted in true positive findings being rejected.

As with previous methods of investigation, including candidate gene and linkage analysis, results have been difficult to replicate and population heterogeneity is again likely to be a factor in this. The small effect size of the variation in genes that have been implicated must make their effects more easily obscured in the different genetic and environmental milieu of a replicate study population. Gene-environment interactions are difficult if not impossible to study using GWAS approaches, due to the unmanageable number of subjects required. Given that environmental factors play such a crucial role in determining blood pressure phenotype (see section 1.1.3) on the background of genetic susceptibility, this may have reduced the power of these studies to identify culprit genes. Further, given that the accuracy and success of GWA studies depends heavily on the linkage disequilibrium in the population (this allows investigators to choose appropriate “tag” SNPs and impute and interpret results), accurate and precise knowledge of this is essential. The international HapMap project was a significant advance in our understanding of this. However, the 1000 Genome Project (Durbin et al., 2010) aims to detail this in a more complete manner and new sequencing technologies will allow this to be completed more comprehensively and economically. The 1000 Genomes project, using next-

generation sequencing (NGS) or massively parallel sequencing technologies has allowed millions of sequence reads of short lengths (35–250bp) at a time. However, although cost has reduced substantially over the last 5-10 years, sequencing remains expensive. Innovative measures have been suggested e.g. pooling of DNA samples (Lee et al., 2011), to overcome some of these difficulties. These emerging technologies will undoubtedly increase the volume of data that can be gleaned from large scale GWA studies but there remain many other difficulties to be overcome before this approach is likely to produce clinically meaningful results.

Therefore, while GWAS have provided important insights into the genetic architecture of human hypertension, the quest for causative genes and pathways continues and the debate surrounding how best to proceed persists. It seems clear that hypothesis driven research remains fundamental to progress in this area, given the complex nature of gene- environment interactions. While the limitations of candidate gene studies should be borne in mind, focused genotype- phenotype studies and continued use of sub-phenotypes of hypertension, are crucial to improving understanding of hypertension. Loci containing CYP17A1, MTHFR and SH2B3, as well as many other genes mentioned above, are likely to contribute to susceptibility to hypertension. However, there is strong evidence that many other genes and gene regulatory areas are involved; particularly those involved in renal sodium handling, prominent among these are those affecting aldosterone synthesis and action.

1.2 Relative aldosterone excess: a sub-phenotype of hypertension

The multi-factorial mechanisms underlying the pathogenesis of hypertension have led to descriptions of sub-phenotypes, i.e. building a profile of clinical features of sub-groups which might share similar common pathogenic mechanisms. In terms of clinical research, a potential advantage of this approach is that, by enriching study cohorts with patients who share common aetiologies, the causes (particularly genetic) are more likely to be discovered. In addition, identifying homogeneous groups of patient may allow better targeting of therapies to control blood pressure and prevent complications. (A practical example of this is the advice regarding treatment for hypertension issued by the

British Hypertension Society which suggests the use of diuretics as first line therapy in older adults and those of African decent, and ACE inhibition in younger patients (Williams et al., 2004))

The sub-phenotype under further investigation in this work is that of relative aldosterone excess. In order to investigate this further, an understanding of the role of aldosterone in health and disease is necessary and this will be discussed in the following sections.

1.2.1 Discovery of aldosterone

Aldosterone was first identified by Silvia (nee Simpson) and James Tait in 1952 and crystallised in 1953 in collaboration with the pharmaceutical company Ciba and the Reichstein group in Basel (Tait et al., 2004). This was achieved by extracting 60-70mg of pure aldosterone from a colossal 1500kg of pig adrenal glands (Heusler and Kalvoda, 1996). Initially named electrocortin, prior to its structure being known, this substance has long been recognised to be involved in the retention of sodium and water and is the most potent (although not the most abundant) mineralocorticoid in humans. We now know that aldosterone acts on the classic mineralocorticoid receptor in the cortical collecting duct of the kidney to increase activity of the epithelial sodium channel (ENaC), resulting in net reabsorption of sodium and water; electrical neutrality is maintained by loss of potassium and hydrogen ions from the renal cell to the tubular fluid.

1.2.2 The mineralocorticoid receptor

The classical actions of aldosterone are mediated by the mineralocorticoid receptor (MR) which, like the glucocorticorticoid receptor (GR), belongs to the nuclear hormone receptor (NHR) family, a group of ligand- activated transcription factors. When unoccupied, these receptors are present in the cytosol but when bound by their ligand, translocate to the nucleus, interact with their respective response elements and induce a change in transcriptional activity leading to an increase in activity of the ENaC.

The structure of both the MR and GR receptors are typical of this group of receptors, possessing an N-terminal domain, a central DNA-binding domain, and a hinge region that links to a ligand-binding domain (LBD) within the C-terminal

half of the receptor. The DNA-binding domain, which comprises two zinc finger structures, binds to specific DNA sequences on its target gene. The MR DNA-binding domain is 94% identical to that of the glucocorticoid receptor (GR) and 90% identical to that of the progesterone receptor (Lombes et al., 2000). Subtle differences in the structure of aldosterone and cortisol (discussed in greater detail in section 1.2.4) lead to greater affinity for their relative receptors; nevertheless, there is considerable overlap in the types of steroid that can be bound by MR and GR. Thus, in spite of its name, the MR is able to bind cortisol, which circulates in plasma at levels a thousand fold higher than mineralocorticoids, with equal affinity to aldosterone. Therefore an alternative mechanism is required to maintain the tissue specificity of aldosterone. The enzyme 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2), acts to convert cortisol to cortisone; without this activity, cortisol, which binds with high affinity to the mineralocorticoid receptor, will activate the receptor and prevent its regulation by aldosterone (White et al., 1997)(Figure 1-6).

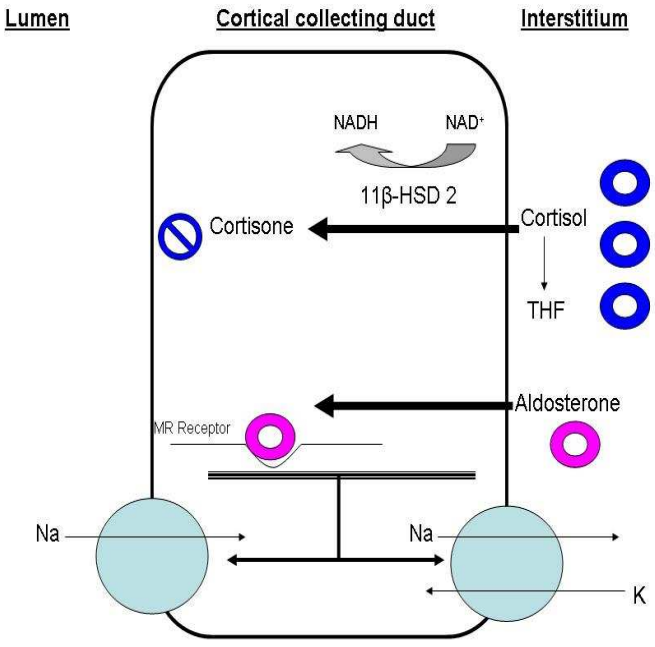


Figure 1-6. Schematic representation of kidney cortical collecting duct.

Cortisol (blue open circle) is inactivated to cortisone (blue lined circle) by the NAD+ dependant "chaperone" enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). This prevents promiscuous occupation and activation of the MR by cortisol and allows regulation by aldosterone (pink open circle).

11ß-HSD is a bidirectional enzyme, and it has been proposed that the redox state of the cell determines whether it functions as a dehydrogenase (cortisol to cortisone) or reductase (cortisone to cortisol) (White et al., 1997).

1.2.3 Mechanism of action of aldosterone

Epithelial sodium channel

In epithelial tissues, aldosterone primarily acts via the epithelial sodium channel (ENaC) to excrete potassium and retain sodium and water. Activation of the mineralocorticoid receptor by aldosterone results in increased expression of ENaCα subunit, which combines with ENaCß and γ (which are constitutatively expressed) and this complex moves to the apical cell surface. The molecular mechanism of regulation of ENaC is now better understood as a result of the study of the monogenic disorder, Liddle's syndrome. This is a rare autosomal dominant condition resulting in a gain of function of ENaC, and is clinically associated with moderate to severe hypertension presenting in childhood (see Appendix 1). The genetic abnormality causing Liddle's syndrome lies on chromosome 16 and mutations identified to date affect the cytoplasmic C-terminal tails of the ß- and γ-subunits of ENaC (Hansson et al., 1995). These result in loss of an adaptor motif that interacts with Nedd4-2 (neural precursor cell-expressed, developmentally down-regulated 4-2). Nedd4-2 ligates a ubiquitin "tag" to the ENaC which targets it for internalisation and subsequent destruction (Soundararajan et al., 2010)(Figure 1-7). This induces constitutative activity of the epithelial sodium channel (ENaC) in the cortical collecting duct (Shimkets et al., 1994), as if activated by aldosterone (see Figure 1-7). Importantly, in this condition, spironolactone (a mineralocorticoid receptor antagonist) is not effective because activation of ENaC is not due to excessive aldosterone levels and is independent of the mineralocorticoid receptor. However, the ENaC is amiloride-sensitive making this the treatment of choice in these patients.

Other components of the ENaC regulatory complex (as shown in Figure 1-7 and discussed in the figure legend) are clearly also of physiological importance. Although no monogenic disorders of SGK1 affecting blood pressure have yet been identified, an SGK1 knock out mouse model demonstrated higher levels of aldosterone under normal conditions compared to wild type animals under

normal salt intake. When examined under reduced salt intake, the SGK1 knock out animals demonstrate a salt losing, aldosterone resistant phenotype, with lower blood pressure and reduced glomerular filtration (Wulff et al., 2002). While polymorphisms of SGK1 have been associated with hypertension in a candidate gene study (Busjahn et al., 2002), this has not been replicated in genome wide association studies (as discussed in section 1.1.7 and 1.1.9).

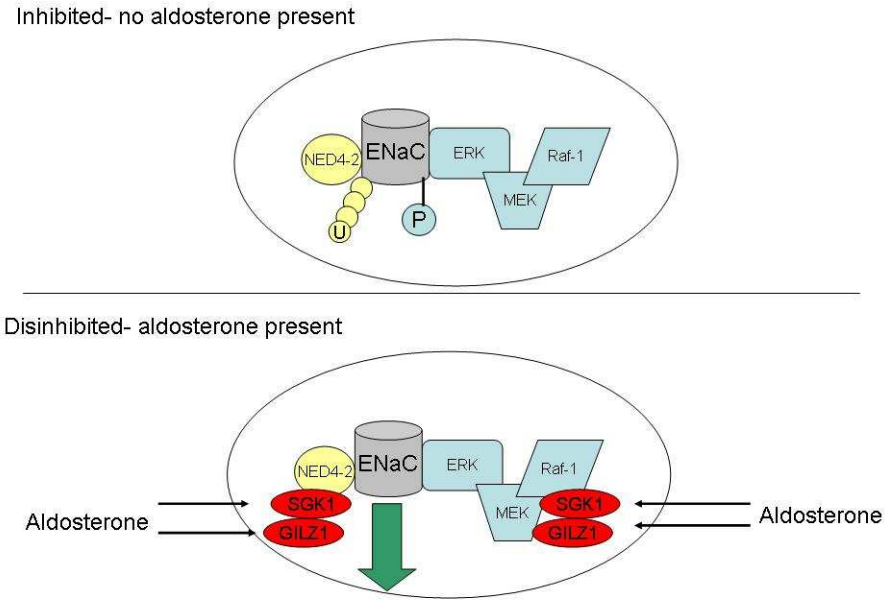


Figure 1-7 The ENaC regulatory complex under the control of aldosterone.

In the absence of aldosterone (top panel) the ENaC regulatory complex composed of NED4-2, ERK, MEK and Raf-1 results in ubiquitination (U) and phosphorylation (P) of ENaC. This means the channel is degraded and endocytosed. In the presence of aldosterone (bottom panel) increased expression of SGK1 and GILZ1 is seen and these compounds prevent the inhibition of ENaC via NED4-2 and the ERK/MEK/Raf-1 complex, resulting in increased surface expression and increased sodium flux through the channel. Adapted from Soundararajan et al (Soundararajan et al., 2010)

Aldosterone has other actions that increase ENaC expression or action. For example, aldosterone induces the expression of small, monomeric Kirsten Ras GTP-binding protein (Ki-Ras). The mechanism of action is not clear but Ki-Ras appears to both keep ENaC open and decrease the number of channels in the plasma membrane (Stockand, 2002). In addition, PI3K is a possible point of "cross talk" between ADH, insulin and aldosterone signalling (Stockand, 2002) as aldosterone increases PI3K activity and reduction in PI3K reduces the effects of aldosterone.

Other actions in the distal nephron

While the main effect of aldosterone induced proteins in the distal nephron is to stimulate ENaC subunits or proteins that modify ENaC (as described above), aldosterone also regulates the Na-K-ATPase pump on the basolateral membrane via increased transcription of the pump subunits and activation of signalling cascades, including upregulation of SGK-1 (Thomas et al., 2008). The Renal Outer Medullary Potassium (ROMK) channel is also modulated by aldosterone, providing a mechanism for the secretion of potassium ions. These combined actions are known as the "aldosterone paradox"; that is, that the distal nephron has the ability to independently regulate salt reabsorption in a state of hypovolaemia versus potassium secretion in a state hyperkalaemia, given the same stimulus (increased aldosterone). The answer to how these two processes are differentially regulated despite the same initial stimulus arose from further study of pseudohypoaldosteronism type 2 (PHAII, see section 1.1.6). In PHAII, an inactivating mutation of WNK4 results in increased reabsorption of sodium without increased potassium secretion. It has also been demonstrated that phosphorylation of WNK kinases in response to hypertonic stress and possibly decreased chloride concentration, increases WNK4 activity and suppressing ROMK, providing a mechanism for uncoupling of this system in normal physiology (Kahle et al., 2010).

Non-epithelial tissues

Mimeralocorticoid receptors are present in a number of non-epithelial sites. However, it is not clear whether activation of these receptors can be attributed to aldosterone rather than cortisol *in vivo,* given the absence of co-localising 11ßHSD at these sites. Nevertheless, in experimental models of aldosterone excess, clear detrimental actions have been demonstrated. Cardiac fibrosis is seen in animals exposed to excess aldosterone, independent of blood pressure (Brilla and Weber, 1992). Histological features of aldosterone-induced cardiac fibrosis include proliferation of cardiac myocytes and fibroblasts and although the mechanism is unclear, an increase in collagen I and III synthesis is seen in response to aldosterone in cardiac fibroblasts (Robert et al., 1994). Excess aldosterone is also associated with evidence of increased myocardial inflammation (Brilla and Weber, 1992) and early increases in inflammatory

markers for example, tumour necrosis factor α and ED-1 positive macrophages has been observed in a rat model of mineralocorticoid hypertension (Young et al., 2003).

Aldosterone excess is associated with reduced vascular reactivity (Farquharson and Struthers, 2002) and increased markers of oxidative stress; aldosterone regulates NADHP subunit expression and this can be ameliorated by blockade of the mineralocorticoid receptor (Young and Rickard, 2011). Further, aldosterone excess (in combination with salt loading) causes an increase in redox sensitive NFκB (Sun et al., 2002) as well as reduced glucose-6-phosphate dehydrogenase (G6DP) (Leopold et al., 2007), which plays an important part in determining the redox state of the cell.

Mineralocorticoid receptors in the central nervous system (CNS) are unprotected by 11ßHSD but aldosterone specific effects have been demonstrated (Gomez-Sanchez, 1997). In addition, blockade of CNS receptors by RU28318 attenuates the hypertensive effects of systemic administration of aldosterone (Gomez-Sanchez et al., 1990) but not the cardiac fibrosis and hypertrophy (Young and Funder, 1996).

Nongenomic actions

It has been proposed that aldosterone can also triggers rapid, non-genomic effects in both traditional aldosterone target tissues (cortical collecting duct) as well as in non-epithelial tissues. In epithelial tissues it is evident that there is increased ENaC activity measured by sodium flux within a few minutes of exposure to aldosterone (Zhou and Bubien, 2001). The rapidity of this response suggests actions that are not mediated by “genomic” mechanisms i.e. do not provoke increased gene transcription. In non-epithelial tissues rapid non-genomic effects have also been observed in heart rate (Schmidt et al., 1999) baroreflex sensitivity (Yee and Struthers, 1998) and central (Schmidt et al., 1999) and peripheral (Romagni et al., 2003) vascular resistance. These non-genomic effects may not act entirely via the mineralocorticoid receptor. For example recent work by Gros et al (Gros et al., 2011) ascribes the rapid action of aldosterone in vascular smooth muscle cells to a G protein coupled receptor, GPR30. These rapid effects are diminished but not abolished by the classical

mineralocorticoid receptor antagonists, spironolactone and eplerenone. Although remaining controversial, this recent data raise the possibility of developing novel pharmaceutical agents to block the rapid, non-genomic actions of aldosterone. If such agents were available, it would allow a more precise analysis of the mechanism of action of aldosterone as well as an improved understanding of the benefits of aldosterone blockade (as described in section 1.3.2 and 1.3.3).

1.2.4 Aldosterone synthesis

The production of steroid hormones within in the adrenal gland and the gonads involves a pathway of sequential enzymatic reactions beginning with cholesterol as substrate. Steroid hormones share a common basic configuration of four carbon rings known as a cyclopentanoperhydrophenanthrene structure, shown in Figure 1-8. The universally accepted numbering of the carbon atoms of the cholesterol molecule is shown in Figure 1-9.

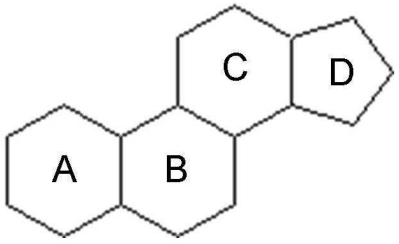


Figure 1-8 The cyclopentanoperhydrophenanthrene structure.

This structure is present in all steroid hormones. The rings are identified by letter according to the universally recognised International Union of Pure and Applied Chemistry (IUPAC).

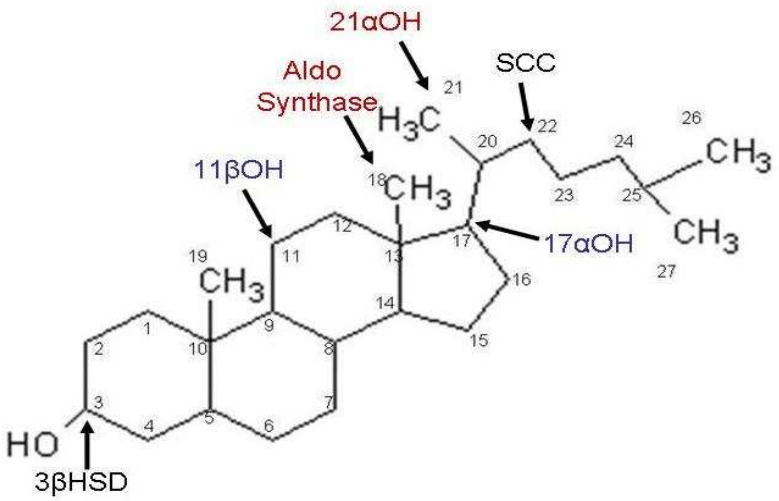


Figure 1-9 Site of action of steroidogenic enzymes on cholesterol molecule.

Cholesterol is the basic molecule from which steroid hormones are derived. The numbering of the carbon atoms is according to the universally recognised International Union of Pure and Applied Chemistry (IUPAC). Enzymes which confer changes that induce mineralocorticoid activity are shown in red while enzymes which confer changes associated with glucocorticoid activity are shown in blue. 3 β HSD, 3β-hydroxysteroid dehydrogenase, 17αOH, 17α-hydroxylase, 21αOH, 21α-hydroxylase,11βOB, 11β-hydroxylase, aldo synthase, aldosterone synthase.

The first step is the transfer of hydrophobic cholesterol across the aqueous mitochondrial membrane, and this rate limiting process is regulated by steroidogenic acute regulatory protein (StAR) (Stocco, 2001). Cholesterol is then converted to pregnenolone by the action of a cytochrome P450 enzyme (Side chain cleavage or CYP-450$_{scc}$, encoded by the gene *CYP11A1*) which cleaves the 6 carbon unit form the original cholesterol molecule, leaving a 21 carbon compound, pregnenelone.

The fate of the pregnenolone molecule then depends on which enzyme it is then exposed to. The enzymes involved in steroidogenesis perform either a hydroxylase reaction (the conversion of a -CH group to a -COH group) or hydroxysteriod dehydrogenase (the conversion of a hydroxyl –OH to carboxyl C=O group) as can be seen from Figure 1-10. This is discussed further in section 1.2.5.

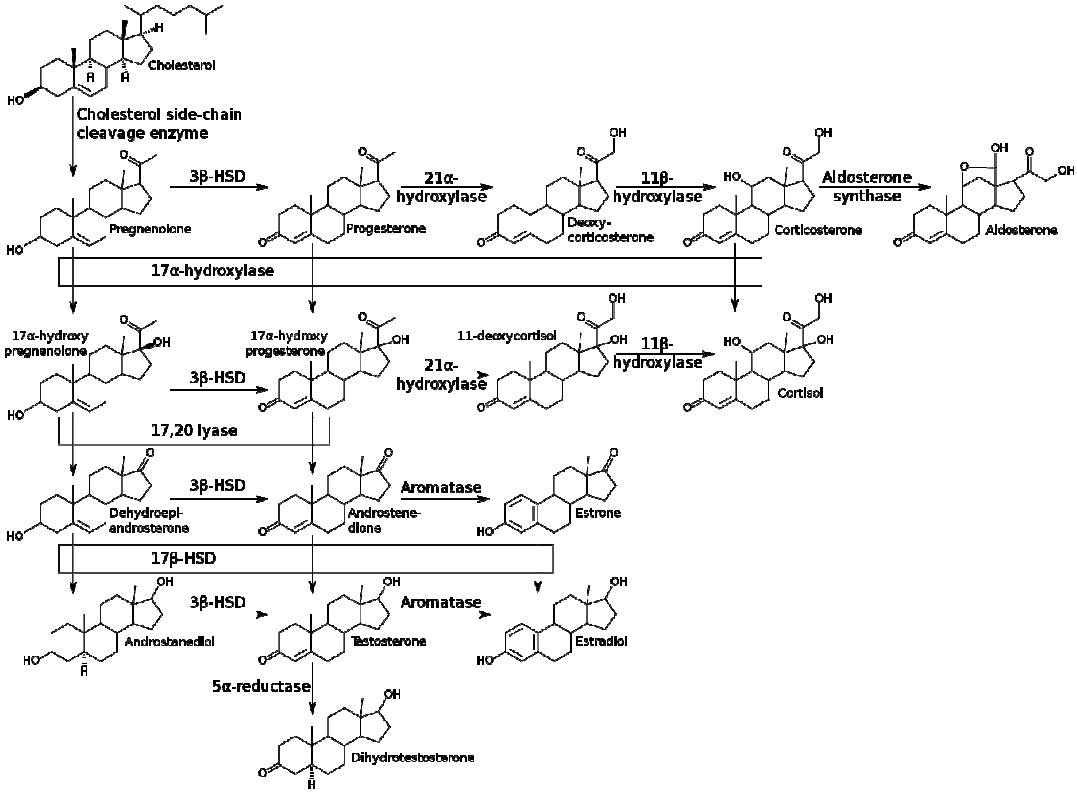


Figure 1-10 Corticosteroid biosynthesis.

Enzymes, substrates and products in human steroidogenesis.

With reference to the production of the corticosteroids, cortisol and aldosterone, there are 5 further key enzymes involved and the next steps take place in the endoplasmic reticulum. 3ß-hydroxysteroid dehydrogenase (encoded by *3BHSD*) oxidises the hydroxyl group at the 3rd carbon atom to from progesterone. If then exposed to 17 α-hydroxylase (encoded by *CYP17A1)*, pregnenolone is the substrate for a hydroxylation reaction at carbon 17 to form 17 α-hydroxypregnenolone and this step is necessary for the production of both androgens (prior to the action of 17,20 lyase (also encoded by *CYP17A1*) and glucocorticoid compounds. The carbon at position 21 is hydroxylased by 21 α-hydroxylase (encoded by *CYP21B*) converting progesterone to deoxycorticosterone and 17 α-hydroxyprogesterone to 11-deoxycortisol.

Like the initial enzymatic reaction involving P-450$_{scc}$, the final steps aldosterone productions are undertaken in the mitochondria by a cytochrome P450 enzyme. The conversion of deoxycorticosterone to aldosterone involves three consecutive

reactions catalysed by aldosterone synthase (*CYP11B2*). This enzyme is the focus of this work and will be discussed in depth in future sections. It catalyses the formation of a hydroxyl group at position 11 to produce corticosterone, followed by 18-hydroxylation to yield 18-hydroxycorticosterone and finally 18-methyloxidation to aldosterone (Connell and Davies, 2005) (see Figure 1-11).

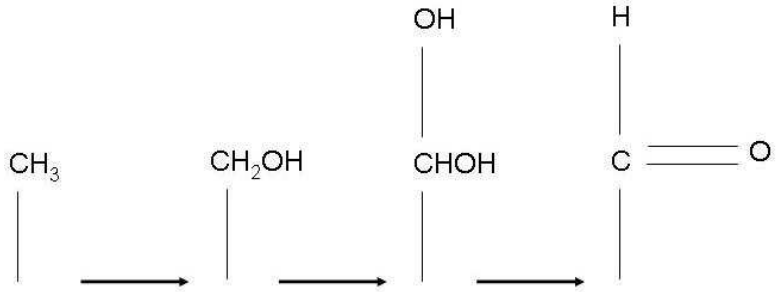


Figure 1-11 Reactions undertaken by aldosterone synthase at C18 of deoxycorticosterone

Aldosterone spontaneously converts to the hemiacetal form (shown in Figure 1-12) and this change is not associated with any change in activity. However, this structure does protect aldosterone from the action of 11 ß-hydroxysteroid dehydrogenase which converts cortisol to the inactive cortisone (as discussed in section 1.2.2).

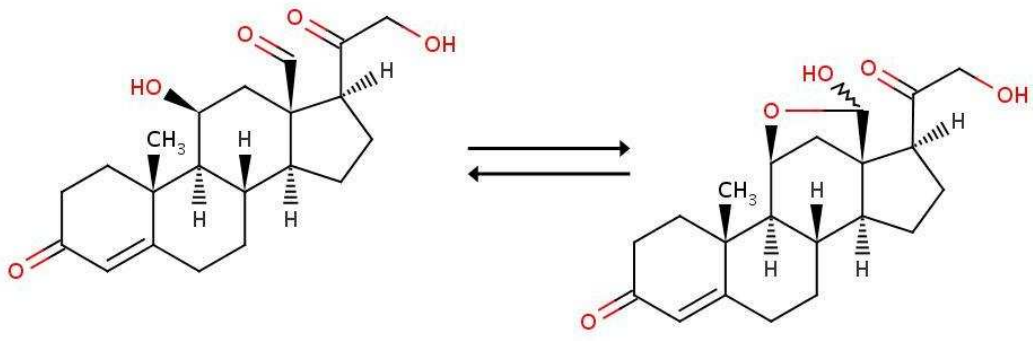


Figure 1-12 Structure of aldosterone and the (predominant) hemiacetal form.

The enzyme 11 ß-hydroxylase (encoded by *CYP11B1*) can also undertake a hydroxylation reaction at position 11 and 18 (Freel et al., 2004). However, the 18-methyloxidation reaction is an exclusive function of aldosterone synthase and this is the final rate limiting step in the synthesis of aldosterone.

1.2.5 Regulation of aldosterone production

In order to further investigate the mechanisms of control of aldosterone synthesis, both genetic and environmental, it is necessary to spend some time describing the normal stimuli to aldosterone production and the points at which production can be regulated.

Structural and anatomical mechanisms

The structure and anatomy of the adrenal gland plays a crucial role in the mechanisms of regulation of production of aldosterone as well as of the other corticosteroids. Processes which disrupt this elegant arrangement provide clear examples of the importance of the structural integrity of the gland in maintaining an ability to differentially produce the physiologically relevant steroids in response to their trophins. The following section will address the functional relevance of the anatomical structure of the adrenal gland.

The adrenal glands consist of an inner adrenal medulla and an outer cortex. These distinct regions arise from separate embryological origins; mesenchymal cells which form the cortex originate from the coelomic cavity lining, adjacent to the urogenital ridge, while invasion of neuroectoderm cells into this region give rise to the adrenal medulla (Wilson J.D. and Foster D.W., 1992). The adrenal cortex is further subdivided onto histological distinct zones which have separate functional properties. The outermost glomerulosa cells produce aldosterone, the fasciculata produces cortisol and corticosterone and the reticularis is the main site of adrenal androgen production. It is suggested that development of the vascular system in crucial in developing the functional zonation of the adrenal gland with blood vessels from the outer cortex draining inwardly into venules of the adrenal medulla. Thus, glomerulosa cells differentiate on the arterial side and reticularis cells on the venous side.

However, the crucial functional difference in the adrenal gland zonation is the differential expression of steroidogenic enzymes, i.e. aldosterone synthase (encoded by *CYP11B2* gene) in the glomerulosa and 17 α- hydroxylase (encoded by *CYP17A1*) and 11 ß-hydroxylase (encoded by *CYP11B1* gene) in the fasciculata.

However, it is now recognised that the distinct histological zones of the adrenal gland are not static; *in utero*, the foetal adrenal consists of a smaller outer zone but it is the larger, inner zone that appears to be the dominant source of steroids, mainly DHEA and DHEAS. There is also a so called "transitional zone" between these two areas that appears to produce cortisol near the end of the gestational period (Kempna and Fluck, 2008). After birth, the large inner zone involutes and the three zoned adult adrenal begins to develop, and it is not until adrenache (a specifically human phenomenon that occurs around the age of 6-8) that adrenal androgens again begin to rise. The processes which initiate and maintain this functional zonation are not clear. It has been proposed that common stem cells exist within the capsule and differentiate while migrating in a centripetal direction to form these layers but this remains a matter of debate (Wood and Hammer, 2011). Recent advances in this area stemmed from the identification of the crucial role of steroidogenic factor 1 (SF-1) in the development of the adrenal gland (Luo et al., 1994). The importance of this molecule in humans was confirmed when the monogenic disorder resulting from a mutation in *SF-1* in humans was described, giving rise to a phenotypic female with an XY genotype as a result of gonadal dysgenesis and primary adrenal failure (Achermann et al., 1999). However, the multitude of signalling pathways involved in the maintenance of adrenal stem cells as well as their differentiation to functioning steroidogenic cells is only beginning to be understood.

The importance of the structure of the adrenal gland in regulating steroid production can be observed in a further monogenic condition, Glucocorticoid Remedial hyperaldosteronism (GRA). This is an autosomal dominant monogenic disorder caused by a hybrid gene comprising the regulatory element of 11 ß-hydroxylase and the coding region of aldosterone synthase gives rise to a phenotype of hypertension and mineralocorticoid excess (Lifton et al., 1992). Aldosterone is produced in response to ACTH rather than its usual trophins (as described later in this section), but importantly, the chimeric gene is expressed

ectopically in the fasciculata, which is a much higher output system that the glomerulosa. This allows the gene product inappropriate access to greater quantities of 11-deoxycorticosterone as a substrate for substantial aldosterone production. Importantly therefore, it is not only that the gene is under regulation by ACTH that produces a state of mineralocorticoid excess, but also the aberrant locus of enzyme expression and structure of the gland that causes dysregulated corticosteroid production, thus highlighting the necessity of strict anatomical and functional zonation.

For many years, further study of zonation in the human adrenal has been limited by the high degree of homology between aldosterone synthase and 11 ß-hydroxylase, making the development of specific antibodies difficult and thus reliable separation of the of the zones challenging. But recent success in the development of specific antibodies has been successfully achieved by Nishimoto et al (Nishimoto et al., 2010), who have confirmed the pattern of conventional zonation with *CYP11B1* expressed constitutively in the fasciculata and *CYP11B2* expressed sporadically in the glomerulosa. However, they also demonstrated a novel pattern of *CYP11B2* expression with clusters of aldosterone producing cells expressing *CYP11B2*, *3BHSD* and no *CYP17A1*, which appeared to be constitutively active in terms of aldosterone production, in that they were present even in tissue adjacent to aldosterone-producing adenomas. This is in contrast to the previously held belief that there would be suppression of *CYP11B2* expression due to the negative feedback of the renin- angiotensin system. This novel pattern of *CYP11B2* expression was confirmed by Boulkroun et al (Boulkroun et al., 2010) who examined the structure of the zona glomerulosa in normal adrenals and compared this to aldosterone producing adenoma as well as tissue from around the adenoma. They provided further evidence that the tissue from around the adenoma was hyperplastic with persistent expression of *CYP11B2*, in some cases encompassing the entirety of the zona glomerulosa. This work adds further to the body of evidence confirming both the non-static nature of the structure of the adrenal gland, and specifically that the zona glomerulosa can enlarge and steroidogenic enzymes be switched on and off, with consequent implications for regulation of aldosterone.

There remain many unanswered questions regarding the development and maintenance of adrenal zonation. Is the fate of an adrenal stem cell

predetermined or can it be altered by environmental factors? What causes the hyperplasia seen in adjacent tissue in aldosterone producing adenomas and how can this be influenced? What causes *CYP11B2* to be constitutively expressed in this context and become unshackled from the control of the renin- angiotensin system? It is clear that the structure of the adrenal gland as well as the processes which regulate it are crucial to its function. While advances have been made, further investigations into the regulation of geographical expression of steroidogenic enzymes, as well as how this relates to the regulation of steroid production, would be enlightening.

Cytochrome P450 enzymes and electron transport

As previously discussed, the enzymes involved in steroid synthesis fall into either cytochrome P450 enzymes (encoded by *CYP11A1, CYP17A1, CYP21A2, CYP11B1* and *CYP11B2*) or hydroxysteroid dehydrogenase enzymes (e.g. *3ΒHSD*). The cytochrome P450 enzymes can be further classified according to their location in the mitochondria or endoplasmic reticulum. The distinction is important because these two classes of enzymes use different strategies for electron transfer and this may be an important method of regulating enzyme function, distinct from enzyme expression.

Mitochondrial enzymes receive electrons from reduced nicotinamide adenine dinucleotide (NADPH) via an electron transfer chain. NADPH binds to adrenodoxin (ferredoxin) reductase, which then interacts with and transfers a pair of electrons to adrenodoxin (ferredoxin). Adrenodoxin accepts and also interacts with the redox partner binding site of the mitochondrial P450 enzyme to donate electrons to it (Miller, 2005). Adrenodoxin reductase is encoded by a single gene on chromosome 17q24-q25 but recent evidence suggests that there are two forms of adrenodoxin/ ferredoxin, encoded by *FDX1* on chromosome 11q22 and *FDX2* on chromosome 19p13.2, each with distinct functions. FDX1 appears to be specific for electron transfer in steroidogenesis while FDX2 is essential for Fe/S protein biogenesis and haem A formation (protein co-factors essential for a variety of cellular functions) but is unable to reduce cytochrome P450 enzymes (Sheftel et al., 2010) (Figure 1-13). Steroid synthesis may be limited by the availability of reducing agents (or the relative composition). However, the

relative quantities of adrenodoxin 1/ 2, adrenodoxin reductase and CYP P450 enzymes in humans are not currently known.

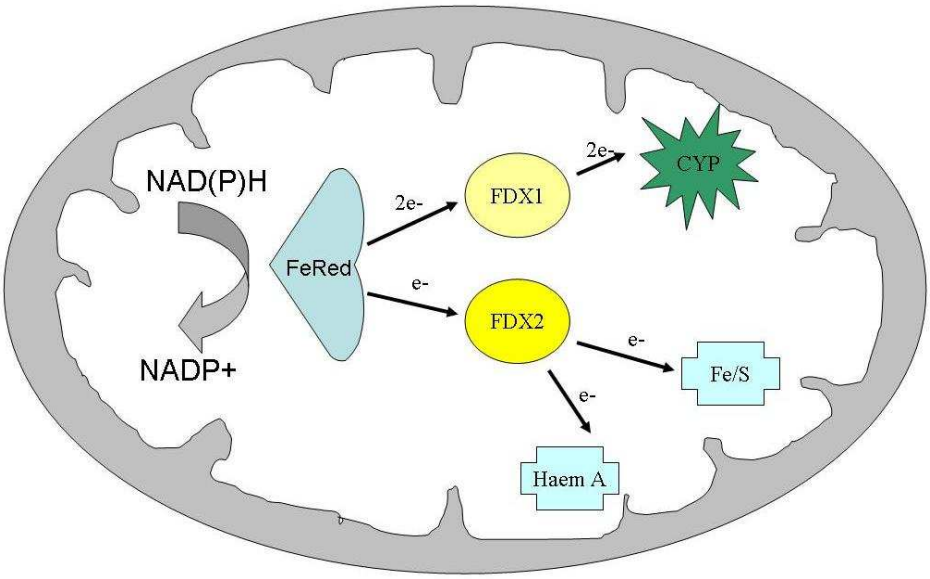


Figure 1-13 Electron transport of ferredoxin and ferredoxin reductase in the mitochondria. FDX1, ferredoxin 1, is involved in electron transfer in steroidogenesis and FDX2, ferredoxin 2, involved in electron transfer in Fe/S protein biogenesis and haem A formation. FeRed, ferredoxin reductase; CYP, Cytochrome P450 enzyme.

In contrast, CYP P450 enzymes located in the endoplasmic reticulum receive electrons from NADPH via the electron chain involving P450 oxidoreductase (POR), sometimes with the assistance of cytochrome b_5 (Miller, 2005). This electron transfer system is a mechanism by which the lyase/ hydroxylase dual action of the product of *CYP17A1* is regulated. Cytochrome b5 acts as an allosteric factor to promote the interaction of CYP17 with POR, selectively increasing 17,20 lyase activity (Lin et al., 1993). Further, increased POR increases 17, 20 lyase activity (Lin et al., 1993) and greater amounts of POR are observed in testicular tissue (where C19 steroids are produced) rather than adrenal tissue (where steroidogenesis is predominantly limited to C21 steroids).

While *POR* knock out in mice is embryonically lethal, the first *POR* mutations in humans were described in 2004 (Fluck et al., 2004). These individuals had disordered sexually development and steroid production consistent with partial deficiencies of CYP17 and CYP21. In addition, in individuals carrying severe

mutations, a disorder of skeletal development is also observed, similar to Antley-Bixler syndrome (http://www.omim.org/entry/207410). The *POR* gene is highly polymorphic and there is much interest in examining whether less severe mutations may be associated with clinical syndromes associated with milder alterations in steroid profiles. In addition, it is worth remembering that as well as being involved in crucial reactions in steroidogenesis, POR is the system by which electron donation is achieved for cytochrome P450 drug metabolising enzymes. Thus, the possibility of a greater understanding of pharmacogenetics may be possible with deeper knowledge of the effects of variation in *POR*. To date, no defects of adrenodoxin or adrenodoxin reductase have been described in humans but clearly, given the example of POR, this is a potential area of regulation of aldosterone production as yet largely uninvestigated.

Trophins of aldosterone production

As has been previously mentioned, increased aldosterone secretion is an outcome of activation of the renin-angiotensin system, and has the effect of restoring circulating volume as well as being a mechanism for excreting potassium ions. Increases in angiotensin II and serum potassium levels are thus the major physiological regulators of plasma aldosterone concentration. Both utilise changes in intracellular free calcium to increase transcription of the key steroidogenic enzymes, but it is clear that there are discrete and separate mechanisms involved in the responses to these two trophins, as the rise in intracellular free calcium invoked by potassium could be blocked with calcium channel antagonists but this was not the case for angiotensin II (Capponi et al., 1984). This difference in action was further exposed by Pezzi et al (Pezzi et al., 1997) who demonstrated the essential role of calmodulin and calmodulin-dependant protein kinases (CaMK) in potassium stimulated increase in intracellular calcium but inhibition of calmodulan and CaMK had little effect on angiotensin II stimulated increases in aldosterone synthase transcripts. This suggests that the role of angiotensin II is more complex than potassium and relies on several mechanisms to stimulate increased aldosterone synthase transcription. These factors as well as other trophins of aldosterone production will be discussed in the following sections.

Angiotensin II

As discussed above, angiotensin II-stimulated aldosterone production via the AT1 receptor rather than the AT2 receptor (Section 1.1.4). Activation of this G protein receptor leads to a cascade of intracellular signalling mechanisms which are incompletely understood. Activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) is observed and this leads to release of intracellular calcium ions, 1,2 diacylglycerol (1,2 DAG) and activation of protein kinase C (PKC) (Spat and Hunyady, 2004) (see Figure 1-14). PKC does not appear to directly increase CYP11B2 expression (Denner et al., 1996) but does appear to reduce expression of CYP17 (Bird et al., 1996), thus preventing substrate from entering the glucocorticoid/ androgen pathway.

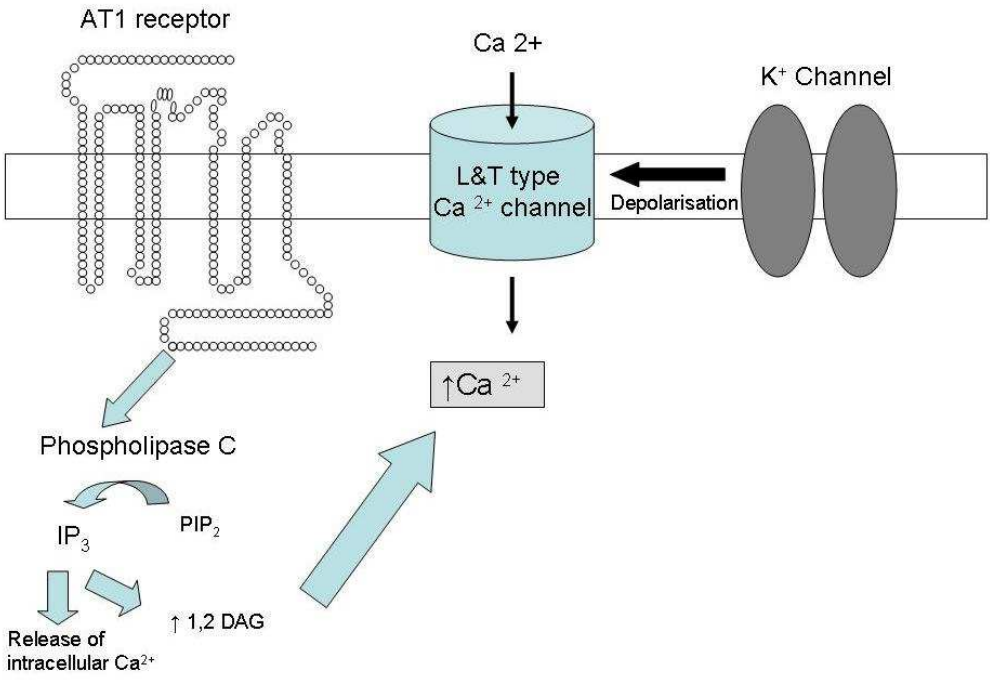


Figure 1-14 Angiotensin II and potassium regulation of intracellular calcium leading to increased aldosterone production.

A rise in intracellular free calcium activates CaMKI and possible CaMKIV (Condon et al., 2002), feeding into the same mechanism utilised by increased calcium via potassium-dependant channels but the effect of angiotensin II on transcription of CYP11B2 is not limited to this mechanism.

Potassium

The role of potassium as a regulator of aldosterone secretion is crucial to physiological homeostasis, the mechanisms were considered to be well understood and straightforward. However, recent investigations have suggested a novel molecular system of potassium-mediated regulation of mineralocorticoids. TASK (TWIK-related acid sensitive K+) channels are two pore, four trans-membrane domain potassium channels, first characterised in the early 1990s and are expressed predominantly in cells where modification of the cell membrane potential is essential to function such as neurotransmitters, hormone secretion and neuronal and muscular excitability (for a review see Lesage et al (Lesage and Lazdunski, 2000), Bayliss et al (Bayliss and Barrett, 2008)). The importance of these channels in aldosterone secretion was highlighted by the development of a *TASK* subunit knock out mouse which exhibited features of autonomous aldosterone production (Davies et al., 2008). These animals had increased plasma aldosterone concentrations apparently not stimulated by plasma renin which was unchanged or lower than in control animals. Plasma aldosterone concentrations were not suppressed by salt or by blockade of the AT1 receptor with candesartan. Elimination of functional TASK channels caused the membrane potential of the glomerulosa cells to be significantly more depolarised, and it is proposed that this leads to continuous calcium channel activity and increased sensitivity to angiotensin II. It is particularly interesting to note that there appears to be sexual dimorphism in the physiological consequences of manipulation of the TASK channels. Heitzmann et al (Heitzmann et al., 2008) produced a *TASK 1* knock out mouse model in which impaired mineralocorticoid homeostasis was restricted to female animals. Both male and female animals exhibited abnormal adrenal cortex zonation i.e. ectopic expression of aldosterone synthase in the high capacity fasciculata layer, but in male mice, this regressed following puberty. This finding highlights two key points, firstly the crucial importance of geographic expression of steroidogenic enzymes within the adrenal cortex (as discussed in more detail earlier in this section) and secondly the multiple influences, in this case presumably sex hormones, that can influence steroidogenesis.

The discoveries regarding the role of potassium channels have been further extended by the recent publication of a study identifying an inherited mutation in the *KCNJ5* (potassium inwardly-rectifying channel, subfamily J, member 5)

gene in a family with severe hyperaldosteronism and bilateral adrenal hyperplasia. In addition, 2 different somatic mutations in the potassium channel gene *KCNJ5* were found in 8 out of 22 aldosterone producing adrenal adenomas examined (Choi et al., 2011). The authors suggest that the mutations identified lead to the channel to be more permeable to sodium, resulting in sodium entry to glomerulosa cells, chronic depolarisation, and consequent activation of calcium channels, thus leading to constitutive stimulation of aldosterone production. This is an exciting development in the understanding of the aetiology of aldosterone excess and aldosterone-producing adenomas in particular, although further work is required to ascertain the contribution of this pathway to patients with less severe phenotypes.

Adrenocorticotrophin stimulating hormone

Conventionally recognised as the principle trophin of cortisol, adrenocorticotrophin (ACTH) is released from the pituitary as part of a classical endocrine feedback mechanism as shown in Figure 1-15.

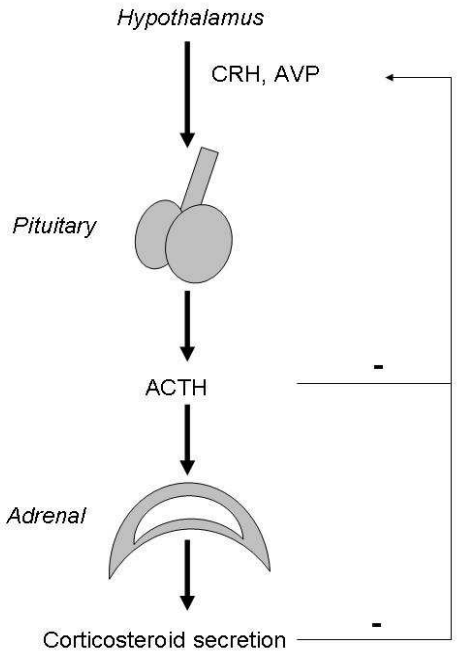


Figure 1-15 Hypothalamic pituitary axis.

Hormones released from the hypothalamus (CRH, corticotrophin releasing hormone, AVP, Vasopressin) regulate the release of pituitary hormone production which in turn stimulates

release of active hormone from the adrenal gland. Hypothalamic inhibition is achieved by glucocorticoids as well as a direct effect of ACTH.

It is clear that the acute effects of increased ACTH stimulate aldosterone production. This is predominantly achieved via increased intracellular cyclic adenosine monophosphate (cAMP), which occurs after ACTH binds to cell surface receptors and activates adenlyate cyclase. However, this effect also depends on the movement of calcium into the cell, as no effect of ACTH stimulation is observed in the absence of calcium (Spat and Hunyady, 2004). It is important to remember that the mechanism by which ACTH leads to increased aldosterone may not simply be via increasing activity of the final steps in steroidogenesis. By increasing the generation of precursors to aldosterone as a result of increased activity of steroidogenic enzymes further upstream, the production of aldosterone may be increased, as long as aldosterone synthase is not the rate limiting step. Indeed ACTH stimulation leads to increased activity of StAR (Stocco, 2001), an early, rate limiting step in steroidogenesis (as described in 1.2.4). However, chronic administration of ACTH suppresses aldosterone secretion (Rayfield et al., 1973). The mechanism behind this does not appear to be by down regulation of the renin-angiotensin system but rather by some direct effect on the ACTH signal either via its receptor or second messenger pathway (Abayasekara et al., 1989). In clinical practice, patients with hypopituitarism do not demonstrate signs of mineralocorticoid insufficiency, nor do patients with ACTH excess (i.e. Cushing's disease) have high aldosterone levels, which would lend support to the theory that ACTH does not play a major role in regulating aldosterone.

Nevertheless, there remain unanswered questions regarding the role of ACTH in regulating aldosterone production. In particular, it is striking that there is a diurnal variation in aldosterone secretion in man (Richards et al., 1986; Freel et al., 2008) consistent with some degree of regulation by ACTH. Recently published data described abnormally elevated aldosterone production in circadian clock–deficient Cry-null mice (Doi et al., 2010), a mechanism that may be at least partly mediated by ACTH. Further, some individuals with salt losing adrenal hyperplasia and mineralocorticoid insufficiency have been shown to carry severe loss of function mutations of the adrenocorticotrophin receptor

(MCR2) (Lin et al., 2007), suggesting ACTH is important in the maintenance of normal levels of aldosterone.

Other pituitary factors

There is some evidence for the involvement of other pituitary factors in the regulation of aldosterone. Corticotrophin releasing hormone and other ACTH secretagogues e.g. vasopressin are released from the hypothalamus and stimulate ACTH release in the form of a precursor molecule pro-opiomelanocortin (POMC). This 241-amino acid peptide is cleaved to form ACTH which is only 39 amino acids in length, by the enzyme pro-hormone convertase 1 (PC1/3) (Nillni, 2007). This cleaves the peptide at lysine-arginine to form biologically active products as shown in Figure 1-16.

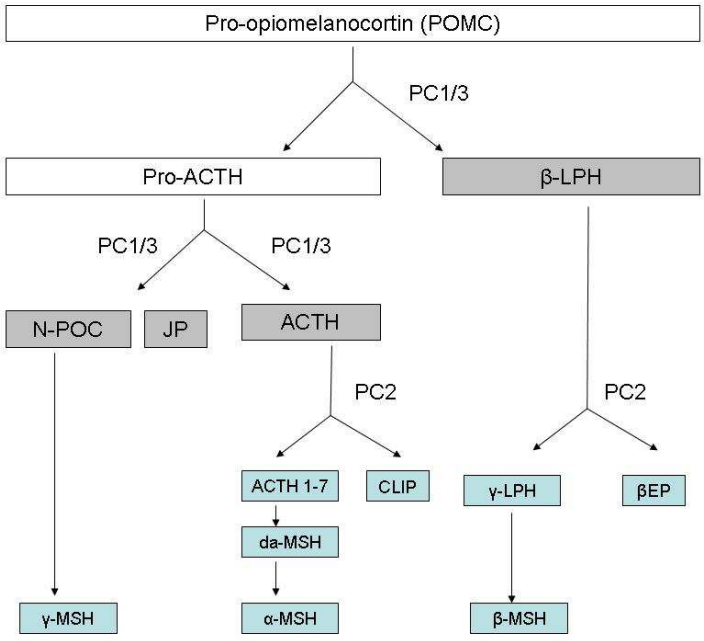


Figure 1-16 Processing of POMC to form ACTH and other anterior pituitary factors.

POMC is cleaved in the anterior pituitary to form β-lipotrophin and pro-ACTH by the actions of pro-hormone convertase 1 (PC1/3). Pro-ACTH is further cleaved to form ACTH, N-teminal proopiocortin (N-POC) and joining protein (JP). These are anterior lobe peptides and boxes are coloured grey. Further cleavage takes place in the intermediate lobe which is not active in normal humans. Intermediate lobe peptides are coloured blue. These are not present in significant amount in normal human plasma, but may be present in tumours as well as in the foetus and during pregnancy. ACTH 1-7 and corticotrophin- like- intermediate lobe-peptide (CLIP) is generated from ACTH cleavage by pro-hormone convertase 2 (PC2), which also acts on β-lipotrophin (β-LPH) to produce γ-LPH and β-endorphin (β-EP). PC2 also cleaves γ-LPH to produce β-melanocyte stimulating hormnone (β-MSH). Carboxypepdidase E and peptidyl α-amidating monooxygenase (PAM) act to produce deascetyl MSH (da-MSH), prior to the action of N-acetyl transferase (NAT) which converts this to α-MSH. Adapted from (Pritchard and White, 2007) and (Wilson J.D. and Foster D.W., 1992).

McCaa et al demonstrated that patients with pituitary insufficiency exhibit altered response to ACTH and angiotensin II which was not corrected by administration of ACTH (McCaa et al., 1981). POMC knock out mice have been demonstrated to have absent or low levels of aldosterone by some (Coll et al., 2004; Karpac et al., 2005; Yaswen et al., 1999) but not all (Linhart and Majzoub, 2008) investigators, and this phenotype is not reversed by administration of ACTH. Some specific POMC products other than ACTH stimulate aldosterone production e.g. ß-endorphin (ß-EP) and, to a lesser extent, joining peptide (JP), have the capacity to stimulate aldosterone production in human adrenal cell suspensions (Molloy et al., 1998) and the actions of ACTH are potentiated by α-MSH in rats (Vinson et al., 1980; Szalay, 1993). However, as these factors are present in negligible amounts in humans, it seems unlikely that they will contribute significantly to the regulation of aldosterone.

Other Factors

There is *in vitro* evidence to suggest that numerous other factors may be involved in the stimulation or inhibition of aldosterone. However, there is little *in vivo* evidence to suggest that they play a major physiological role. These will not be discussed further but a list can be found in Table 1-4(adapted from (Wilson J.D. and Foster D.W., 1992; 1992))

INHIBITING FACTORS	STIMULATING FACTORS
Atrial natriuretic hormone* Calcitonin gene-related peptide Dopamine Nitric oxide Platelet-derived growth factor Somatostatin Transforming growth factor-β Unsaturated fatty acids	Acetylcholine Angiotensin II* ATP Bradykinin Cholecystokinin Corticotrophin (ACTH)* β-Endorphine Enkephalins Endothelin Epidermal growth factor 12-Hydroxyeicosatetraenoic acid K^+* Melanocyte stimulating hormone Neuropeptide Y Neurotensin Norepinephrine Parathormone Prolactin Prostaglandins Serotonin Substance P Vasoactive intestinal polypeptide Vasopressin

Table 1-4 Inhibitory and stimulatory factors regulating plasma aldosterone *in vitro.* Starred factors are thought to be physiologically most relevant.

1.2.6 Aldosterone in circulating plasma and its metabolism

Once aldosterone synthase catalyses the final reaction in the production of aldosterone, the steroid is not stored but immediately released from the adrenal gland into the circulation. Thus, in addition to the rate of production, the amount of "free" hormone (i.e. not bound by plasma proteins) and the rate of metabolism and clearance also affect the concentration of the steroid. The half life of aldosterone is much shorter than that of cortisol; some possible reasons for this are described in the following section.

In contrast to cortisol which is 90-97% protein bound, aldosterone is only weakly associated with corticosteroid binding globulin (20%) and albumin (40%) (Wilson J.D. and Foster D.W., 1992), with the remainder circulating freely in the plasma. It is proposed that this may make plasma concentrations of aldosterone more

susceptible to variation than cortisol as being bound to plasma proteins provides a “reservoir” of cortisol, preventing rapid swings in plasma concentrations.

The metabolism of aldosterone is achieved via reactions that take place in the liver: aldosterone is reduced by 5β-reductase and 3α-dehydrogenase enzymes and conjugated with glucuronic acid to render it more water soluble. The major urinary metabolite of aldosterone is tetrahydroaldosterone (this comprises around 35%) but aldosterone-18-glucuronide is formed from direct conjugation of the unreduced aldosterone (making up around 20%). There are acquired conditions associated with altered aldosterone metabolism and clearance; as aldosterone metabolism takes place almost exclusively via hepatic enzymes, these are predominantly due to alteration in liver function. For example, patients with liver failure, or patients with cardiac failure have a degree of functional liver failure due to hypo perfusion. Both these patient groups can have increased levels of aldosterone.

There are no reported cases in the literature to date of genetic abnormalities of metabolism of aldosterone. However, in the case of androgen metabolism, an exciting advance in understanding has highlighted how this aspect of control of steroid regulation may be important. Dehydroepiandrosterone (DHEA) is the principle precursor of adrenal androgens. Rather than being conjugated with glucuronic acid as is the case with aldosterone, DHEA is sulphated to form DHEAS. As DHEAS, it can be excreted in the urine and in humans this is the most abundant steroid. However, only DHEA can be converted to active androgens. Therefore, the control of DHEA sulfation is a mechanism by which androgen production can be regulated. Noordam et al (Noordam et al., 2009) described a patient with low levels of DHEAS and a phenotype of precocious puberty in childhood and features consistent with polycystic ovary syndrome in adulthood. They demonstrated that this was associated with a mutation in a key enzyme involved in the DHEA-DHEAS sulfation system. This enzyme, 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2) is an activated sulphate donor used by the sulfotransferase SULT2A1. Functional assays of these mutations in the *PAPSS2* gene reconstructed the DHEA-DHEAS sulfation system and confirmed inactivation of the PAPSS2 enzyme. This important discovery of a functional mutation in metabolism of adrenal androgens leading to a clinical syndrome of androgen excess highlights the potential role at this level of regulation. While these

mechanisms are clearly important in the regulation of androgens, little is known about the regulation of metabolism of aldosterone.

1.2.7 CYP11B1 and CYP11B2

As discussed above, the final steps of aldosterone and cortisol synthesis are catalysed by the enzymes aldosterone synthase encoded by *CYP11B2* and 11β hydroxylase encoded by *CYP11B1*. Transcriptional regulations of these two rate limiting enzymes are crucial regulating mechanisms in the control of aldosterone and cortisol production. This thesis, in particular the experimental work, will focus on the effect of variation in *CYP11B2* on regulation of transcription of aldosterone synthase. Current evidence regarding the regulation of *CYP11B2* will be discussed in section 1.2.8 but in order to put this into context, a short section will follow on the general considerations to be taken into account when studying these two genes, including their origins, as well as a brief overview of general principles of transcriptional regulation.

Evolution of CYP11B1 and CYP11B2

Thought to arise from a gene duplication event around 400 million years ago around the time of colonisation of land (Colombo et al., 2006), *CYP11B1* and *CYP11B2* lie in tandem on chromosome 8 have 9 exons and share around 95% homology in their coding regions (Mornet et al., 1989) and this homology contributes to significant technical difficulties in laboratory experiment. Their structures diverge in the 5' untranslated region of the genes as can be observed in Appendix 7.3. For some years there was controversy over whether one multifunctional gene was responsible for 11-hydroxylation, 18-hydroxylation and 18-oxidation step in man (Globerman et al., 1988), as in the bovine adrenal (Hashimoto et al., 1989), or whether two separate genes were responsible (Mornet et al., 1989; Kawamoto et al., 1992). We now the second option is correct. The different biochemical reactions undertaken by these two enzymes are discussed above (section 1.2.4); however some consideration must also be given to the different mechanisms of regulation of gene transcription as maintaining independent function clearly depends on the ability to discriminate between signals to produce more cortisol without necessarily increasing aldosterone synthesis, and vice versa.

Gene transcription

The principles of gene transcription are integral to the central dogma of molecular biology; that is the concept of DNA transcribed to RNA, and this then translated to protein. The control of these processes involves numerous mechanisms that ensure tight regulation of gene expression. RNA transcription must be controlled and regulated, and in contrast to DNA replication, processes must be present which allow selective transcription of certain genes and not others in order to maintain distinct cellular properties and functions. In order for transcription to take place, a pre-initiation complex is formed comprising of transcription factors including TATA box binding protein and RNA polymerase II (Pol II). The hydrogen bonds between complementary nucleotides of DNA are broken and Pol II moves along the DNA strand pairing RNA nucleotides with complementary DNA bases with the exception that thymines are replaced with uracils and the nucleotides are composed of a ribose (5-carbon) sugar where DNA has deoxyribose in its sugar-phosphate backbone. Finally, the hydrogen bonds of the untwisted RNA+DNA helix break, freeing the newly synthesized RNA strand.

The simplest transcriptional unit contains a core promoter element (usually a TATA sequence) around 20-30bp from the transcriptional start site, an upstream activator sequence, and silencer element both within 100-200 bp of the TATA box. However, a typical eukaryotic gene has more complex regulatory mechanisms, with a transcription factor complex containing numerous transcription factors which can be tissue specific. In addition, eukaryotic genes can possess several enhancer regions which can be located within introns, 3' or 5' regulatory regions. Further, many genes contain binding sites for proximal regulatory factors which may not function as classical activators but rather tethering elements that act as anchors for other transcription factors to bind and act in a enhancing or repressive manner (Levine and Tjian, 2003). It is also now understood that distal regulatory elements (up to 1Mb away) can control gene transcription and this may be achieved through physical proximity if the DNA loops round to lie close to the transcriptional start site (Maston et al., 2006).

The chromatin structure of DNA is known to influence transcription as chromatin can prevent the transcriptional machinery from accessing the DNA. Chromatin-

modifying activities and histone-modifying complexes, which add or remove covalent groups (e.g., acetyl groups, methyl groups, and phosphates) from histone tails can alter gene transcription and provide an alternative mechanism of control (Maston et al., 2006).

DNA methylation is another mechanism by which transcription is regulated and is associated with transcriptional silencing. This can be achieved in two ways. Firstly, methylation at CpG dinucleotides (GC rich areas of the genome) can repress transcription by blocking the ability of transcription factors to bind their recognition sequences. Secondly, methylation-specific binding proteins can specifically bind methylated CpG dinucleotides and recruit histone deacetyltransferases which can alter the chromatin structure to make it available to transcriptional binding complexes (Clouaire and Stancheva, 2008).

1.2.8 Transcriptional regulation of CYP11B2

The first observations regarding the transcriptional regulation of *CYP11B2* via protein: DNA interactions in a human cell line were made by Clyne et al, using reporter constructs for promoter deletion assays, DNAse foot printing experiments and electromobility shift assays (Clyne et al., 1997). Functionally important regions of the human *CYP11B2* promoter were identified by this group as -776/756 (NBRE-1), -129/144 (Ad5) and -71/64 (CRE).

This last site, CRE, is also present in the *CYP11B1* promoter (Bassett et al., 2004b) and therefore it is likely that the NBRE-1 and Ad5 sites are more important for differential regulation of *CYP11B1* and *CYP11B2*. It was observed that the CRE site was similar to other sites that bound CREB, ATF-1 (activating transcription factor 1) and ATF-2 (activating transcription factor 2) and therefore, DNA- protein complexes were interrogated for the presence of these transcription factors using electromobility shift assays and a "super-shift" approach with antibodies to these proteins (Bassett et al., 2000). More recently, their role was further confirmed by siRNA knock down as well as transfection with constitutively active vectors for of ATF1, ATF2 and CREM and CREB (Nogueira and Rainey, 2010).

Transcription factors belonging to the NGFIB family were previously known to be expressed in the human adrenal gland and implicated in the transcriptional

control of other steroidogenic enzymes e.g. *CYP21* (Davis and Lau, 1994). The presence of a consensus sequence for NGFIB (AAAGGTA) within one of the functionally important regions supports the hypothesis that they are likely to be involved in the transcriptional regulation of *CYP11B2*. Indeed, NGFIB (Nerve Growth factor 1B, also known as NURR77 and NR4A1) and NURR1 were shown to form complexes with oligonuctiotides spanning the sequences for the NBRE-1 and Ad5 sites (Wilson et al., 1993).

The Ad5 element has also been shown to bind COUP-TF (chicken ovalbumin upstream promoter transcription factor), a transcription factor that is thought to inhibit transcription of *CYP17* but to promote that of *CYP11B2*. It has been suggested that this tissue specific regulation is mediated by co-regulator proteins, for example, Ubc9, small ubiquitin-related modifier-1 (SUMO-1)-conjugating enzyme and PIAS1 (protein inhibitor of activated STAT 1) SUMO-1-conjugating ligase (Shibata et al., 2004; Kurihara et al., 2005).

A summary of these transcription factors and binding sites within the *CYP11B2* promoter are shown in Figure 1-17. It is worth noting that these discoveries have arisen as a result of focused, hypothesis-driven experiments. However, they do not exclude the possible role of other factors in controlling *CYP11B2* transcription. Recent advances in molecular biology techniques allow a more biologically agnostic approach, without *a priori* assumptions which may limit the discovery of novel mechanisms. In addition, to date, there has been no research published regarding the influence of mechanisms such as chromatin modification or methylation (as described above) on the transcriptional regulation of *CYP11B2*. In short, there remain many unanswered questions around the control of this important process.

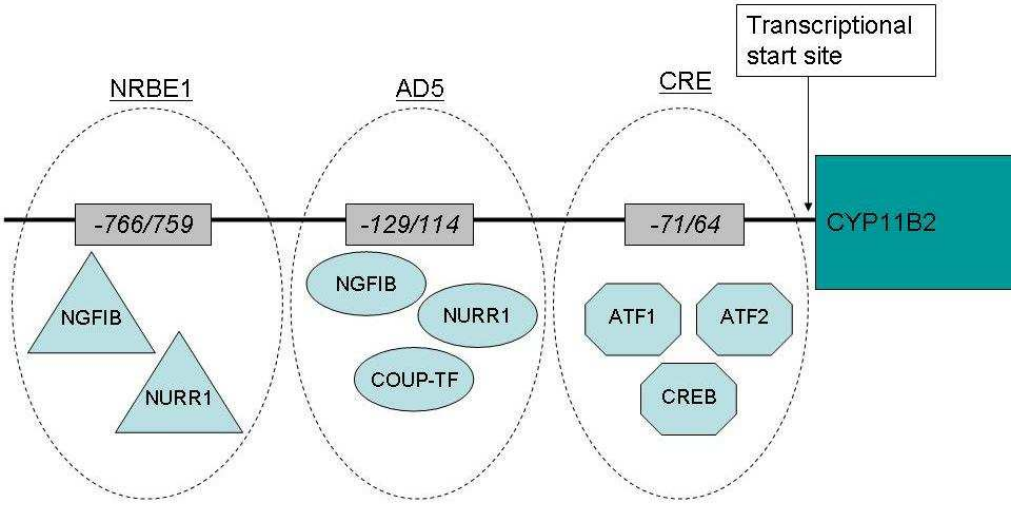


Figure 1-17 Transcription factor binding sites characterised in the *CYP11B2* promoter region.

DNA binding sites denoted by grey rectangles, position relative to transcriptional start site given in italics. Transcription factor proteins denoted by blue shapes below. NGFIB, Nerve Growth factor 1B; NURR1, Nuclear receptor related protein 1; COUP-TF, chicken ovalbumin upstream promoter transcription factor; ATF1, activating transcription factor 1; ATF2, activating transcription factor 2; CREB, cAMP response element-binding protein.

1.2.9 Angiotensin II and CYP11B2 gene transcription

In order to further investigate the effect of angiotensin II on aldosterone producing cells, both Rainey (Nogueira et al., 2007) and Gomez-Sanchez (Romero et al., 2007) et al have carried out microarray analyses of RNA from H295R cells (an adrenal cortical carcinoma cell line) exposed acutely to angiotensin II and compared the response to cells under basal conditions. This technique allows quantitative changes in expression of thousands of gene to be measured across the human genome. Despite experimental differences (angiotensin II concentrations were not the same, nor was the time incubated with the trophin), these two groups identified a core of 6 transcription factors in common. Included in these were transcription factors from the NGFIB family (Nurr1 and Nor1). In addition, members of the AP1 (activator protein 1, i.e. FOS and JUNB) family of transcription factors, which are known to be induced in the adrenal gland by angiotensin II (Viard et al., 1992): Egr (early growth response), BTG2 and ATF3 (activating transcription factor 3). It is interesting that mechanisms previously suspected to be involved in regulation of aldosterone synthase transcription have now been confirmed using this unbiased approach.

However, there are problems with this experimental method. Only a fraction of the volume of data produced in these experiments is published and although some investigators use publicly available repositories for depositing their data, methodological details are not always available or not similar enough to allow valid comparisons with other experiments. In addition, given the pleotrophic effects of angiotensin II, including its effects on other steroidogenic enzymes, it is not possible from microarray data alone to identify transcription factors which directly influence aldosterone synthase transcription and further experiments are required to confirm the role of transcription factors identified by this method. However, this does not always result in a clear answer. For example, both groups demonstrated up-regulation of FOS by angiotensin II, and the Gomez-Sanchez group indicated that up-regulation of FOS was associated with increased aldosterone synthase promoter activity (Romero et al., 2007). It would be tempting to speculate that increased angiotensin II leads to increased FOS causing increased aldosterone synthase promoter activity and increased production of aldosterone. However in the experimental model used by Nogueira et al this was not found to be the case in H295R cells (Nogueira et al., 2009a). It is worth noting that there is evidence that increased FOS leads to down-regulation of CYP17 (possibly via an interaction with SF-1) (Sirianni et al., 2010). This effect may not be seen in H295R cells as they express all the characteristics of androgen, glucocorticoid and mineralocorticoid producing cells (Gazdar et al., 1990); in fact their principle product is adrenal androgens, thus a relative excess of CYP17 compared to CYP11B2 may obscure this phenomenon in H295R cells.

Both groups have published subsequent work in order to further dissect these pathways (Nogueira et al., 2009b; Romero et al., 2010a). However, the relative contributions of these novel transcription factors in steroidogenesis in general and the regulation of aldosterone synthase in particular remains to be further elucidated.

The effects of potassium and ACTH on the transcription of human *CYP11B2* are even less clear. This is predominantly due to limitations of the model system. H295R cells respond to potassium but only at supra-physiological concentrations and there is no effect of ACTH (although the effect of ACTH can be mimicked by the use of dibutyryl cyclic AMP or forskolin) due to low levels of expression of

the ACTH receptor. To date, there are limited data regarding the mechanisms of their actions on transcription of *CYP11B2*.

1.3 Cardiovascular consequences of disruption of aldosterone production

The main physiological role of aldosterone and the mechanisms by which its production is regulated have been discussed in the preceding sections. How disruption of these mechanisms may lead to adverse cardiovascular consequences will be discussed in the following sections.

1.3.1 Blood pressure effects of mutations in steroidogenic genes: Congenital adrenal hyperplasia

As discussed previously, the perturbation of normal mechanisms of homeostasis observed in monogenic disorders can provide useful insights into normal function and the steroidogenic pathway offers many such examples.

The commonest condition seen in clinical practice is 21 -hydroxylase deficiency, which is described as either classical, commonly presenting in the neonatal period with salt wasting +/- ambiguous genitalia (rare: incidence 1:14 000 (New, 2004)), or non-classical, presenting in adulthood with symptoms of androgen excess and often asymptomatic in men (more common: incidence estimated 1:100 in Caucasian population (New, 2004) and http://omim.org/entry/613815). The biochemical phenotype, while covering a wide spectrum, arises from a reduced ability to convert progesterone and 17 α-hydroxyprogesterone (17-OHP) to 11-deoxycorticosterone (DOC) and 11-deoxycortisol (Compound S) and thence to the end products, aldosterone and cortisol (Compound F) respectively. This leads to increased ACTH drive in an attempt to restore homeostasis; however, this results only in androgen excess. Indeed, the diagnosis is made on the basis of elevated 17- OHP levels in response to ACTH. Treatment with exogenous steroids to suppress ACTH ameliorates many of the metabolic manifestations. However, mutations in other genes in the steroidogenic pathway can lead to congenital adrenal hyperplasia (CAH), and provide clear examples of the influence of these genes on steroid, and cardiovascular phenotypes. These are described in Table 1-5.The syndrome of aldosterone synthase deficiency is associated with low blood pressure and salt loss but a condition associated with

a “gain of function” would be of interest in the development of hypertension and relative aldosterone excess. Of particular relevance to studies of the pathogenesis of hypertension, it is worth noting that 17α-hydroxylase deficiency can present with perturbations of blood pressure and it has already been observed that a SNP in the locus of this gene has been identified as associated with blood pressure phenotypes in genome wide association studies of essential hypertension (See section 1.1.8,). No further direct association studies have been carried out with variants in this gene in populations of hypertensive patients, nor has any functional work to identify “milder” mutations yet been published. This would be an area of much interest for future work.

ENZYME	***21 hydroxylase***	***11-beta-hydroxylase***	***Aldosterone synthase***	***17-alpha-hydroxylase***	***3-beta hydroxysteroid dehydrogenase***
GENE AFFECTED	CYP21A	CYP11B1	CYP11B2	CYP17	HSD3B2
AMBIGUOUS GENETALIA	Females	Females	No	Males No pubertal development in females	Males Mild in females
INCIDENCE	1: 14 000	1:10 000	Rare	Rare	Rare
GLUCO-CORTICOIDS	↓	↓	Normal	↓	↓
MINERALO-CORTICOIDS	↓	↑	↓	↑	↓
ANDROGENS	↑	↑	Normal	↓	↓Males ↑Females
BLOOD PRESSURE	↓	↑	↓	↑	↓
NA BALANCE	↓	↑	↓	↑	↓
K BALANCE	↑	↓	↑	↓	↑
ACIDOSIS	Yes	+/- alkalosis	Yes	+/- alkalosis	Yes
ELEVATED STEROIDS	17OHP	DOC, 11-deoxycortisol	Corticosterone +/- 18-hydroxy-corticosterone	DOC, corticosterone	DHEA, 17OHPreg

Table 1-5 Characteristics of different forms of CAH.

Adapted from(146). 17OHP, 17 α-hydroxyprogesterone, DOC, deoxycorticosterone, DHEA, dehydroepiandrosterone, 17OHpreg, 17α- hydroxypregnenelone.

1.3.2 Aldosterone and Hypertension

10-15% of patients with hypertension have an aldosterone level that is elevated in relation to the level of renin (Rossi et al., 2006; Lim et al., 1999; Lim et al., 2000a; Fardella et al., 2000). A proportion of these patients will have autonomous aldosterone secretion. From a clinical perspective, classical Conn's syndrome or an aldosterone-producing adenoma remains the most important diagnosis to make in this context, as patients who have no contraindications to surgery may be cured of their high blood pressure by adrenalectomy, as initially described by Jerome Conn in 1956 (Conn and LOUIS, 1956). Since this

phenomenon was first described by Conn, there has been significant controversy regarding the contribution of aldosterone excess to the incidence of hypertension. However, it quickly became apparent that relatively few patients with hypertension harboured a large, unilateral, surgically resectable adenoma. Difficulties interpreting biochemical analyses including difficulties establishing normal reference ranges, the confounding effects of antihypertensive medication and other environmental factors all contributed to the difficulty in arriving at a diagnosis of aldosterone excess and establishing the prevalence of aldosterone excess in hypertension. The greater use of the aldosterone to renin ratio (ARR) as a screening test meant that the diagnosis could be considered and excluded in a significant proportion of hypertensive patients (unlike serum potassium which may be normal). This lead to a number of investigators contributing data to suggest the prevalence of aldosterone excess was greater than previously recognised (Gordon et al., 1994; Fardella et al., 2000; Calhoun et al., 2002; Lim et al., 2000b; Lim et al., 1999; Loh et al., 2000; Rossi et al., 2006). The largest and most comprehensive of these studies was the PAPY study (Rossi et al., 2006). This prospective study carefully established the biochemical features of aldosterone excess using suppression testing after an initial ARR screening test, and attempted to lateralise the source of aldosterone excess with adrenal vein sampling where available. The rates of aldosterone secreting adrenal adenoma in this study were 4.8% and the overall rate of aldosterone excess was 11%. Similar rates have been described in Scottish primary (Lim et al., 1999) and secondary care (Lim et al., 2000a) settings.

In patients with aldosterone excess but no evidence of a unilateral adenoma, the underlying pathophysiology is less clear and the majority of cases are attributed to “idiopathic bilateral hyperplasia”. The spectrum of pathological change from a normal adrenal gland to an aldosterone-producing adenoma is not well understood and there remains much controversy as to whether the development of abnormal bilateral hyperplastic adrenals precedes the emergence of a single dominant nodule. Some aspects of this have been discussed in section 1.2.5. While this topic raises interesting and important points, it is clear that regardless of the precise process of pathological change, there is a relationship between plasma aldosterone concentration and risk of high blood pressure, even when levels are within the so-called normal range and

secretion does not exhibit automaticity as in primary aldosteronism. Evidence for this comes from the Framingham cohort where, in normotensive subjects, a plasma aldosterone level at the top end of the normal range was associated with a higher incidence of hypertension at 4 year follow up than a lower plasma aldosterone level (Vasan et al., 2004). Further, data from Reynolds et al (Reynolds et al., 2009) demonstrated in an elderly population a positive correlation between plasma aldosterone concentration after stimulation, and suppression and blood pressure. The efficacy of spironolactone as a blood pressure lowering agent in resistant hypertension (Chapman et al., 2007) has also contributed to the body of evidence which places aldosterone in a central role in the pathogenesis of hypertension.

1.3.3 Cardiovascular damage independent of blood pressure

Animal models from Rocha et al (Rocha et al., 1998; Rocha et al., 2000; Rocha et al., 1999) and Weber et al (Brilla et al., 1990) elegantly demonstrated the detrimental effect of aldosterone on a range of cardiovascular tissues, including the heart, brain and the kidney in rat models of mineralocorticoid excess and hypertension. Vascular damage was prevented by mineralocorticoid blockade and was independent of blood pressure. It is of interest that the presence of aldosterone proved necessary but not sufficient for the development of vascular pathology as the detrimental effects required the presence of a high salt diet (Rocha et al., 1998; Rocha et al., 2000; Blasi et al., 2003). A murine model of aldosterone induced vascular damage did not develop the cardiovascular phenotype in the absence of high salt diet (Wang et al., 2004), confirming that this phenomenon is constant across species. In man, the crucial role of dietary sodium has been confirmed in a cohort of hypertensive patients, where the blood pressure lowering effect of a restricted sodium intake was considerable (Pimenta et al., 2009). These data from Pimenta *et al* suggests that elevated aldosterone (without confirmation of autonomous aldosterone production) in combination with a high salt intake, leads to increased urinary protein excretion, independent of blood pressure. Further evidence of end organ damage caused by the combination of high salt and high aldosterone can be found in work by Du Cailar *et al*, who demonstrate that in a group of patients with hypertension, left ventricular hypertrophy progressively increased across

sodium tertiles only in patients with high plasma aldosterone concentration (du Cailar G. et al., 2010).

Further evidence for the adverse effects of aldosterone is provided by findings that patients with primary aldosteronism have higher rates of left ventricular hypertrophy (Rossi et al., 1996) and other adverse cardiovascular events including stroke, non-fatal myocardial infarction (MI), atrial fibrillation (Rossi et al., 1996; Milliez et al., 2005) and renal dysfunction (Sechi et al., 2006) than patients with similar levels of blood pressure where aldosterone is not increased. The increased proteinurea associated with primary aldosteronism is partly reversible following medical therapy with mineralocorticoid antagonist or surgical resection (Sechi et al., 2006).

Aldosterone excess has also been associated with poor outcomes in heart failure as well as adverse events post myocardial infarction (Beygui et al., 2006). It is known that the degree of neurohumoral activation and specifically, increased aldosterone concentrations in plasma, are associated with increased mortality; both the SAVE trial (Vantrimpont et al., 1998) (post MI) and CONSENSUS (Swedberg et al., 1990) (heart failure) demonstrated that high aldosterone levels predict poor cardiovascular outcome. In addition, further data have confirmed that this association of increased mortality is present in patients across all NYHA classifications of heart failure (Guder et al., 2007). These adverse effects are likely to reflect specific actions of aldosterone on the heart, given that the hormone in excess provokes myocardial fibrosis and remodelling in the rat heart, independent of blood pressure and angiotensin II (Brilla et al., 1990). Blocking aldosterone using mineralocorticoid receptor antagonists is associated with less remodelling both in animal models (Fraccarollo et al., 2003) and in a human cohort post MI (Weir et al., 2009).

Finally, the importance of mineralocorticoid receptor activation on development of cardiovascular dysfunction is illustrated by studies in patients with cardiac failure and myocardial infarction. A large number of clinical trials have investigated the utility of inhibition of renin/angiotensin system the in cardiovascular conditions, but the focus was predominantly on inhibition of angiotensin converting enzyme (ACE) or blockade of the angiotensin II receptor. However, pilot data from the RESOLVD trial (McKelvie et al., 1999)

demonstrated that even the combination of an ACE inhibitor and angiotensin receptor blocker does not chronically suppress aldosterone secretion - a phenomenon described as “aldosterone escape” - prompting the evaluation of inhibition of aldosterone action as an alternative approach in cardiovascular therapy. The Randomised Aldactone Evaluation Study (RALES) (Pitt et al., 1999) provided the first crucial evidence that blockade of aldosterone receptors decreased mortality in patients with left ventricular systolic function, leading to a re-evaluation of the perception of aldosterone antagonists and their clinical applications. This study had a major influence on clinical practice and indeed, prompted a degree of concern regarding the increased incidence of adverse events associated with spironolactone, specifically hyperkalaemia. The EPHESUS study used the selective mineralocorticoid-receptor antagonist eplerenone added to recommended medical therapy. This reduced the rates of death from any cause and hospitalization for cardiovascular reasons among patients with acute myocardial infarction complicated by left ventricular systolic dysfunction and heart failure (Pitt et al., 2003). Most recently, EMPHASIS-HF evaluated the effects of eplerenone in patients with systolic heart failure and mild symptoms (NYHA class II) and also confirmed a mortality benefit as well as a reduction in hospitalisation (Zannad et al., 2011).

In summary, it is clear that inappropriate aldosterone for the level of renin and salt intake can be detrimental and that blocking the action of aldosterone via mineralocorticoid receptor antagonists ameliorates this effect. The mechanisms behind inappropriate aldosterone production are not clear. However, aldosterone levels (Inglis et al., 1999) and the aldosterone to renin ratio (Alvarez-Madrazo et al., 2009; Newton-Cheh et al., 2007) (the most commonly used screening test for aldosterone excess) are heritable, consistent with an underlying genetic mechanism. The aldosterone synthase gene catalyses the final rate limiting steps in the production of aldosterone and is a logical candidate gene for hypertension and the sub-phenotype of relative aldosterone excess. Further discussion of the aldosterone synthase gene and the effect of variation within it, leading to altered regulation of plasma aldosterone concentration will now follow.

1.4 Structure of CYP11B2: Mutations and variations

CYP11B2 lies on chromosome 8 q24.3 and is a gene with 9 exons, all of which are transcribed. It is highly polymorphic with high linkage disequilibrium across the locus (White and Slutsker, 1995). However, this varies according to ethnicity with a greater degree of polymorphism and less strong LD in African populations, presumable due to greater frequency of recombination events (Alvarez-Madrazo S et al., 2009). *CYP11B2* lies just 40kb from *CYP11B1* (11 ß-hydroxylase). These two enzymes are structurally very similar, with around 93% homology, reflecting their shared origin (they are thought to be as a result of a gene duplication event). However, there is less similarity between their 5' untranslated regions, presumable reflecting their separate mechanisms of regulation.

Mutations in aldosterone synthase which alter the protein code can result in decreased or absent aldosterone synthase activity. As aldosterone synthase catalyses both the 18-hydroxylation of corticosterone to 18-hydroxycorticosterone (18-OHB) as well as the final biochemical step of aldosterone biosynthesis, the 18-hydroxylation of 18-OHB to aldosterone, an inability to undertake either or both of these reactions can occur.

To test the hypothesis that variation in aldosterone synthase could lead to a phenotype of hypertension and inappropriate aldosterone level for the level of renin, Smithies group (Makhanova et al., 2008) generated a mouse model with increased expression of the aldosterone synthase gene. This was achieved by inserting the more stable 3' end of the human growth hormone gene instead of the endogenous 3' end of aldosterone synthase gene. The group confirmed that the experimental mouse had greater levels of expression of aldosterone synthase and demonstrated that the experimental mouse had less activation of the RAAS under low salt conditions with less deactivation of the RAAS under high salt conditions than control animals. In addition, the experimental mice became hypertensive in response to high salt conditions and demonstrated greater cardiac hypertrophy in response to angiotensin II infusions, a phenomenon not exhibited by control mice. These important experiments are a proof of principle that increased expression of aldosterone synthase can lead to minor elevations in plasma aldosterone concentrations, but that phenotypic differences in the

form of hypertension and other adverse cardiovascular phenotypes are only evident under specific environmental conditions.

Polymorphic variations in *CYP11B2* (i.e. variation that is more frequent than 5% of the population and do not affect protein coding) have been described. White et al first described a polymorphism at position -344 in the 5'untranslated region of the gene (White and Slutsker, 1995). This corresponds to a binding site for steroidogenic factor 1 (SF-1), a transcription factor implicated in the transcriptional regulation of many steroidogenic enzymes. The C allele at position -344 binds SF-1 four-five times more avidly than the T allele (White and Slutsker, 1995; Bassett et al., 2002) but, this site appears to have no functional effect *in vitro* as deletion has no effect on transcription (Clyne et al., 1997) and reporter constructs carrying to two contrasting alleles are expressed at the same level (Bassett et al., 2002).

In addition, a variation in the intron 2 was also first described by White et al (White and Slutsker, 1995), where part of the sequence in the *CYP11B2* intron 2 corresponds to that of the intron 2 in *CYP11B1*. It is though that this has arisen from a gene conversion event. However, its functional significance is unclear and has as yet not been assessed *in vitro*, or indeed, clearly delineated *in vivo*. As we are now recognising the possible functional roles of what was previously thought of as "junk DNA" it may be worth investigating the possibility that the intron conversion polymorphism may play a functional role resulting in altered regulation of the gene.

1.5 CYP11B2 and hypertension phenotypes

There has been much interest in the polymorphic variation in *CYP11B2* in relation to hypertension and the sub-phenotype of inappropriately elevated aldosterone levels. The T allele at -344 has been associated with increased excretion of urinary metabolites of aldosterone (Davies et al., 1999) and plasma aldosterone (Paillard et al., 1999). There is also evidence that, amongst hypertensive subjects with an elevated ARR, the T allele is more frequent (Lim et al., 2002). However, there are conflicting reports in the literature as to whether the T or the C allele is more prevalent in patients with hypertension. The discrepancy between different populations (not an uncommon finding in

candidate gene studies as discussed above in section 1.1.6) may be as a result of variation in linkage disequilibrium in different populations, a phenomenon that we know varies with ethnicity. A meta-analysis conducted in 2007 concluded that, overall the -344 C allele was associated with around a 17% reduced relative risk of hypertension than the -344 T allele (Sookoian et al., 2007).

An interesting phenomenon associated with genetic variation at -344 is that of an alteration in index of 11ß- hydroxylation. Elevated deoxycorticosterone and 11-deoxycortisol in relation to corticosterone and cortisol has been consistently found in T allele carriers compared to C allele carriers (Davies et al., 1999; Davies et al., 2001). Why alteration in *CYP11B2* is associated with a phenotype of reduced efficiency of the enzyme encoded by *CYP11B1* is not clear. However, it has been demonstrated that the polymorphism at -344 in *CYP11B2* is in strong LD with variation in the promoter region of *CYP11B1* which is associated with reduced transcriptional activity in response to ACTH in *in vitro* studies (Barr et al., 2006). The mechanism may have some similarities to that observed in individuals with 11 ß-hydroxylase deficiency i.e. that increased ACTH as a result of an absence of negative feedback from cortisol, leads to increased drive to the adrenal cortex. In the case of congenital adrenal hyperplasia caused by the monogenic disorder 11 ß-hydroxylase deficiency, this increased ACTH drive is associated with increased deoxycorticosterone (see section 1.3.1, Table 1-5) which acts as a mineralocorticoid leading to increased sodium and water retention and thus suppression of renin. In contrast, in the case of the more minor reduction in 11 ß-hydroxylation described here, in which cortisol levels are entirely normal, it has been suggested that increased ACTH drive (as evidenced by increased morning plasma ACTH concentrations (Freel et al., 2008) and increased adrenal androgens (Freel et al., 2007), leads to increased synthetic capacity for aldosterone production either by causing hyperplasia of the zona glomerulosa or by increasing expression of *CYP11B2*. Thus, according to this hypothesis, the phenotype may not be driven by variation in the *CYP11B2* gene but rather that the -344 polymorphism is merely a “passenger” SNP which is inherited along with the “driver” SNPs in the promoter region of CYP11B1. However, as described above (see section 1.2.5), the role of ACTH as a trophin of aldosterone production is controversial. Indeed, in the case of CAH due to 11 ß-hydroxylase deficiency hypertension is caused not by increased aldosterone

but rather by increased deoxycorticosterone secretion, and aldosterone levels are low.

Another possible alternative hypothesis is that the entire *CYP11B1/ CYP11B2* locus is necessary for the development of the phenotype, with variation in the CYP11B1 promoter driving the elevated DOC: S, B: F ratio and that this is co inherited with variation within the *CYP11B2* promoter which causes increased aldosterone. Recent data from within the Connell/ Davies group suggest that the haplotype containing the T allele at -344 along with A/G at -1881/ 1851 in CYP11B1 is associated with increased transcription of aldosterone synthase and reduced transcription of 11 ß-hydroxylase *in vitro* and an increased risk of hypertension *in vivo*.

Having described some of the current knowledge in the field relating to the regulation of aldosterone levels in general and the regulation of transcription of aldosterone synthase in particular, a programme of work has been undertaken to investigate the hypotheses discussed in the following section.

1.6 Hypothesis: Aldosterone and essential hypertension

1.6.1 Hypothesis

The principle hypotheses to be explored in the following work is firstly, that there is a genotype-dependant increase in basal aldosterone production and/ or increased responsiveness of aldosterone production to its principle trophins and secondly, that this phenotype also displays evidence of inefficient 11ß-hydroxylase activity as evidenced by an increase in the ratio of 11-deoxycortisol to cortisol and an increase in the ratio of 11-deoxycorticosterone to corticosterone. Further, I propose that the molecular mechanisms that produce this phenotype are a result of polymorphisms that are inherited in a non-random fashion in the two genes controlling the final stages of corticosteroid metabolism. Specifically, I aim to test whether increased aldosterone production is a result of variation in the promoter region of *CYP11B2* and corresponds to increased promoter activity and/ or increased sensitivity to trophins. Thus, a mechanism for increased aldosterone in some individuals may be independent of

relative inefficiency of 11ß-hydroxylase which is co-inherited as a result of high linkage disequilibrium within the locus.

1.6.2 Aims

To identify the pattern of linkage disequilibrium across the promoter region of aldosterone synthase.

To investigate and validate the H295R adrenocortical cell line as an appropriate model for the study of the regulation of steroidogenesis in general and aldosterone production in particular.

To investigate the functional effects of candidate polymorphisms in the promoter region of aldosterone synthase in order to determine their likely effects on aldosterone production.

To explore in detail the effect of variation in the promoter region of aldosterone synthase on corticosteroid production and regulation in normal adult volunteers studied under standardised salt conditions.

Methods

2. Methods

2.1 Genotyping of polymorphisms in the CYP11B2 and CYP11B1 locus

2.1.1 Subjects

Oxford cohort (HTO) has been previously described (Baker et al., 2005) and consists of 1773 individuals from 255 families where the proband was found to have essential hypertension. Subjects were recruited from 1993-1996 and families were ascertained from the hypertension clinic at the John Radcliffe Hospital, Oxford, or through general practices in the Oxford area. Hypertension was defined as mean SBP> 140mmHg and DBP>90mmHg on daytime ambulatory monitoring or more than three office BP readings of SBP>160mmHg DBP>95mmHg. All patients were 60 or younger at time of onset of hypertension. Subjects aged more than 30 years with a diastolic blood pressure <90 mmHg and a systolic blood pressure <140 mmHg were considered unaffected. Exclusion criteria were a BMI >30 kg/m2, use of oral contraception or a diagnosis of a secondary causes of hypertension. All probands and family members were UK residents of self reported Caucasian ethnicity. For the purposes of this work, 279 individuals were selected at random from this cohort for genotyping across the CYP11B2 promoter region.

2.1.2 PCR of CYP11B2 promoter region

The 2kb region of the CYP11B2 promoter was amplified by polymerase chain reaction (PCR) followed by sequencing. A number of primers were designed based on published sequences of the CYP11B2 gene (http://www.ensembl.org/index.html) and were between 20 -24 bp in length. They had less than 60% Guanine/ Cytosine (G/C) content and were screened for their ability to form secondary structures and dimers. In addition, they were checked for specificity using the BLAST database, (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers, which had been purified by the High Purity Salt Free (HPSF) purification method, were commercially obtained (Eurofins MWG Operon, Ebersberg, Germany). The optimum primer combination was selected and used for PCR amplification of the region. Primer sequences are shown in the following table.

PRIMER DIRECTION	SEQUENCING/ PCR	PRIMER NAME	SEQUENCE	BASE PAIRS	TM (˚C)
Sense	PCR	B2_F2	GCC AAT AGA ACT GAC TTG CC	20	57.3
Antisense	Sequencing	B2_R1	AGG ATT T GG GCT GAA CA G GGT GGA	24	64.4
Antisense	Sequencing	SF1R	AGG CGT GGG GTC TGG ACT	18	60.5
Antisense	Sequencing	B2Pro494	TTC AAG CAA TTC TCC CGC CT	20	57.3
Antisense	PCR	B2 5' 783	AGA TCA TCC CAC TGC ACT CC	20	59.3

The Taq polymerase that was used was a mixture of Thermostable Taq DNA Polymerase and a polymerase with proof reading (3'-5' exonuclease) activity, Expand High Fidelity PCR systems (Roche Diagnostics, Mannheim, Germany). The PCR conditions were optimized using a temperature gradient and the most successful annealing temperature was chosen. PCR reactions were set up in 96 well plates and the PCR was performed on Tetrad PTC-225 Thermo Cycler (MJ Research Waltham, Massachusetts, USA). 5 µl DNA (5ng/ µl) was added to the following reaction mixture:

Reagent	**Volume added per well**	Final concentration
dNTPs	2 µl	80µM
Forward Primer	0.75µl	400nM
Reverse Primer	0.75µl	400nM
Expand High Fidelity Taq DNA polymerase	0.75µl	
Expand High Fidelity Buffer, 10x conc. with $MgCl_2$	2.5 µl	
Nuclease free water	13.25 µl	
Total	**20 µl**	

The PCR conditions were as follows:

1. 94°C for 2 mins	Denaturing step
2. 94°C for 15 secs	Denaturing step
3. 61.4°C for 30 secs	Annealing step
4. 68°C for 4 mins	Elongation step
Repeat steps 2-4 for 9 cycles	
5. 94°C for 15 secs	Denaturing step
6. 61.4°C for 30 secs	Annealing step
7. 68°C for 4 mins (+ 5 secs per cycle)	Elongation Step
Repeat step 5-7 for 19 cycles	
8. 72°C for 7 mins	Final Elongation

Selected samples, including a negative control, were resolved on a 1% agarose gel. Agarose (1g) was added to 100ml TBE buffer and heated to boiling point in a 950W microwave oven for 50 seconds. 1 µl ethidium bromide was added and gently mixed. This was allowed to cool in gel moulds with Teflon combs until set. To confirm the presence of PCR product and the absence of contamination, 10 µl of PCR product was loaded into the wells and run at 90 volts for 40 mins. DNA bands were visualised under UV light at 302nm and results were recorded using Multi-Analyst software v1.1 (Bio-Rad, Hertfordshire, UK) and a 2kb band was visualised.

The PCR product was cleaned up prior to sequencing using the AMPure system (Agencourt, Beverly, MA, USA.) removes unincorporated primers, dNTPs, DNA polymerases and salts used during PCR amplification that can interfere with the sequencing reaction. AMPure was re-suspended by vortexing before adding to the PCR reaction products. The plate was spun down and incubated for 3 min before being placed on a magnetic block for 10 min. 200µl of 70% ethanol was added to each well and after 30 sec, the supernatant was discarded by inverting the plate, whilst still on the magnet, onto absorbent paper. The inverted plate and magnet were spun down at 76x g for 30 sec. The plate was removed form the magnet and left to air dry for 20 min before the addition of 40µl of NF water. The plate was returned to the magnet for 10 min and 2µl of this final solution was the substrate for the sequencing reaction.

2.1.3 Sequencing reactions for CYP11B2 promoter region

PCR products were sequenced using Applied Biosystems Big Dye v3.1 cycle sequencing chemistry (PE Applied Biosystems, Foster City, California, USA), a

modification of the chain terminator method. This process requires a primer which is complementary to the beginning of the sequence of DNA previously amplified by PCR. This is annealed to the DNA and then extended by DNA polymerase using modified substrate which includes labeled 2'-3'-dideoxynucleotides. Each base is labelled with a different colour; these ddNTPs are incorporated into the replicate DNA however, once incorporated further extension is not possible due to the lack of a 3' hydroxyl group. PCR products of varying lengths are produced, which can be fractionated on a sequencing column. Fluorescent sensors detect the colour of each DNA strand, allowing the nucleotide sequence to be interpreted.

Sequencing reactions were set up in 96 well plates with the following components:

Reagent	Volume added per well
PCR product	2 µl
Primer (3.2pmol/ µl)	1 µl
Sequencing Buffer (5x)	3.5 µl
ABI PRISM BigDye Termination v3.1 Ready Reaction Mix	0.5 µl
Nuclease free water	13 µl
Total	**20 µl**

Sequencing primers are shown in the first table. The sequencing reaction was performed on 96 well PTC 225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) and the conditions were as follows:

1. 96°C for 45 seconds
2. 50°C for 25 minutes
3. 60°C for 4 minutes
4. Repeat from step (1) x 24
5. Incubate at 94°C for 15 minutes

The CleanSEQ purification method (Agencourt, Beverly, MA, USA) was utilised to remove reagents of the sequencing reaction prior to automated sequencing. The CleanSEQ reagent was resuspended by vortexing before adding 10µl was added to the sequencing product. 62µl of 85% ethanol was also added to each well. The plate was placed on the magnet for 5 min before the solution was removed and

discarded. 150µl of 85% ethanol was added to each well and this was incubated at room temperature for 30 secs. The plate and magnet were inverted on absorbent paper and centrifuged at 210 x g for 30 secs. The plate was removed from the magnet and left to dry for 20 min before the addition of 40µl of NF water. The plate was vortexed, spun down at 210 x g for 30 sec before being placed back on the magnet when 20µl was transferred to a bar coded plate for automated sequencing.

Automated sequencing was preformed on ABI 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA)

Results were analysed using SeqScape v2.2 genotyping software (Applied Biosystems, Foster City, CA, USA) and sequences were aligned to a reference sequence based on published sequences of the CYP11B2 gene (Entrez Gene database, NCBI http://www.ncbi.nlm.nih.gov/gene). Sequence annotated with location of SNPs and primers used is given in Appendix 7.2. Samples of poor quality were excluded from further analysis.

2.1.4 PCR and sequencing of CYP11B2 intron 2 region

The intron 2 polymorphism was genotyped according to a protocol previously optimised in the laboratory. The PCR primers are shown in the following table.

PRIMER DIRECTION	SEQUENCING/ PCR	PRIMER NAME	SEQUENCE	BASE PAIRS	TM (°C)
Sense	PCR	ICTAQMAN F	GATGGCATGAAGCA CAAAGCT	21	57.9
Antisense	PCR	ICTAQMAN R	CCTTGGGCGACAG CACA	17	57.6
Antisense	Sequencing	INTCONR (B1B2)	GTGTTCGAGCTGC AGCCTTTTC	22	62.1

PCR reactions were set up in 96 well plates and the PCR was performed on Tetrad PTC-225 Thermo Cycler (MJ Research Waltham, Massachusetts, USA).10µl of DNA (5ng/µl) was added to the following master mix:

Reagent	Volume added per well	Final concentration
dNTPs	5 µl	200µM
Forward Primer	0.5µl	400nM
Reverse Primer	0.5µl	400nM
$MgCl_2$(25mM)	2	2.0mM
Thermostart Taq, Abgene	1.25µl	
Thermostart Buffer, 10x conc.	2.5 µl	
Nuclease free water	6.25 µl	
Total	**15 µl**	

The PCR conditions were as follows:

1. 95°C for 15 mins
2. 95°C for 15 secs
3. 62°C for 30 secs
4. 72°C for 2 mins

Repeat steps 2-4 for 44 cycles

5. 72°C for 7 mins

Selected samples, including a negative control, were resolved on a 1% agarose gel as previously described and a 600bp band was visualised. The PCR products were cleaned up as previously described.

2.1.5 PCR and sequencing of CYP11B1 promoter region

The polymorphisms in the promoter region of CYP11B1 were genotyped according to a protocol previously optimised in the laboratory. The PCR primers are shown in the following table.

PRIMER DIRECTION	SEQUENCING/ PCR	PRIMER NAME	SEQUENCE	BASE PAIRS	TM (°C)
Sense	1ST PCR	B1 5'UTR	TCCTTCGCATCCCTTG TAAGTT	22	58.4
Antisense	1ST PCR	B1 PROM-260	CTTGGATTATTCAT CTCCTTGCAAGG	26	61.6
Sense	2ND PCR	B1 5' 732	GCATCCCTTGTAAG TTGGATTCCTAA	26	61.6
Antisense	2ND PCR	B1 5' 369-393	AAGCATTCCCTTTG AAAACTGGTAC	25	59.7
Antisense	Sequencing	B1 PROM 250-229	AAGTCAAATTGTCT CTGTTTG	21	52

The first PCR reactions were set up in 96 well plates and the PCR was performed on Tetrad PTC-225 Thermo Cycler (MJ Research Waltham, Massachusetts, USA).5µl of DNA (5ng/µl) was added to the following master mix:

Reagent	**Volume added per well**	Final concentration
dNTPs	5 µl	200µM
Forward Primer	1µl	400nM
Reverse Primer	1µl	400nM
$MgCl_2$(25mM)	1.5 µl	2.0mM
Thermostart Taq, Abgene	0.25µl	
Thermostart Buffer, 10x conc.	2.5 µl	
Nuclease free water	8.75 µl	
Total	**20 µl**	

The PCR conditions were as follows:

1. 95°C for 15 min
2. 95°C for 30 sec
3. 60°C for 30 sec
4. 72°C for 3 min

Repeat steps 2-4 for 34 cycles

5. 72°C for 7 min

The PCR products were then diluted 1:10 in nuclease free water and 1µl used as a template for the second PCR reaction using the following master mix:

Reagent	**Volume added per well**	Final concentration
dNTPs	5 µl	200µM
Forward Primer	1µl	400nM
Reverse Primer	1µl	400nM
$MgCl_2$(25mM)	1.5 µl	2.0mM
Thermostart Taq, Abgene	0.125µl	
Thermostart Buffer, 10x conc.	2.5 µl	
Nuclease free water	12.875 µl	
Total	**24 µl**	

The PCR conditions were as follows:

1. 95°C for 15 min
2. 95°C for 30 sec
3. 60°C for 30 sec
4. 72°C for 3 min

Repeat steps 2-4 for 34 cycles

5. 72°C for 7 min

Selected samples, including a negative control, were resolved on a 1% agarose gel as previously described and a 387bp band was visualised. The PCR products were cleaned up as previously described.

2.1.6 Data analysis

The data from the sequencing of the CYP11B1 and CYP11B2 region was analysed using Haploview V4.2 (http://www.broad.mit.edu/mpg/haploview)(Barrett et al., 2005). This package was used to determine Hardy- Weinberg equilibrium, percentage of genotyping, pattern of linkage disequilibrium and haplotype structure.

2.2 Cell culture

2.2.1 H295R cell maintenance and subculturing procedure

H295R cells were a gift from Prof W Rainey (Medical College of Georgia). DNA was extracted according to standard techniques and Short Tandem Repeat (STR) profiling was performed by LGC standards (Teddington, Middlesex, UK). Short tandem repeat analysis is a commonly used forensic test and has been proposed for verifying the identity of cell lines (Masters et al., 2001). Shown below Figure 2-1 is the profile of the Strain 2 H295R cells, cultured as described above. This profile matches the expected result from the commercially available H295R cells (H295R CRL-2128) and verifies that the cells have not been cross contaminated or misidentified.

Cell Line Authentication Service – 9 Loci Service Results

Full descriptions of loci names can be found on page 2 of this document. These results are subject to LGC Standards terms and conditions. If you have any questions please contact the LGC Standards office +44 (0)208943 8489 or uksales@lgcstandards.com.

Sample LGC 045

SAMPLE DESCRIPTION	POWERPLEX1.2 STR RESULTS								
H295R	Amel	D5	D13	D7	D16	VWA	THO1	TPOX	CSF1
	XX	12 12	13 13	9 12	11 11	17 18	9.3 9.3	8 8	10 12

Figure 2-1 Short Tandem Repeat analyisis of H295R DNA

Cells were grown in a monolayer in medium consisting of DMEM/ F12 (Invitrogen, Paisley, Scotland) supplemented with 2% Ultroser G serum (Pall scientific, Cergy, France), 1% ITS Universal culture supplement (Insulin, Transferrin, Selenous acid. BD Bioscience, Oxfordshire, UK), 1% Penicillin/streptomycin (Invitrogen, Paisley, Scotland). Cells were cultured at 37°C with 5% CO_2 until approximately 80% confluent. The culture medium was removed and the cells rinsed with phosphate buffered saline (PBS) solution. Trypsin-EDTA 0.25% (Sigma, St Louis, Missoiuri, USA) was added to cover the cells and after incubation for approximately 3 minutes the cells detached from the flask. Complete growth medium was added to inactivate the trypsin and the solution was spun at 1500rpm for 5 minutes. The cells formed a pellet and after removing the growth medium, were re-suspended in fresh complete growth medium and aliquoted to new flasks according to the desired sub cultivation ratio. Cells were fed with fresh media every 3 days and usually spit in a ratio of 1 in 2 to 1 in 3 once per week.

Cells that were frozen down were handled in the same way however, after being spun down to a pellet they were re suspended in growth medium supplemented by dimethyl sulphoxide (DMSO). Aloquots were transferred to cryovials and frozen gradually in a Nalgene cryo freezing container (Thermo Fisher Scientific, NY, USA) before being transferred to liquid nitrogen for long term storage. When cells were removed from liquid nitrogen, they were thawed in a water bath at 37°C, the cell were pelleted by centrifugation and after the

defrosted medium was removed, fresh growth medium was added and the solution was transferred to a 75cm^3 flask and incubated at 37°C with 5% CO_2.

2.2.2 H295R Phenotyping: General

H295R cells were sub-cultured into 100mm tissue culture dishes at a concentration of 5x10^6 per dish. After 24 hours, the cells were rinsed with phosphate buffered saline (PBS) (Invitrogen, Paisley, Scotland) and the medium replaced with serum-free medium (10 ml). After a further 24 hours, this was replaced, either with fresh serum-free medium alone or with medium containing Ang II (10 pM), potassium chloride (6 mM) or dibutyryl cAMP (Bu_2cAMP) (1 mM) (Sigma, St Louis, Missouri). Each experiment was performed in triplicate.

2.2.3 H295R phenotyping: RNA extraction and analysis

Cells were trypsinised and pelleted as described above and the pellet was washed twice in DPBS. The cells were disrupted using Buffer RLT from RNeasy Mini Kit (QIAGEN, Crawley, U.K.) with the addition of 1% ß-mercaptoethanol to inhibit RNAse activity. Lysates were transferred to 2ml lysing matrix tubes (Lysing matrix D 1.4mm ceramic spheres, MP Biomedical, Salon, Ohio, USA) and homogenised for 30 sec on a rotator stator homogeniser (MagNA Lyser Roche, Switzerland). One volume of 70% ethanol was added and mixed well by pippetting. This was passed through an RNeasy spin column, which selectively binds RNA molecules longer than 200 bases to its silica membrane, and centrifuged for 15 secs at > 8000x g. The membrane was washed with 700µl of buffer RW1 followed by 2 washes of 500µl Buffer RPE before being eluted with 30µl of PCR grade water (x2).

To eliminate genomic DNA contamination, the samples were DNased using the TURBO DNA-free kit (Applied Biosystems, Foster City, CA, USA). 0.1 volumes of 10x Turbo DNase buffer and 1 µl TURBO DNase was added to the RNA. This was incubated at 37°C for 30 min before the addition of 0.1 volume of DNase inactivation reagent. This was left for 5 min at room temperature with occasional mixing before being spun down at 10 000 x g for 1.5 min. The supernatant containing the RNA was aspirated and the remaining pellet discarded.

RNA was diluted to a constant concentration using a spectrophotometer (ND-1000 spectrophotometer, Nanodrop Technologies, Washington, USA) before being transcribed to cDNA using the ImProm-II Reverse Transcription System (Promega, Madison, Wisconsin, USA) on a 96 well plate according to the standard protocol. All samples were reverse transcribed alongside an equivalent reaction omitting reverse transcriptase (–RT control) and reactions substituting water for RNA were also performed (blank controls).

Denature

Reagent	Volume per reaction
RNA	2 µl
Random primers(0.5 µg/reaction)	1 µl
Nuclease free water	2 µl
Total	**5 µl**

This was heated to 70˚C for 5 mins then chilled on ice to 5˚C for 5 mins.

Reverse transcription

Reagent	Volume per reaction	Final concentration
Nuclease free water	3.7 µl	
ImProm-II 5x reaction buffer	4.0 µl	
$MgCl_2$	4.8 µl	6 mM
dNTPs	1.0 µl	0.5 mM each dNTP
Inhibitor	0.5 µl	20 U
RT/Water	1.0 µl	
Total	15 µl	

The Reverse transcription conditions were as follows:

1. 25°C for 5 mins	Annealing step
2. 42°C for 1 hour	Elongation step
3. 70°C for 15 mins	Inactivation

Quantitative RT-PCR assays for CYP17 (17α-hydroxylase), CYP11B1 (11ß-hydroxylase) and CYP11B2 (aldosterone synthase) were performed with ß-actin used as a housekeeping gene. All reactions were performed in a 384-well plate format on an ABI 7900 HT Prism Sequence Detection System (Applied Biosystems,

Foster City, California, USA). Primers were obtained from Eurofins MWG Operon, Ebersberg, Germany, ABsolute QPCR ROX Mix from ABgene,Epsom, UK and probes from Universal Human Probe Library, Roche Diagnostics, Burgess Hill, UK.

GENE	PRIMER SEQUENCE	PROBE
CYP11B2	(F) GCACCTGCACCTGGAGATG (R) CACACACCATGCGTGGTCC	#57
CYP11B1	(F) ACTAGGGCCCATTTTCAGGT (R) GGCAGCATCACACACACC	#68
CYP17	(F) CTATGCTCATCCCCCAC AG (R) TTGTCCACAGCAAACTCACC	#67
β-ACTIN	(F) CCA ACC GCG AGA AGA TGA (R) CCA GAG GCG TAC AGG GAT AG	#64

Reagent	**Volume per reaction**	**Final concentration**
Nuclease free water	2.1 µl	
ABsolute QPCR ROX Mix	5.0 µl	
Forward primer	0.4 µl	400nM
Reverse primer	0.4 µl	400nM
Probe	0.1 µl	100nM
Total	8 µl	

8 µl master mix was pippetted into a 384 well plate. This was spun down before the addition of 2 µl cDNA. Reactions were incubated at 95°C for 15 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Results were analysed using the comparative C_T method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

2.2.4 H295R phenotyping: steroid and protein measurements

Medium was removed at 24 hours and stored at -20°C. Steroid extraction and measurement was performed by Miss Mary Ingram. Steroids were extracted from medium (10 ml) using ChemElute cartridges (Varian, CA, USA) and eluted with dichloromethane. The eluates were evaporated to dryness under nitrogen and reconstituted in acetonitrile (60 µl). An aliquot (20µl) was chromatographed on a

reversed phase column (Polaris 5 µm, 150mm x 2mm C18-A) and the effluent analysed by tandem LC:MS (Varian 1200L, California, USA).

2.2.5 Protein assay: Bicinchoninic acid (BCA) assay

Cells were washed with DPBS, (Invitrogen, Paisley, Scotland), 1000µl of Reporter Lysis Buffer (Promega, Wisconsin, USA) was added and the cells left at room temperature for 15 mins with occasional rocking. Culture vessels were then stored at -70°C until analysis. Samples were thawed and protein assays were carried out to correct for cell number using a commercially available bicinchoninic acid (BCA) kit (Pierce Biotechnology, Rockford Illinois, USA). This assay relies on the principle that protein will reduce Cu^{2+} to Cu^{1+} in an alkaline environment. The Cu^{1+} ion can be detected by the reaction of bicinchoninic acid with Cu^{1+} which turns purple. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. 25µl of cell lysate was pippetted into a clear 96 well plate. 200µl of BCA working reagent was added to the sample and after incubation at 37°C for 30 mins the plate was read using a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Scciences, Boston, Massachusetts, USA). A standard curve was made up with bovine serum albumin (BSA) protein standards and Reporter Lysis Buffer, and this was used to determine protein concentration.

2.3 Promoter *in vitro* functional studies

2.3.1 Reporter gene system

The effect of polymorphic variation on the promoter activity was investigated using a reporter gene assay system. The CYP11B2 5' region (1880kb) was cloned into the pGL3 Basic vector (Promega, Wisconsin, USA) by Mrs Christine Holloway. The pGL3 Basic vector contains a modified coding region for firefly (*Photinus pyralis*) luciferase. The construct was sequenced to confirm the insert was present and orientated correctly. Site directed mutagenesis was carried out by a commercial company (Eurofins MWG Operon, Ebersberg, Germany). This resulted in one plasmid containing a T allele at position -1651 and one identical plasmid with the exception of a C allele at position -1651. In order to control for transfection efficiency, the plasmid pGL4.73 was co-transfected with the pGL3 reporter construct at a ratio of 50:1. The pGL4.73 plasmid contains a coding

region for renilla (*Renilla reniformis*) luciferase. Both plasmids were prepared in the same way as described in the following sections.

2.3.2 Transformation of competent cells

The plasmid DNA was transformed into JM109 *E.Coli* competent cells (Promega, Wisconsin, USA). Each construct was transformed alongside a positive control and a no DNA control. 1ml eppendorf tubes were pre chilled on ice and JM109 cells were thawed to 4°C. 50µl of competent cells were dispensed to the pre chilled eppendorfs before adding 50ng of plasmid DNA. This was left on ice for 10 minutes before being subjected to heat-shock treatment at 42°C for 50 seconds. The cells were returned to ice for a further 2 minutes before 900µl cold Super Optimal broth with Catabolite repression medium (SOC) was added. This was incubated for 1 hour at 37°C while shaking. 100µl of cells were plated on Luria broth with ampicillin and incubated overnight at 37°C. Single transformed colonies were used to inoculate incubations which were then shaken overnight at 37°C in 5ml Luria broth with ampicillin.

2.3.3 DNA purification

Plasmid DNA was extracted using the QIAprep Mini Prep kit (QIAGEN Ltd, West Sussex, UK). The cultured Luria broth was transferred to a 1.5ml eppendorf tube and centrifuged at maximum speed for 2 min. The resultant cell pellets were resuspended in 250µl of Buffer P1 (50mM 2-amino-2-hydroxymethyl-1,3-propanediol, pH8; 10mM EDTA, 100µg/ml RNAase A). To lyse the cells 250µl buffer P2 (200mM NaOH; 1% sodium dodecyl sulphate) was added and mixed gently by inverting. 350µl buffer P3 (3 M potassium acetate at pH5.5) was added and mixed by gently inverting the tube. The samples were centrifuged at maximum speed for 10 min using a table top centrifuge. The supernatants were transferred to a QIAprep Spin Column and centrifuged at maximum speed for 1 min. The flow through was discarded and the column washed by adding 0.5 ml buffer PE before being centrifuged at maximum speed for 1 min. This step was repeated with 0.75ml buffer PE before further centrifugation at maximum speed for 1 min. The column was then placed in a clean 1.5 ml eppendorf and the DNA eluted by adding 50µl of nuclease free water to the spin column. This was allowed to stand for 1 min prior to being centrifuged at maximum speed for 1 min. The mini preps were stored at 4°C for further use.

2.3.4 Restriction digestion

To ensure the construct was of the correct size and contained the appropriate insert, restriction analysis was performed. Plasmid DNA was digested with HinDIII restriction enzyme, which linearized the cDNA, producing a single fragment of 6740 bp and BamHI which produced 3 fragment of 2863 bp, 2594 bp and 1283 bp. 2 µl of plasmid DNA was incubated with 0.5 µl of enzyme, 2 µl of buffer, 0.2 µl of BSA and 15.3 µl of nuclease free water. The reaction mixture was incubated for 2 hours at 37°C. The construct was resolved on a 1% agarose gel. Agarose (1g) was added to 100ml TBE buffer and heated to boiling point in a 950W microwave oven for 50 seconds. 1 µl ethidium bromide was added and gently mixed. This was allowed to cool in gel moulds with Teflon combs until set. The gel and digestion products were subjected to electophoresis at 100V for 40 min.

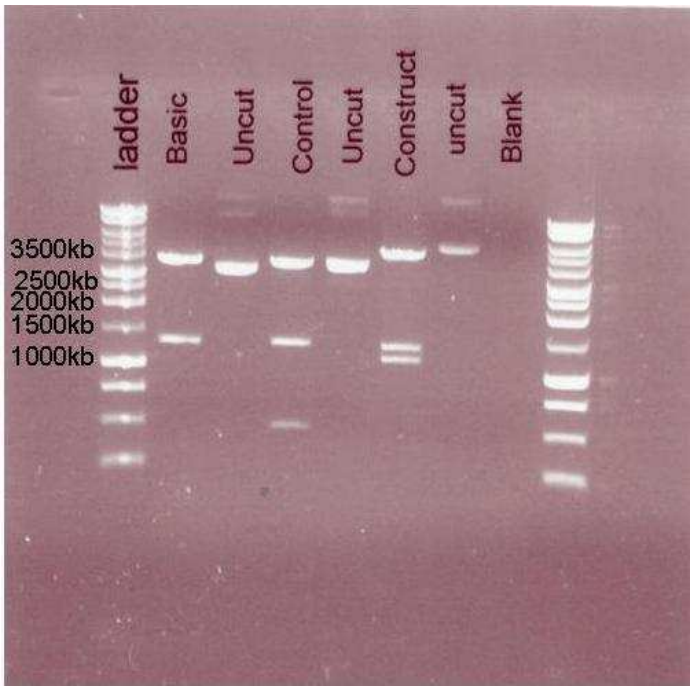


Figure 2-2 Agarose gel electrophoresis of plasmid construct and plasmid control.

Lane 1 ladder, lane 2 pGL3 basic vector following restriction enzyme digestion, lane 3 basic vector incubated with no enzyme, lane 4 pGL3 control vector following restriction enzyme digestion, lane 5 pGL3 control vector incubated with no enzyme, lane 6 pGL3 construct (CYP11B2 5' region) following restriction enzyme digestion, lane 7 pGL3 construct (CYP11B2 5' region) incubated with no enzyme, lane 8 reaction mix, no DNA, lane 9 ladder.

2.3.5 Large scale plasmid preparations

Large scale plasmid preparations were performed using a QIAgen plasmid Maxi kit. A single colony from a freshly streaked ampicillin Luria plate which had been gown overnight was picked and used to inoculate a starter culture. This was grown in 5ml Luria broth containing 100µg/ml ampicillin and shaken in an incubator at 37°C for 8hrs. 1ml of this was diluted in 100ml of Luria broth with ampicillin and incubated overnight in a shaking incubator at 37°C.

The bacterial cells were harvested by centrifugation at 6000 *x g* for 15 min at 4°C. Culture media was removed and pellets were resuspended in 10ml of buffer P1 (50mM 2-amino-2-hydroxymethyl-1,3-propanediol, pH8; 10 mM EDTA, 100 µg/ml). 10 ml of buffer P2 was added (200 mM NaOH; 1% SDS) to initiate the lysis reaction and this was inverted 4-6 times and kept at room temperature for 5 min. 4ml of chilled buffer P3 (3 M potassium acetate at pH5.5) was added to neutralise the lysate and the mixture was inverted again 4-6 times and incubated on ice for 20 min. The solution was centrifuged at 16000 *x g* for 40 min at 4°C. A QUIATIP column was equilibrated with 10 ml of buffer QBT (750 mM NaCl; 50 mM 3-morpholinopropanesulfonic acid pH7 (MOPS); 15% isopropanol; 0.15% Triton-X 100) which was allowed to empty by gravitational flow. The lysate supernatant was applied to the column and allowed to enter the resin. The column was washed twice with 30ml buffer QC (1 M NaCl; 50 mM MOPS pH7; 15% isopropanol), and the plasmid DNA was eluted with 15ml of buffer QF (1.25 M NaCl; 50 mM Tris pH8.5; 15% isopropanol) into polypropylene tubes. The DNA was precipitated with the addition of 10.5ml isopropanol and centrifuged at 15000 *x g* for 20min at 4°C. The supernatant was poured off and pellets resuspended in 5ml of 70% ethanol and aliquoted into eppendorfs. These were centrifuged at 160000 *x g* for 10 min and once the supernatant was discarded, allowed to air dry for around 1 hour and DNA dissolved in 100 µl of nuclease free water.

2.3.6 H295R transient transfection procedure

H295R cells were grown as described in section 2.2. For transfection, cells were sub-cultured onto 24 well subculture dishes at a density of $2x10^5$ cells/ well. Cells which were initially grown in 175cm flasks were counted according to the following protocol. The culture medium was removed and the cells rinsed with

phosphate buffered saline (PBS) solution. Trypsin-EDTA 0.25% (Sigma, St Louis, Missouri, USA) was added to cover the cells and after incubation for approximately 3 minutes the cells detached from the flask. Complete growth medium was added to inactivate the trypsin and the solution was spun at 1500rpm for 5 minutes. The cells formed a pellet and after removing the growth medium, were re-suspended in 1ml of fresh complete growth medium. This was diluted 1:100 and cells counted using a haematocytometer. The cells were diluted to a concentration of $4x10^5$ cells/ ml in a universal tube and placed back in the incubator until ready for use.

Cells were transfected with pGL3 basic (Promega, Madison, Wisconsin, USA) expression vectors containing 1.8kb of the CYP11B2 promoter and plasmid with T allele at position -1651) and C allele at -1651 (mutated by site directed mutagenesis) were compared. pGL4.73 (Promega, Madison, Wisconsin, USA) was co-transfected to control for transfection efficiency. Transfections were carried out using siPORT™ NeoFX™ Transfection Agent (Applied Biosystems, Foster City, California, USA) according to the manufacturers protocols using the reverse transfection method. The protocol was as follows. A solution of containing 1.5 µl of NeoFX™ and 48.5 µl Opti-Mem reduced serum media (Invitrogen, Paisley, Scotland) per well was made and allowed to equilibrate for 10min. Solutions containing 1000ng of pGL3 plus insert plasmid, 20ng pGL4.73 reporter plasmid (50:1 ratio) made up to a total volume of 50 µl per well was made and allowed to equlibrate for 10 min. The Opti-Mem/ NeoFX™ solution and the DNA/ Opti-Mem solution were then combined (50 µl of each per well) and this was allowed to equilibriate for 10min. 100 µl of this soulition was then dispensed to each well and once the cells had been briefly resuspended with pippetting, 400 µl of cell solution was added.

The plate was tipped gently to ensure even coverage. Positive controls were included and these consisted of either control pGL3 plasmid alone (containing the luciferase gene and a viral promoter) or pGL4.73 plasmid alone (containing the renilla gene and a viral promoter). Negative controls were included and consisted of a 50:1 ratio of pGL3 basic vector (containing the luciferase gene but no promoter) and pGL4.73 plasmid. Also, wells with no DNA transfected were included.

The cells were incubated at 37°C and after 24 hours the transfectant was removed and replaced with normal media. Transfection efficiency was not formally measured (but was controlled for by co-transfection with renilla plasmid). However, when green fluorescent protein plasmid was transfected, the efficiency was noted to be low (approximately 50%). When cells were stimulated, the concentrations of trophins used were as follows: angiotensin II ($1x10^{-7}$M), dibutyryl cAMP (1mM) or potassium (22mM). APEX 1 inhibitor was added after 24 hours for the relevant experiments at a concentration of 10μM or vehicle.

After a further 24 hours, the cells were lysed with 100μl passive lysis buffer, before the addition of Dual-Glo assay reagents (Promega, Madison, Wisconsin, USA).

2.3.7 Luciferase assay

Luciferase measurements were carried out using the DUAL luciferase assay (Promega, Madison, Wisconsin, USA). Buffers were allowed to thaw slowly to room temperature. LAR II was reconstituted by adding 10ml of buffer to the substrate.

20 μl of lysate was dispensed into the bottom of a 5ml round bottom polypropylene tube (75 x 12mm) (Sarstedt, Leicester, UK). After priming the tubing of the luminometer (Lumat LB 9507, Berthold Technologies, Herts, UK) with the relevant reagents, the reactions were commenced. The luminometer was programmed to inject 100 μl of LAR II into the tube (firefly measurement) followed by 100 μl of Stop and Glo reagent (renilla measurement) and perform a 2 second pre-measurement delay, followed by a 10-second measurement period for each reporter assay. On completion the tube was discarded and moved on to the next assay. Finally, the primed lines were purged and cleaned with 70% ethanol and distilled water. The ratio of firefly to renilla luciferase was used for analysis as this allowed the variation in transfection efficiency to be controlled for.

2.4 Protein:DNA binding studies

2.4.1 H295R nuclear protein extraction

Nuclear extracts from human adrenal carcinoma cell line (H295R) were isolated using a modified method based on Dignam et al (Dignam et al., 1983).Nuclear protein was extracted under varying salt conditions in the presence of sodium orthovanadate (a tyrosine phosphatase inhibitor). Cells were cultured as described in section 2.2, trypsinised, pelleted by centrifugation at 400g. The pellet was washed with PBS and spun down again before the supernatant was aspirated and discarded. The cells were lysed by the addition of 0.4 ml per tube of Buffer A (10 mM HEPES, pH 7.9, 10 mM potassium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.625% (v/v) Nonidet P-40, 0.5 mM PMSF). The samples were then centrifuged at 48,000g for 30 s at 4°C, and the supernatant was removed. The pellet was resuspended in 50 µl of buffer B (20 mM HEPES, pH 7.9, 100mM – 500 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) Samples were agitated for 15 min at 4°C, centrifuged at 48,000g for 5 min, and the protein content of the supernatant was determined using a Bradford assay.

2.4.2 Protein quantification; Bradford assay

The Bradford Reagent can be used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present. The Bradford Reagent is compatible with reducing agents, in this case, DTT. Other protein assay procedures (e.g. Lowry and BCA) are not compatible with reducing agents. A standard curve was prepared using BSA diluted in Buffer B as follows:

Final concentration	Vol of Dilutant µl (Buffer B)	Volume of BSA µl (2mg/ml)
0	10	0
1	9.5	0.5
2	9.0	1.0
5	7.5	2.5
10	5.0	5.0
15	2.5	7.5
0	10	0

3 µl of sample was added to each tube. 500 µl of water was then added along with 500 µl of Bradford reagent. Absorbance was measured at 595 nm on a spectrophotometer. Samples were diluted to a final concentration of 2 µg/ µl.

2.4.3 Oligonucleotide probes

Oligonucleotides were commercially obtained (Eurofins MWG Operon, Ebersberg, Germany). The sequences are as follows: 5' GGA CGA GAC TAG CCT GGC CAA C 3' which was complementary to 5' GTT GGC CAG GCT AGT CTC GAA CTC C 3', and 5' GGA CGA GAC CAG CCT GGC CAA C 3' which was complementary to 5' GTT GGC CAG GCT GGT CTC GAA CTC C 3'. Oligonucleotides were diluted to a concentration of 10pM and end labelled with γ32P ATP. Oligos were incubated at 37°C for 30 mins with 2ul buffer (70mM Tris-HCl pH 7.6, 10mM MgCl2, 5mM DTT), 2µl oligo, 1 µl γ32P ATP (MP Biomedicals, Illkirch, France) and 1 µl T4 Polynucleotide kinase (Promega, Madison, Wisconsin, USA) and 14 µl Nuclease free water.

Complementary oligonucleotides were annealed by incubating together in 1 ml eppendorf suspended in a water bath heated to boiling temperature and gradually allowed to cool. Labelled oligonucleotides were separated from free radioactivity on an Illustra probe quant G-50 micro column (GE Healthcare, Amersham, UK) according to the manufacturer's instructions.

2.4.4 Electromobility shift assay

DNA: protein binding reactions were carried out in a final volume of 20 µl as follows:

Reagent	Volume
Binding Buffer (HEPES pH 7.9 20mM, MgCl2 10mM, EDTA 0.5mM, NaCl 20mM, 50% glycerol)	5.4 µl
Bovine serum albumin (0.001g/ml)	1 µl
Dithiothreitol	100mM
PolyDI·DC (0.01g/10ml)	1 µl
Radiolabelled probe	1 µl
NF water	6.6 µl
Nuclear protein	5 µl

Nuclear extract was allowed to incubate with the reaction buffer for 20 minutes at room temperature before the addition of radio labelled probe, then for a further 20 minutes also at room temperature. The protein: DNA complex was loaded onto a 6% resolving acrylamide gel. (10ml 30% Acrylamide mix (Sigma, St Louis, USA), 5ml 10x TBE buffer, 35ml dH_2O; following degassing for 3 mins, 500 µl 25% ammonium persulphate and 10 µl TEMED were also added). Loading buffer consisted of 500 µl TE buffer, 500 µl binding buffer, 10% glycerol, Bromophenol blue. The gel was run at 100mV for 3 hours (Hoefer SE600 Ruby, GE Healthcare, Amersham, UK) in Tris-borate EDTA buffer.

The gel was washed twice in 5% ascetic acid and then once in 10% glycerol before being dryed on a gel dryer (Model 583 Gel Dryer, Bio-Rad, CA, USA) for 2 hours. The gel was visualised after autoradiography for 4 days at -80°C.

2.5 In vivo studies in normal volunteers

2.5.1 Recruitment of normal volunteers

The "Role of Genetic Variation in aldosterone synthase- physiological studies" (Adrenal Function Study, AFS) was submitted for ethical review to the West Glasgow Ethics Committee in June 2007 and received ethical approval on the 3rd July 2007 (REC ref no 07/S0703/70).

Subjects were recruited from a number of sources including advertisements and promotional articles in the local media, the University website and alumni association and via advertisements from around the University campus and local area. Initial screening visits were carried out by the investigator and the exclusion criteria are listed in Table 2-1.

Exclusion criteria
Age<18 or >70 at time of recruitment
Resting blood pressure >145/90
Use of any antihypertensive medication
Pregnant or plans to become so in 6 months following participation in study
Use of oral, inhaled or topical steroids in 6 months preceding study
History of severe atopy or asthma
Known renal or cardiac dysfunction
Inability to comply with study instructions

Table 2-1 Exclusion criteria for study examining the effects of variation in the promoter region of aldosterone synthase on corticosteroid production

Study visits took place in the Glasgow Clinical research facility, tenant Memorial building, Church St, Glasgow. The investigator was present at each visit which was undertaken with the help of the Nursing staff in the Clinical research facility (lead study nurse Sister B McLaren).

2.5.2 Study protocol

Subjects were studied during careful dietary sodium control to maintain either a high (200 mmol per day) or low (80 mmol per day) sodium intake. A diet sheet was provided which gave advice on a low salt diet (approx 4.8g per day). Salt loading using sodium supplement (HK Pharma, Bangkok, Thailand) tablets containing 600mg sodium chloride, achieved a total intake of 200 mmol per day (approx 12g) (four tabs 3 times per day). Placebo tablets (Western Infirmary Pharmacy Production Unit, Glasgow, UK) were prescribed for the duration of the low salt week to ensure the subjects and investigators were blinded to the salt status.

Once in balance (after 3 days) subjects were infused with ACTH (1ng/kg/minute for 30 mins); on the following day subjects were given an infusion of angiotensin II (3 ng/kg/minute for 30 mins) (BAChem, Weil am Rhein, Germany). Infusions were prepared by the Western Infirmary Pharmacy Production Unit. Basal, stimulated and 30 minutes post stimulation plasma samples were collected, for renin (baseline) and steroid measurements. Blood pressure was monitored at baseline, after 30 minutes recumbent rest and at 10 minute intervals for the duration of the infusion. A final blood pressure recording was made at the end of the study visit after a final 30 minutes. Blood pressure was recorded using a Mindray VS 800 monitor. In addition, 24 hr urine collections were undertaken and were analysed for steroid measurements by GC:MS. Urine samples were measured for sodium concentration which allowed and estimate of total sodium excretion in 24hrs.

Blood samples were drawn using the Vacutainer system (BD, Oxford Science Park, Oxford UK) at baseline blood tests before the infusion (x2 Lith Heparin and x 1 EDTA), at completion of the infusion, as well as 30 mins after completion (x2 Lith Heparin and x 1 EDTA).Lithium heparin samples were stored on ice and spun down at 4°C and EDTA samples were stored at room temperature and spun down at 25°C for 15 mins at 3000*g*. Plasma was aspirated and stored immediately at -80°C.

The patient information sheet, study protocol and data collection sheet are included in Appendix 7.4, 7.5 and 7.6

2.5.3 Urinary electrolyte measurements

Aliquots of urine were measured in the NHS biochemistry laboratory Gartnavel Hospital for sodium concentration.

2.5.4 Urinary steroid measurements

24 hour urine samples were collected in plain containers and the volume measured before being aliquoted and stored at -20°C without preservatives. Steroid metabolites were measured by Miss Mary Ingram by gas chromatography using the method of Shackleton et al(Shackleton, 1993).

2.5.5 Plasma steroid measurements

Plasma steroids were measured by Miss Mary Ingram. Samples were extracted from 3ml plasma using Chem Elute cartridges (Varian, CA, USA) and eluted with dichloromethane. The eluates were evaporated to dryness under nitrogen and reconstituted in 60ul acetylonitrile. Aliquots (20ul) were injected into a Polaris 5 micron, 150mm x2mm C-18-A reversed phase HPLC column. Identification and quantification were accomplished by tandem mass spectrometry using a Varian 1200L mass spectrometer with a triple quadropole detector. The internal standard was 16ßmethylprednisolone.

2.5.6 Plasma renin measurements

Plasma renin concentration was measured by the Department of Clinical Biochemistry Glasgow Royal Infirmary by means of a Diasorin Liaison® immunochemiluminometric analyser (DiaSorin Ltd, Wokingham, Berkshire, UK)(Dorrian et al., 2010).

2.5.7 DNA extraction and Genotyping

DNA was extracted using an automated method (Gentra System Autopure LS, Large Sample Nucleic Acid Purification Automated DNA extraction, Quiagen, West Sussex, UK) according to the manufacturers instructions by Ms C Brock.

Genotyping of normal volunteer DNA was carried out by Mrs Elaine Friel and subjects were genotyped were determined for the seven polymorphisms in the promoter region of CYP11B2 as well as the presence of the intron 2 conversion and the polymorphisms in the promoter region of CYP11B1 as described in section 2.1.

2.5.8 Data analysis

Data was collected and stored using Microsoft Access and statistical analysis was carried out using Minitab V 12.21. Supplementary graphs were produced using GraphPad Prism V4 and Microsoft Excel.

Characterisation of the CYP11B2 promoter region

3. Characterisation of the CYP11B2 promoter region

3.1 Introduction

Studies linking variation of the promoter region of the aldosterone synthase gene and hypertension have mainly focused on the C/T polymorphism at position -344, with some groups reporting an association with hypertension and a low ARR and the T allele (Davies et al., 1999; Brand et al., 1998; Freel et al., 2007), but other groups have found the association to be with the C allele (Matsubara et al., 2004). A meta-analysis of the literature published up until 2006 concluded that individuals homozygous for the C allele have a 17% lower risk of hypertension (Sookoian et al., 2007). As discussed in the introduction to this work (section 1.5), some of the controversy in the literature may have arisen from the variation in composition of the populations studied. Although smaller numbers of African and Asian subjects have been studied, the association of hypertension with the T allele is less robust in these populations (Sookoian et al., 2007) and it is clear that the allele frequencies vary substantially between ethnic groups. There is strong linkage disequilibrium across the CYP11B2 locus in the Caucasian population (Alvarez-Madrazo S et al., 2009; Barr et al., 2007) however, there is significantly less linkage disequilibrium in the CYP11B2 locus amongst people of African decent (Alvarez-Madrazo S et al., 2009) presumably due to greater degree of recombination in this population and a loss of genetic variation in the Caucasian population due to bottlenecks during migration out of Africa.

In vitro studies have demonstrated that the T allele binds to the transcription factor steroidogenic factor 1 (SF-1, NR5A1) with 4-5 fold greater affinity than the C allele. However, deletion of this site does not alter transcriptional activity suggesting it has no functional effect (Clyne et al., 1997). Given the high degree of linkage disequilibrium exists across this locus (Barr et al., 2007), it has been hypothesised that this polymorphism may be in linkage with alternative, functional polymorphisms. It is known that when a disease causing allele enters the gene pool it is often surrounded by alleles that may have no functional effect. Over time, with recombination, the link between the disease causing allele and surrounding neutral alleles becomes less strong (Conrad et al., 2006;

Barnes, 2006). It may be hypothesised that this is the phenomenon that is observed within the CYP11B2 locus; a disease-causing polymorphism in linkage disequilibrium with the -344C/T has entered the gene pool. In the African population, recombination has reduced the linkage disequilibrium but in the Caucasian population it functions as a "tag" SNP for the disease-causing allele elsewhere in the locus.

Previous studies have identified seven common polymorphisms in the promoter region of the CYP11B2 gene each with a frequency of >1% in the population (Barr et al., 2007). These SNPs were identified following sequencing of twenty six normotensive individuals, selected from the WHO MONICA (multinational MONItoring of trends and determinants in CArdiovascular disease) and stratified according to genotype at -344 and intron conversion. None of these seven novel polymorphisms are in areas of the promoter region that have been previously been shown to be involved in regulation of the CYP11B2 gene. However, previously published work has predominantly focused on an area more proximal to the transcriptional start site and none have directly examined these sites of variation (Bassett et al., 2004b). In addition, more than one polymorphism may alter promoter activity and such variants may promote or inhibit activity. Classical promoter deletion studies may have obscured any differentially regulating effects by removing more than one polymorphic site at a time (Clyne et al., 1997). For these reasons the polymorphic variations in the promoter region deserve further analysis.

Characterising the pattern of inheritance is an important first step, as it is important to ascertain the linkage disequilibrium between the disease-associated polymorphism at -344 and any possible causative polymorphisms. Although publicly available repositories of genotyping information e.g. HapMap (2005) (http://hapmap.gov), and latterly the 1000 genome project (Via et al., 2010) (http://www.1000genomes.org) are available, the pattern of linkage disequilibrium in this region was not described in sufficient detail. Therefore in order to further investigate the linkage between SNPs in the promoter region of the CYP11B2 region in a Caucasian population, a detailed examination of the CYP11B2 promoter region was undertaken in the following study.

3.2 Aims

To sequence seven single nucleotide polymorphisms in the promoter region of CYP11B2 in 300 subjects from the Oxford hypertension study cohort, in order to describe the allele frequencies and the pattern of linkage disequilibrium.

3.3 Methods

3.3.1 Subjects

Three hundred DNA samples selected at random from the Oxford family study were analysed. Probands and their families were recruited in the Oxford region of the UK from 1993-1997. Patients were screened for secondary causes of hypertension according to local practice. Families consisted of at least three siblings (including the proband) and at least one parent, but at least four siblings (including the proband) were required if no parent was available for DNA analysis. DNA was extracted according to standard methods (Keavney et al., 1998).

3.3.2 Genotyping

The CYP11B2 gene from approximately 2kb upstream of intron one to the beginning of intron one was amplified by PCR as described in section 2.1.1. PCR products were identified by resolving on a 1% agarose gel (Figure 3-1)

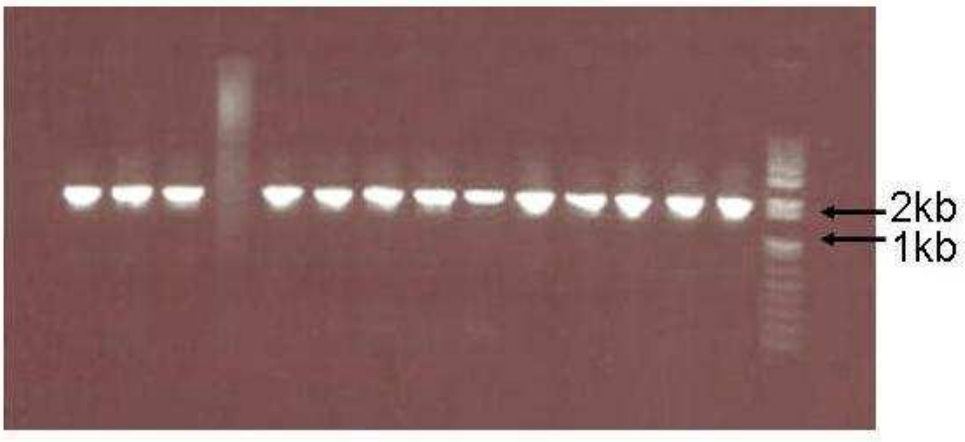


Figure 3-1 PCR product of CYP11B2 promoter region (2000bp) length

The product was resolved on 1% agarose gel. 1kb ladder is shown and Lane 4 is negative control.

Following PCR, three separate sequencing reactions were carried out and the seven polymorphisms identified as described in section 2.1.2. The resultant sequences were analysed using SeqScape V2.2 software (Applied Biosystems, Foster City, CA, USA) which displayed electropherograms of the sequencing sample aligned to a reference sequence (Figure 3-2). The reference sequence is included in Appendix 7.2.

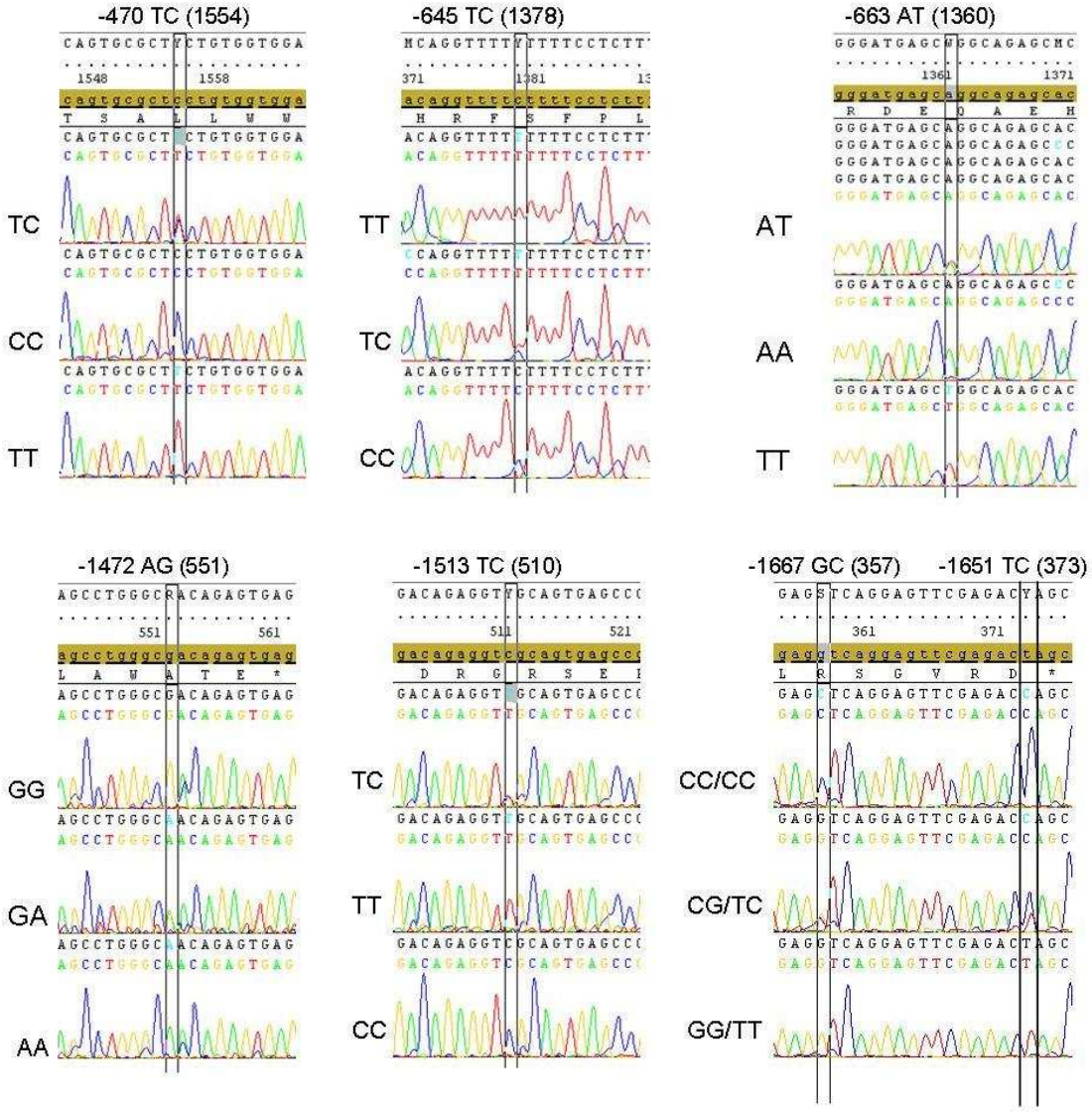


Figure 3-2 Electropherogram of polymorphisms

Variation at position -470T/C, -645T/C, -663T/C, 1472A/G, -1513T/C, -1667G/C, upstream of CYP11B2 transcriptional start site are shown.

3.3.3 Statistical Analysis

Data were collated on an Excel spreadsheet before being imported to Haploview v. 4.2. This software was used to analyse genotype frequencies and calculate Hardy Weinberg equilibrium as well as interpret and visualise haplotype structure (Barrett et al., 2005; Barnes, 2006).

3.4 Results

3.4.1 CYP11B2 promoter polymorphisms

The 5' promoter region was amplified as described to produce a 2kb fragment shown in Figure 3-1. Following direct sequencing, electropherograms were constructed to identify alleles at the sites of polymorphic variation. The 280 DNA samples sequenced from the Oxford cohort were from 37 families. The data were analysed using Haploview 4.2 software.

Characteristics of the polymorphisms are shown in Table 3-1. All polymorphisms were in Hardy-Weinberg equilibrium using a p value cut off of 0.001. As the percentage of samples genotyped for the rs62524561 (-1513 T/C) and rs62524560 (-1472 A/G) was below 80%, they were excluded from further analysis.

SNP number	Location (5' end)	SNP (rs number)	Alleles	MAF	Percentage genotyping	Observed Heterozygocity	Hardy Weinberg p-value
1	-1667	rs13254375	G/C	0.421	82.6	0.463	0.7409
2	-1651	rs13268025	T/C	0.442	88.1	0.481	0.9112
3	-1513	rs62524561	T/C	0.377	67.2	0.457	0.9455
4	-1472	rs62524560	A/G	0.364	59.7	0.455	1
5	-663	rs28659182	T/A	0.439	98	0.472	0.7363
6	-645	rs11781082	C/T	0.266	98.8	0.468	0.0455
7	470	rs10087214	T/C	0.432	99.2	0.48	0.9133
8	-344	rs1799998	C/T	0.432	99.6	0.48	0.9133

Figure 3-3 Characteristics of SNPs genotyped in the promoter region of CYP11B2

Patterns of linkage disequilibrium (LD) and haplotype structure were assessed for 6 SNPs. The LD plot for the CYP11B2 promoter region for the Oxford cohort is shown in Figure 3-4 where *D'* values are given. In Figure 3-5 the r^2 values for the

population are shown. It can be seen from both measurements that there is high LD across the region. From these data, three haplotypes were constructed and are shown in Figure 3-6. These three haplotypes account for 95.6% of the population. These data have been combined with sequencing results from across the CYP11B1 and CYP11B2 locus and the entire pattern of linkage disequilibrium across this locus is included in Appendix 7.2.

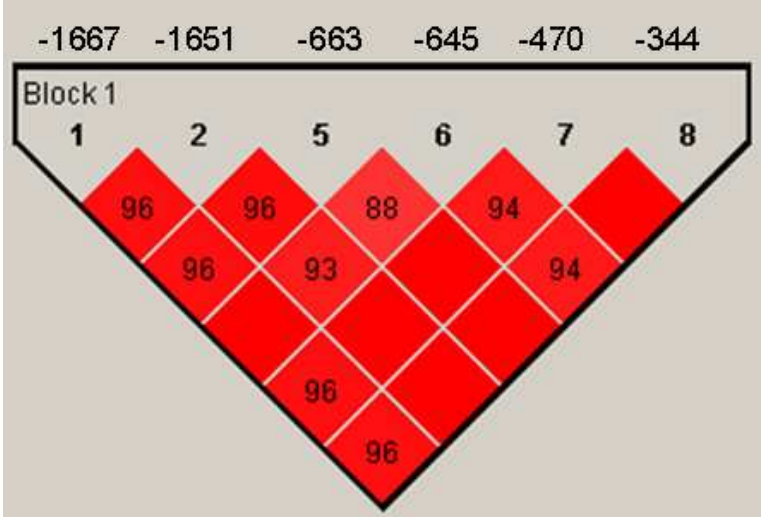


Figure 3-4 Linkage disequilibrium plot of CYP11B2 promoter polymorphisms *D'* values

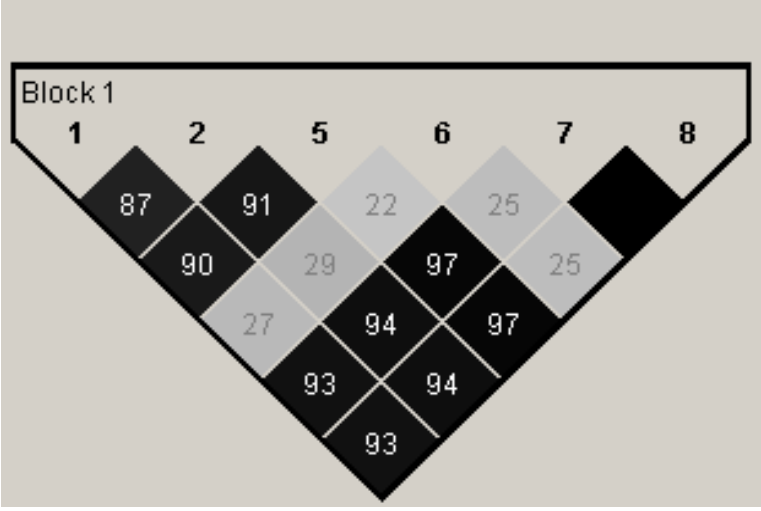


Figure 3-5 Linkage disequilibrium plot of CYP11B2 promoter polymorphisms r^2 values

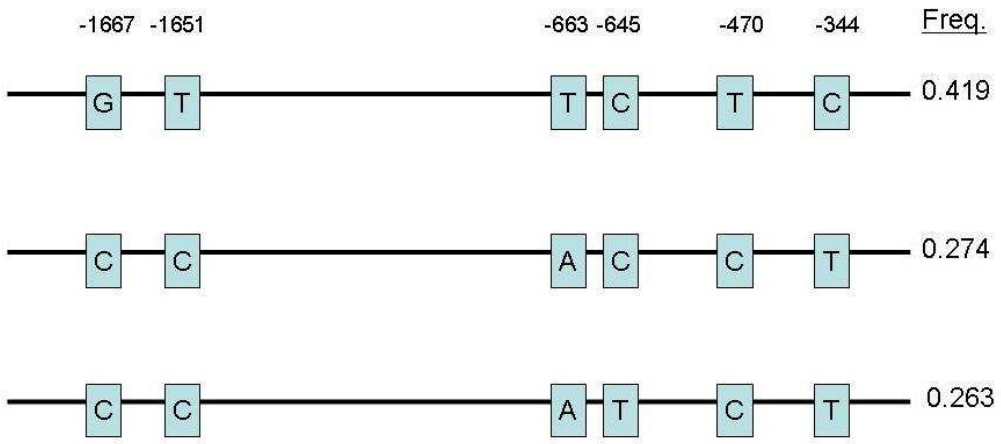


Figure 3-6 Three common haplotypes and their frequencies for CYP11B2 promoter polymorphisms representing 95.6% of the study population.

3.5 Discussion

Linkage disequilibrium (LD) is an extremely useful concept in the area of analysis of single nucleotide polymorphisms and statistical genetics. LD refers to the association between alleles when their frequencies are significantly different from their predicted individual allele frequencies if they were to occur together (in contrast to "linkage" which refers to the tendency of markers, genes or DNA sequences at a specific locus to be inherited together due to their proximity on a single chromosome). This concept allows certain observations to be made, because the linkage disequilibrium between alleles declines over generations with increasing recombination and mutation events. Thus, it can be used to map population evolution as well as a tool for genetic mapping of particular diseases and reduces the amount of genotyping required for association studies.

The statistical concepts *D'* or r^2 are measurements of linkage disequilibrium and both are displayed in the LD plots for the promoter region of CYP11B2 (Figure 3-4, Figure 3-5). For further discussion, some explanation of these values is necessary. The value *D* describes the deviation of haplotype frequencies from the equilibrium. *D* is calculated thus: If a haplotype formed by two SNPs with alleles (A,a) and (B,b) there would be four possible haplotypes: AB, Ab, aB and ab. The frequency of each haplotype can be calculated by the product of the frequencies of the alleles $f(A)f(B)$, where $f(A)$ is the frequency of allele A at the first locus and $f(B)$ is the frequency of allele B at the second locus and the

frequency of haplotype AB is *f*(AB). From this *D* can be calculated using the following simple equation:

$$D = f(AB) - f(A)f(B)$$

LD can be said to be occurring when the value of *D* is significantly greater than zero. The value of *D* is important because both *D'* and $r^2$are calculated from *D*.

D' is the absolute ratio of D compared with its maximum value, D_{max} (D_{max} = f(A)f(B)). Complete equilibrium would mean a D' of 1. D' is a useful concept for the assessment of historical recombination in a population as in the absence of any recombination, there would be no deviation from linkage equilibrium. Therefore statistically significant values of D' that are near 1 provide a useful indication of minimal historical recombination, but intermediate values are not useful either for comparison of the strength of LD between studies or to measure the extent of LD. In terms of the data presented here it can be seen that in the population studied, D' values were high in the CYP11B2 promoter region, suggesting little historical recombination in this locus. Given that this is the case, it is unlikely that the two SNPs that were not genotyped successfully would demonstrate LD that deviates significantly from the rest of this locus.

In contrast, r^2 is a measure of correlation between two alleles, i.e. how much does one allele predict the other; thus when r^2=1, the genotypes of alleles of one SNP will perfectly predict the genotypes of another SNP.

The r^2 value is more useful in the context of association studies and can provide an estimate of the sample size required to provide the power to detect an association between a causal SNP and a disease (calculated as the inverse of the r^2 value). For example, the region of the promoter with less strong LD is the polymorphism at position -645. The r^2 value for this polymorphism and -344 is 0.25 which means that if only one were to be genotyped in an association study, the sample size would have to increase by a factor of 4 to maintain the same power (Wang et al., 2005).

It is useful to remember that D' and r^2 are both derived from D and in fact, r^2 describes the upper limit of D'^2 and the maximum potential level of useful LD for the purposes of association mapping.

This current study was undertaken to map polymorphisms in the promoter region of the CYP11B2 gene and assess the LD of the novel SNPs in the promoter region with the extensively studied polymorphism at position -344. This region has been implicated in previous case control/ association studies which have essentially used the polymorphism at position -344 as a "tag" SNP. The CYP11B2 gene is highly homologous to the CYP11B1 gene, making sequencing across this region difficult. Given the high linkage disequilibrium elsewhere in this locus (see Appendix 7.7.) and the close geographical proximity of the SNPs in the promoter region seemed likely that they were in LD with the SNP at -344 but the pattern of LD and the haplotype blocks remained to be established. From these results, it can be seen from both the *D'* and r^2 values that there is a high degree of linkage disequilibrium across the promoter region. As a consequence, three haplotypes account for greater than 95% of the population and, by genotyping only one SNP, the pattern of inheritance of the remaining SNPS can be predicted with reasonable accuracy. This has certain advantages in an association study as it reduces the amount of genotyping required; however, it does not provide any evidence of which SNP in the haplotype is driving the phenotype. The high degree of LD in the promoter region as well as across the entire CYP11B1 and CYP11B2 locus make it impossible to identify causative SNPs at this stage. Ongoing work within our group is investigating this issue in large population cohorts but, the 5'promoter region remains a likely region within the locus for a functional polymorphism to be found. Studies of the functional effect of polymorphisms within the CYP11B1 promoter region have demonstrated altered transcriptional activity and this is a potentially interesting area to study in more depth in the CYP11B2 gene in both in vivo and in vitro investigations.

3.6 Conclusions

This study confirms a low degree of historical recombination in this locus in a Caucasian population and high degree of linkage disequilibrium between all SNPs in the promoter region of CYP11B2. Although data from large genotyping studies which have examined polymorphisms within this locus can be extrapolated to

variation in the promoter region by imputation, this provides no information regarding selecting polymorphisms for further functional work.

The high degree of linkage disequilibrium also means that there is a small number of common haplotypes that make up the majority of the genetic variation at this locus in the Caucasian population, and this has implications for recruitment of normal subjects for dynamic studies as discussed in Chapter 6.

Characterisation of the H295R cell line

4. Characterisation of the H295R cell line

4.1 Introduction

The previous chapter described in detail common polymorphisms in the promoter region of *CYP11B2* and their pattern of linkage disequilibrium in a Caucasian population. In order to assess *CYP11B2* promoter function and examine DNA: protein interactions, and assess any functional effects of these polymorphisms, an appropriate model system of adrenocortical function is necessary. A number of different model systems have been used in previous investigation of steroidogenesis, but all have shortcomings. These shortcomings are an important consideration and have limited studies of many aspects of adrenocortical function.

Normal human adrenal tissue is rarely available for primary cell culture in laboratory studies and while recent progress has been made regarding generating differentiated steroidogenic cells from mesenchymal (MSC) and embryonic stem cells (ESC), this technique has not yet developed sufficiently to be widely used. Indeed, while treatment of MSC and ESC with steroidogenic factor 1 (SF-1) and other transcription factors have been reported to induce an adrenocortical phenotype, the pattern of steroidogenic enzyme expression is more consistent with adrenal fasciculata cells (Miyamoto et al., 2011) and further work is required before the expression and functionality of aldosterone synthase in induced stem cells can be confirmed.

An alternative approach is to use animal models. However, they can differ anatomically and physiologically from their human counterparts, for example, bovine adrenal cells have been an attractive system to study as fresh tissue is readily available and the size of the gland is suitable for most practical purposes. However, the bovine genome contains only *CYP11B1* and, although there are multiple forms of this gene present (Kirita et al., 1990), the gene product catalyses both the conversion of 11-deoxycortisol to cortisol and DOC to aldosterone (Morohashi et al., 1990). Rats have four *CYP11B* genes (Mukai et al., 1993) with *CYP11B1* and *CYP11B2* functioning in a similar way to those of humans. *CYP11B3* is only expressed *in utero* and in the early postnatal period (Mellon et al., 1995). The *CYP11B4* gene appears to be non-functional and is

referred to as a pseudo-gene. Rodent steroidogenesis differs further from human steroidogenesis because it lacks 17α-hydroxylase so that the predominant glucocorticoid generated by *CYP11B1* is corticosterone rather than cortisol. Despite these important interspecies differences, both rodent primary cell cultures as well as immortalized cell lines have provided valuable insights into the understanding of steroidogenesis (Rainey et al., 2004). The Y1 cell line was developed from an irradiated mouse and, after repeated sub-culturing, a clone with steroidogenic properties that displayed responsiveness to ACTH was obtained (Yasumura et al., 1966). The murine genome is similar to that of humans, in that it expresses *CYP11B1* and *CYP11B2*. However like the rat, it lack the capacity of 17-hydroxylation and does not produce cortisol as its main glucocorticoid and instead, the main product of *CYP11B1* is corticosterone.

Clearly, given this interspecies variation, a human adrenal cell line would be an advantage. To this end, the H295 cell line was developed from a human adrenocortical carcinoma (Gazdar et al., 1990). It synthesises a range of steroid hormones; over thirty steroids have been identified in its secretion including the principal corticosteroids. However, over one third of its steroid output is adrenal androgens. This is much higher than would be expected in normal tissue, but is consistent with the clinical pathology of the donor patient. The cells respond to the zona glomerulosa trophins, angiotensin II and potassium, although ACTH-dependent function must be tested with dibutyryl cAMP (Bu_2cAMP) as expression of ACTH receptors is negligible (Rainey et al., 1994). This cell line grows in suspension and has a phenotype of loosely adherent clumps. A number of H295 subtypes have been developed by different investigators with the aim of cultivating adherent cells or of favouring production of particular steroids by selectively growing in specific medium. One such subtype that has been widely used to study many aspects of adrenocortical function is the H295R strain. It grows in a monolayer and the ACTH dependant pathway responds well to cAMP analogues. However, aldosterone production is less responsive than in the original H295 cells. This sub-strain (H295R Strain 1 ATCC CRL-2128) was deposited in a publically available repository (American Type Culture Collection, available in the UK via LGC Standards, Middlesex) by Prof W Rainey; his group have also developed alternative sub-strains (Strain 2 and Strain 3) with greater aldosterone responses to agonists. Strain 2 responds to angiotensin II and

potassium but is cultured in a serum substitute that is difficult to obtain, leading to the development of a third strain. Strain 3 grows in a more easily available commercial calf serum and maintains its response to aldosterone agonists, although this decreases over time in culture (personal communication, Prof W Rainey, Medical College Georgia, Augusta, Georgia). The H295R cell line has made important contributions to the understanding the second messenger systems operating in the gland and the complex pattern of transcription factors that governs gene expression (Bird et al., 1993). It has also simplified the assessment of the effects of a wide range of xenobiotics (Muller-Vieira et al., 2005). However, to use these cells for quantitative studies of the dynamics of corticosteroidogenesis may present problems given suggestions that the potency of the cells and the profile of the secretion change over time and varies according to medium composition and culture conditions. As the H295R cells have a relatively long doubling time and must be cultured for an extended time in order to generate sufficient numbers for experimental purposes, this may be a significant cause of variation between *in vitro* studies. In the following experiments, the steroid profile of the Strain 2 H295R cells was assessed and changes in the profile of C21 steroid production over time were followed. These were examined in basal culture and during stimulation with a number of trophins. These changes were compared with concurrent variation in the level of expression of steroidogenic genes. For comparison, the profile and effects of trophins on plasma steroid concentrations were followed in healthy human volunteers.

4.2 Aims

To assess the H295R (strain 2) cell line as a paradigm for human steroidogenesis by:

1. Confirming the capacity of cells in culture to produce mineralocorticoid and glucocorticoid at basal levels and comparing the corticosteroid profile of the *in vitro* cell line model to *in vivo* steroid production

2. Confirming the capacity of the cells to respond to trophins of aldosterone and cortisol synthesis.

3. Quantifying the variability of these parameters over time in culture, in order to assess the stability of the cell line as a model of corticosteroid synthesis for future experiments.

4.3 Methods

Strain 2 H295R cells (gift from Prof W Rainey, Medical College Georgia, Augusta, Georgia) were sub-cultured in 100mm culture vessels as described in Methods Chapter 2.2. Initial assessment of H295R cell phenotype (steroid production and mRNA expression of steroidogenic enzymes at basal levels and in response to trophins) was made with cells at passage 10. This was compared to cells that had been passaged a further 20 times to assess the effect of time in culture. Cells were cultured for 24 hours in serum free media before being stimulated at both these time points with angiotensin II ($1x10^{-7}$M), Bu_2cAMP (1mM) and potassium (22mM). For comparison, the plasma measurements of 6 normal volunteers selected at random from the cohort described in Chapter 6 are shown. Coefficient of variation and limits of detection for the LC:MS method are shown in Appendix 8.4.

Steroid concentrations were measured in media after incubation with cells for 24 hours. Measurements were made by LC:MS after solid phase extraction as described in Chapter 2. Steroid measurements were obtained from 4 samples under each condition and total amount of steroid was normalised to total protein content to account for variation in cell number.

RNA was obtained from 3 samples under each condition. Messenger RNA (mRNA) was first reverse transcribed to produce complementary DNA (cDNA) before quantifying with real time PCR (qRT-PCR) to measure the relative amounts. An oligonucleotide probe with a fluorescent reporter dye and quencher moiety attached, binds to the amplicon created by the PCR reaction. When the Taq polymerase replicates the template (amplicon bound to probe), the quencher moiety is cleaved off the probe, allowing the fluorescence to be detected. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. The crossing point (C_t) is the PCR cycle number at which a sample reaches the threshold level of fluorescence. The C_t is inversely proportional to the amount of target material in any given sample: the lower the C_t, the higher

the amount of target material within a sample (Figure 4-1). Each sample was analysed in technical triplicate.

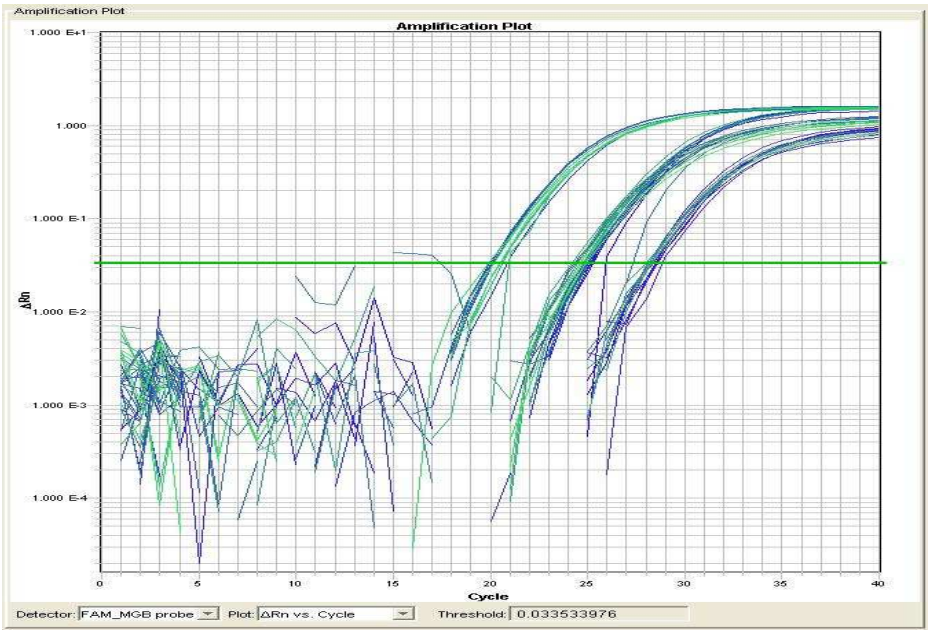


Figure 4-1 Example of amplification plot showing serial dilutions of pCMVB2 plasmid

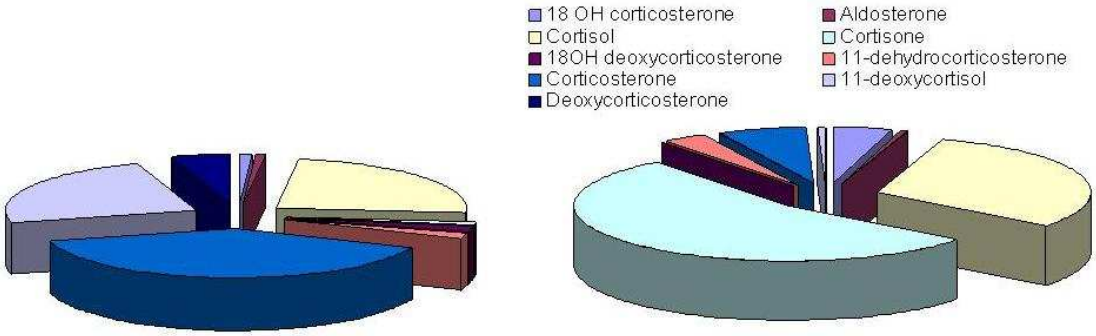
The qRT-PCR data are presented as change in mRNA level relative to a reference gene and normalized to a housekeeping gene ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). Given the high degree of homology between the *CYP11B1* and *CYP11B2* genes, optimisation was required to ensure that no cross reactivity of the assays was present. Plasmids containing *CYP11B1* and *CYP11B2* were a gift from Dr P. C. White (University of Texas, Southwestern Medical Centre). Serial dilutions were made spanning a 10^5 range and assessment of the assay efficiency was made. In addition, *CYP11B1* samples were spiked with *CYP11B2* plasmid and vice versa to assess any cross reactivity of the assays.

4.4 Results

4.4.1 Steroid production and enzyme expression the H295R cell

The steroid profile of the H295R cell line was assessed under basal conditions. The results are shown in Figure 4-2 where the amount of each compound measured is expressed as a percentage of the total steroid measured. In interpreting these values it is important to remember that androgens were not

measured and do not form part of this data, although it is clear from previous reports that they form a significant part of the steroid output of H295R cells. It was confirmed that the cells produced aldosterone and cortisol. The proportion of cortisol relative to the total steroid measured was similar to that of human plasma. Similarly aldosterone was a small proportion of the total steroid output of the H295R cells, a situation mirrored in human plasma. However, as expected given that adrenal cells do not express 11ßHSD, a clear difference is that cortisone is detected in human plasma but was not present in significant amounts in the steroid output of the H295R cells. A further contrast to the plasma pattern is that 11-deoxycorticosterone, corticosterone and 11-deoxycortisol were proportionally much higher in the H295R cells than *in vivo*. Indeed, in normal subjects these compounds form a very minor part of the steroid levels measured.



	Human (n=6) % total Mean (StDev)	H295R cells (n=3) % total mean (StDev)
18OH corticosterone	4.49 (1.02)	1.06 (0.02)
Aldosterone	0.45 (0.10)	0.90 (0.05)
Cortisol	30.68 (4.88)	24.47 (4.64)
Cortisone	53.73 (6.09)	0.94 (0.01)
18OH deoxycorticosterone	0.11 (0.07)	1.65 (0.05)
11-dehydrocorticosterone	3.18 (0.99)	1.96 (0.24)
Corticosterone	6.89 (1.74)	40.01 (2.10)
11-deoxycortisol	0.45 (0.11)	23.94 (0.73)
Deoxycorticosterone	0.01 (0.01)	4.98 (0.11)

Figure 4-2 Steroid production of human plasma (6 normal volunteers) and H295R cells (P10, basal conditions).

Pie charts give visual representation of differences between H295R steroid production (left) and human plasma (right). Results in table represent the mean values of the percent of total steroids measured and standard deviation (brackets).

These differences between human plasma and the steroidogenesis of the H295R cells can be further interrogated by examining the ratio of a steroid to its precursor compound, as seen in Table 4-1. The ratio of the concentration of 11-deoxycortisol to cortisol (S:F) is used as an index of 11β-hydroxylation (CYP11B1) efficiency. The ratio of corticosterone to cortisol (B:F) can be used as an index of 17α-hydoxylase activity (CYP17). Aldosterone production is the best available

index of aldosterone synthase (CYP11B2) activity. The ratios of S: F and B: F were much higher in the H295R steroid output than in human plasma, reflecting less efficient enzyme activity. Quantification of the expression of steroidogenic genes is displayed in Table 4-2. In this table, the crossing thresholds (C_t values) are shown along with the values obtained from RNA extracted from a normal human adrenal gland. The $\Delta\Delta C_t$ value is not used; only one measurement was made for the adrenal tissue as it was included as a positive control. Nevertheless, it provides useful values for comparison. Lower levels of expression (i.e. higher C_t values) in H295R cells than adrenal tissue was seen for all enzymes measured.

	11-DEOXYCORTISOL: CORTISOL S:F	CORTICOSTERONE: CORTISOL B:F
Human plasma basal	0.001	0.02
H295R cells basal	0.970	1.636
H295R cells Angiotensin II	0.356	1.624
H295R cells Bu_2cAMP	0.639	1.144
H295R cells potassium	0.468	1.438

Table 4-1 Ratio of steroids to precursor compounds in human plasma and H295R cells

Human plasma obtained from 6 normal volunteers. H295R cells at P10 under basal and stimutated conditions.

ENZYME	H295R n=4 C_T Mean (StDev)	Adrenal n=1 C_T
B-actin	20.75 (0.32)	21.24
StAR	20.20 (0.07)	16.06
CYP11A1	20.66 (0.07)	15.57
CYP21A2	22.74 (0.61)	17.46
CYP17A1	23.76 (0.32)	17.47
CYP11B1	31.19 (0.40)	18.11
CYP11B2	27.19 (0.80)	23.71

Table 4-2 qRT-PCR of steroidogenic enzymes in H295R cells and normal adrenal sample. Results for the H295R cells at P10 (basal conditions) represent a mean (n=3) and standard deviation. Human adrenal results were only performed once.

4.4.2 Response of the H295R cell to trophins

Aldosterone production by the H295R cell responds to stimulation by angiotensin II and potassium although not to ACTH (Bird et al., 1993). In order to stimulate cortisol production, intracellular cAMP must be increased by using dibutyryl cAMP or forskolin. The response to trophins can vary with time and culture conditions and therefore it was important to establish response parameters under our own conditions. The steroid response of the H295R cells at passage 10 can be seen in Figure 4-3. Three biological replicates were measured from the same batch of cells. Aldosterone production was increased by almost 6-fold by angiotensin II and potassium. While Bu_2cAMP had an effect on aldosterone production, it was not as potent as angiotensin II or potassium. This is mirrored in the changes in *CYP11B2* expression as shown in Figure 4-5. The trophins that produced the greatest increase in aldosterone synthase (*CYP11B2*) were potassium and angiotensin II with Bu_2cAMP producing a less potent rise in *CYP11B2* expression.

However, cortisol production was stimulated to an equal extend by all three trophins tested, despite dibutyryl cAMP causing a more substantial increase in *CYP11B1* expression than angiotensin II or potassium.

CYP17 expression was affected only minimally by angiotensin II but was stimulated to some extent by both potassium and dibutyryl cAMP. This is consistent with the steroid data which show a slight increase in 11-deoxycortisol production, a reduction in 11-deoxycortisosterone production (Figure 4-3) and a reduction in the B:F ratio (Table 4-1) with these two trophins.

Finally, it is interesting to compare the responses of the H295R cell line to those seen *in vivo* by plasma analysis (Figure 4-4). While it must be remembered that in comparing the response in vivo to the cell model that different doses were used, it is relevant that angiotensin II produced a very specific rise in plasma aldosterone concentration, whereas a more pleiotrophic effect was observed in the H295R cells. *In vivo* ACTH produced a rise in cortisol concentration as well as in the concentrations of corticosterone, 11-deoxycortisol and 11-deoxycorticosterone but not aldosterone. Again, this is in contrast to the response of the H295R model.

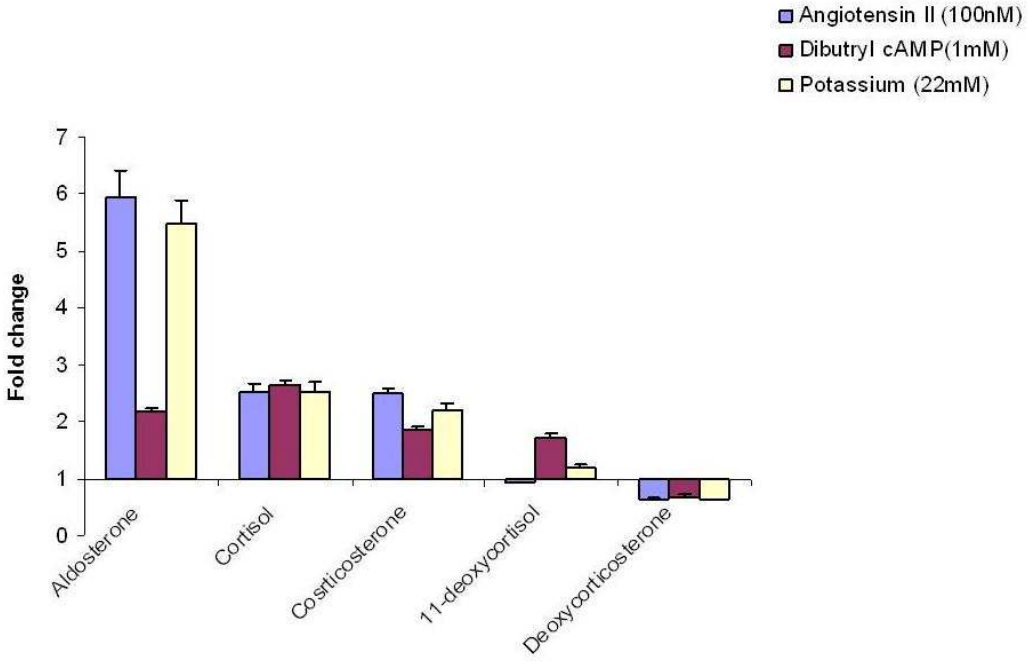


Figure 4-3 H295R cell (P10) steroid response to trophins

Data expressed as fold change in steroid production, normalised to total protein content.

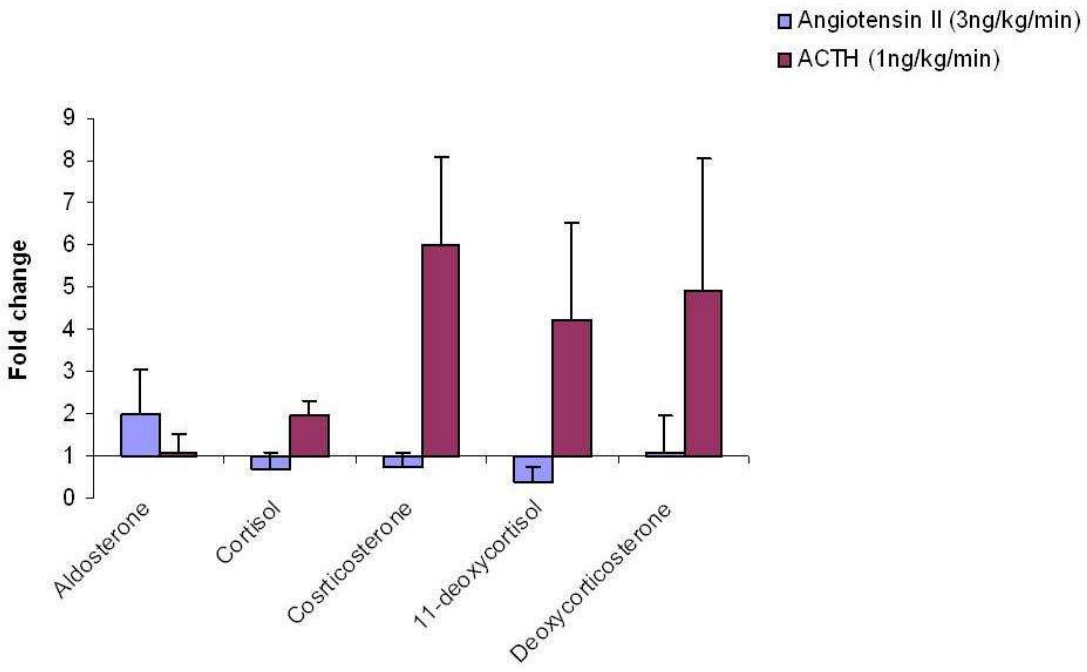


Figure 4-4 Human steroid response to ACTH and Angiotensin II in 6 normal volunteers

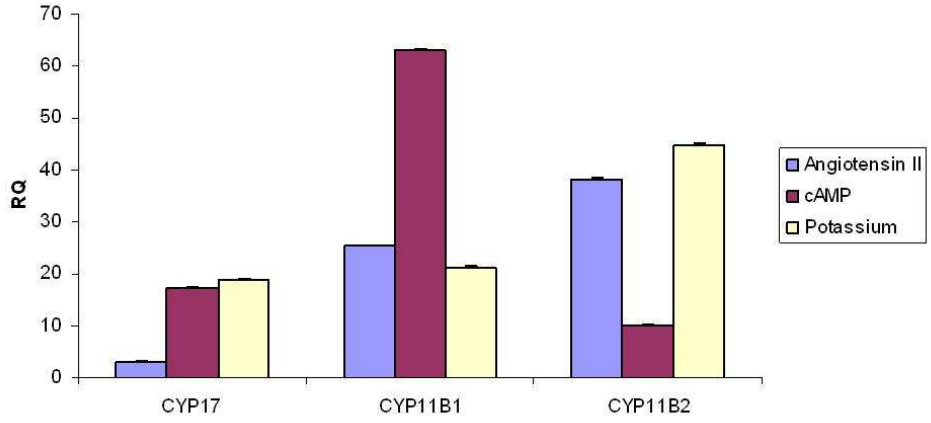


Figure 4-5 H295R cell (P10) change in enzyme expression in response to trophins
Data expressed as fold change compared to basal samples (normalized to β-actin).

4.4.3 Effect of time in culture on basal and stimulated aldosterone and cortisol production

Figure 4-6 shows cortisol production in H295R cells at passage 10 (labelled "Young") and passage 30 (labelled "Old"). Three biological replicates were measured from the same batch of cells. Under basal as well as stimulated conditions, cortisol production was greater in older cells. Figure 4-7 shows aldosterone production for the same cells. Aldosterone production was less in cells passaged more times. In Figure 4-8 and Figure 4-9, the expression of *CYP11B1* and *CYP11B2* in the younger cells are displayed relative to old cells. The relative expression under basal and angiotensin II stimulated conditions of *CYP11B1* and *CYP11B2* was greater than 1, i.e. higher in young cells than in older cells for both genes. For both *CYP11B1* and *CYP11B2*, it is the response to potassium and even more, the response to angiotensin II, which was more pronounced in young cells. In contrast, the expression of CYP17A1 did not differ significantly between young and older cells (Figure 4-10).

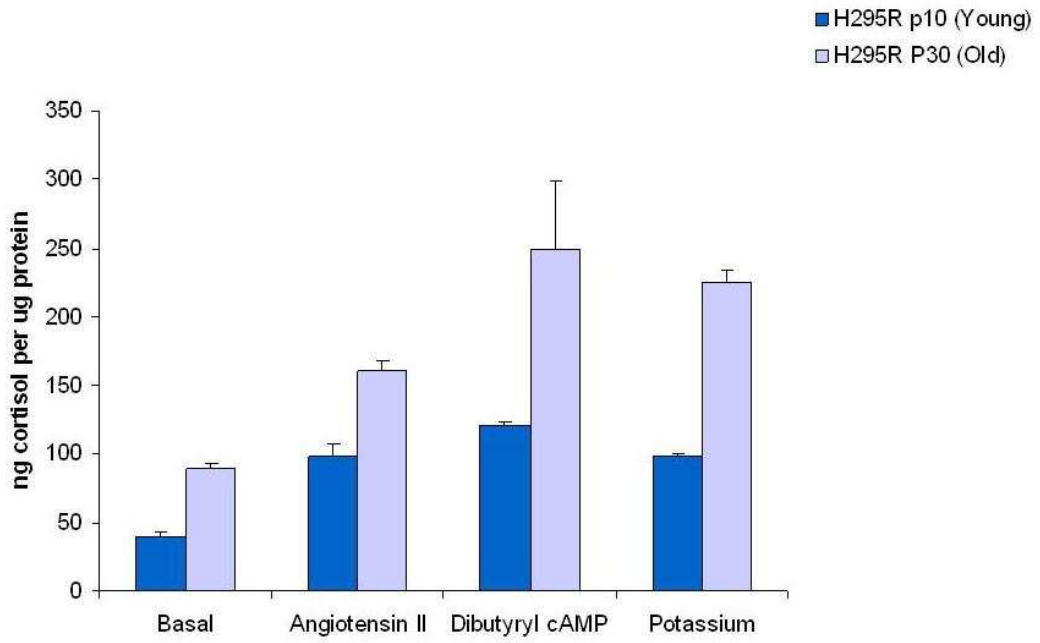


Figure 4-6 Cortisol production in young (P10) and old (P30) cells

Data normalised to total protein. Results represent mean and standard deviation (n=3).

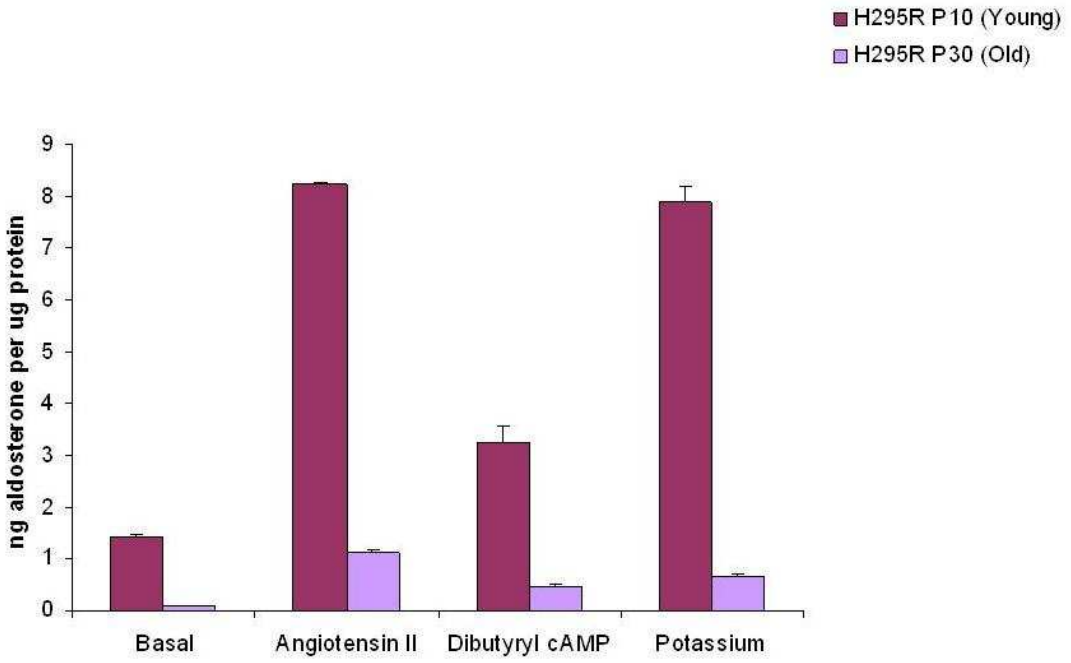


Figure 4-7 Aldosterone production in old (P10) and young (P30) cells
Data normalised to total protein. Results represent mean and standard deviation (n=3)

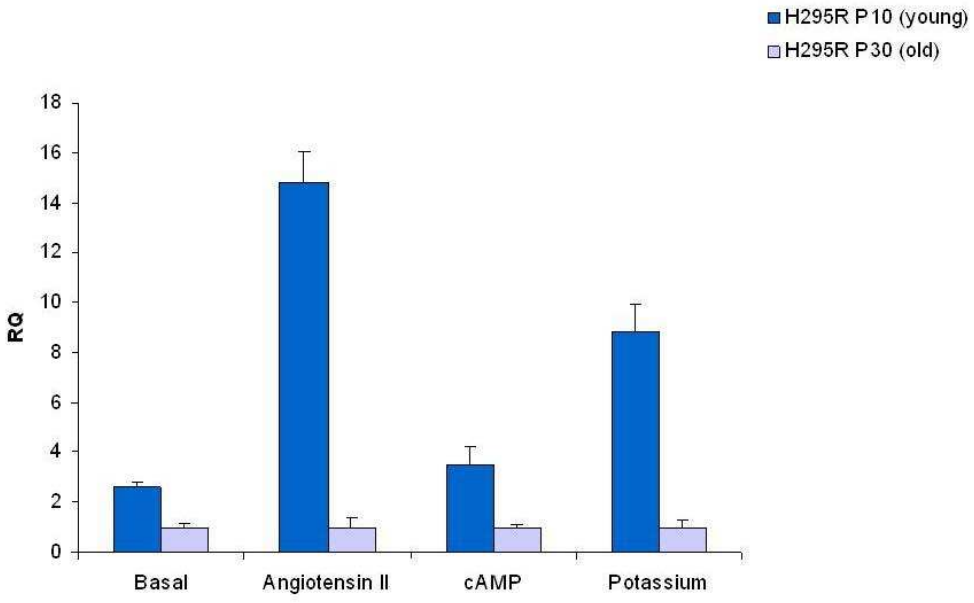


Figure 4-8 CYP11B1 expression in young (P10) cells relative to old (P30) cells.
Results represent mean and standard deviation (n=3).

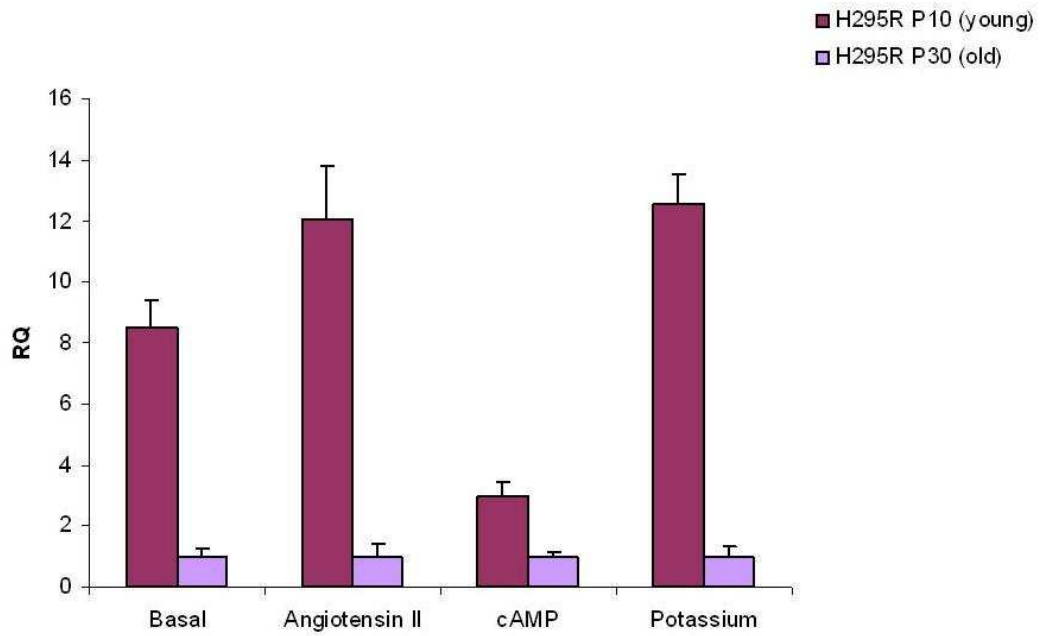


Figure 4-9 CYP11B2 expression in young (P10) cells relative to old (P30) cells. Results represent mean and standard deviation (n=3)

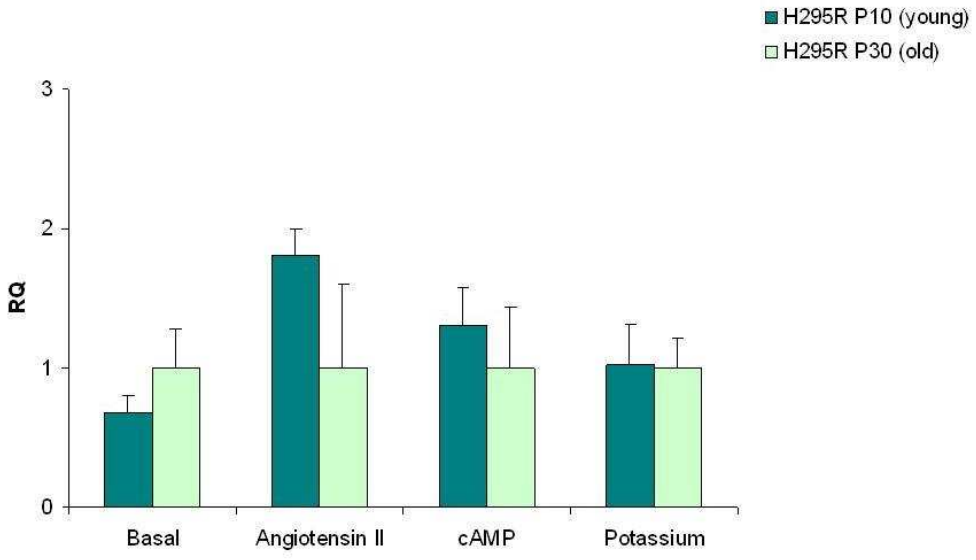


Figure 4-10 CYP17A1 expression in young (P10) cells relative to old (P30) cells. Results represent mean and standard deviation (n=3)

4.5 Discussion

The purpose of these studies was to explore the function of the H295R cell line in order to assess its utility as a model of human aldosterone production. To be acceptable as a surrogate for human adrenocortical secretion, it was necessary to assess the steroid production of the cell line under basal conditions and in response to stimulation and compare this to the *in vivo* steroid profile. In addition, it was important to establish any change in the H295R cell steroid pattern and response over time.

Results have confirmed that the H295R cell line expresses the necessary enzymes to produce both aldosterone and cortisol and that these compounds are secreted from the cells in measureable quantities. In addition, both aldosterone synthase and aldosterone synthesis increased in response to angiotensin II and potassium, as did 11ß hydroxylase and cortisol synthesis in response to Bu_2cAMP. From this perspective, it has been demonstrated that the cell line is an appropriate model of mineralocorticoid and glucocorticoid production. This was the main purpose of these experiments and the data shown here is robust in this regard.

However, there are characteristics of the H295R cell line that are less desirable in a model system. Firstly, the steroid profile of the H295R cell line showed clear differences from the human plasma steroid profile. The relative amounts of the C21 precursor compounds corticosterone, deoxycorticosterone and 11-deoxycortisol were significantly higher in H295R cells than in human plasma where they were a minor constituent. This pattern of elevated precursors as compared to end products is somewhat analogous to the clinical picture of adrenal malignancy in general (Kikuchi et al., 2000) and the clinical presentation of the donor patient in particular (Gazdar et al., 1990). This pattern could be explained by a reduced level of expression of *CYP11B1* and *CYP11B2* relative to other steroidogenic genes in the H295R cells as compared to normal adrenal expression. Limited conclusions can be drawn from the comparison of normal adrenal mRNA compared to H295R mRNA profile as only one normal adrenal sample (originally included simply as a positive control for the reaction) was only measured once. Nevertheless, the results do suggest that while *CYP11B1* and *CYP11B2* are expressed at a lower level than in normal adrenals, the same can also be said of the other steroidogenic genes measured. This part of the study

should be repeated to increase confidence in the results but it may suggest that it is not simply expression of *CYP11B1* and *CYP11B2* that determines the enzyme activity. This will be discussed in more detail later in this section.

Secondly, cortisone makes up a significant proportion of the human plasma steroid profile but in the H295R cell this is present in very small quantities. This is not surprising as 11ß-hydroxysteroid dehydrogenase (11ß-HSD), the enzyme which catalyses the conversion of cortisol to inactive cortisone *in vivo*, is not expressed in the adrenal cortex and was not found to be expressed in measurable amounts in the H295R cells.

Finally, the pattern of steroidogenesis changed over time in culture, with both aldosterone and cortisol production diminishing. It should be borne in mind that due to the scale of these experiments, the three biological replicates was from the same batch of cells and repetition in an independent experiment would give greater confidence, nevertheless, this lack of consistency over time in culture is clearly an important consideration when planning large scale or repeated experiments with this cell line. In this regard, one of the most interesting findings of these studies has been the discordance between enzyme expression and steroid production. This phenomenon was observed in two of the experiments described in this chapter. When the cells were stimulated with Bu_2cAMP, a brisk increase in *CYP11B1* was measured, greater than the increase in *CYP11B1* measured in cells stimulated with either potassium or angiotensin II. Although cortisol rose in response to Bu_2cAMP treatment, the rise was no greater with this trophin than with potassium or angiotensin II. Further, cells that had been in culture for a longer period of time and subjected to more passages expressed less *CYP11B1* but incongruously secreted more cortisol than those that had been in culture for less time and expressed more *CYP11B1*. The mechanisms behind this phenomenon are not clear. Some forms of congenital adrenal hyperplasia, e.g. patients with 3ß-hydroxysteroid dehydrogenase (3ß-HSD) deficiency, present with a lack of cortisol and aldosterone despite normal *CYP11B1* and *CYP11B2* as a consequence of not being able to generate substrate for 11ß-hydroxylase and aldosterone synthase. Alternatively, an increase in 17,20 lyase activity relative to hydroxylase activity of CYP17A1 (even in the context of a constant amount of mRNA levels of *CYP17A1* as was demonstrated in these experiments) would result in more androgen production and less substrate

for glucocorticoid production. In fact, changes in expression of *3βHSD* and *CYP17A1* have been proposed to be the mechanism for increased androgen production in the H296R compared to the H295A cell line (Samandari et al., 2007). However, in these experiments it is not likely that lack of substrate was the rate limiting factor in the H295R cells as 11-deoxycortisol was always present in relatively high amounts.

It has been previously recognised that the characteristics of the H295R cells can vary with time in culture. Wang et al described a reduction in expression of CREB in H295R cells of late passage compared to earlier passage cells (Wang et al., 2000). Some investigators have found that H295R cells in their hands do not express CREB at all. CREB is implicated in the regulation of transcription of *CYP11B1* (Wang et al., 2000; Bassett et al., 2000) and *CYP11B2* (Nogueira and Rainey, 2010) (see section 1.2.8). This may be an importance mechanism contributing to the altered pattern of transcription of steroidogenic genes over time in culture.

The increase in *CYP11B1* transcripts measured implies that the mechanisms controlling the up regulation of transcription of the gene in response to trophins is intact. The dissonance between amount of *CYP11B1* mRNA measured and the amount of product of the 11β-hydroxylase enzyme in the form of cortisol may be as a result of mechanisms that regulate translation of the mRNA to functional protein, or as a result of processes that affects the function of the 11β-hydroxylase enzyme itself. It would clearly be useful to be able to measure the levels of 11β-hydroxylase protein in order to establish if there is reduced translation of mRNA to protein, but due to the high degree of homology between the coding regions of the *CYP11B1* and *CYP11B2* genes there are no antibodies currently available that can reliable distinguish between the 11β hydroxylase and aldosterone synthase protein products. Therefore it is not possible to conclude whether the unexpectedly low cortisol occurred as a result of not enough enzyme or of an inefficient enzyme.

Once mRNA is transcribed in the nucleus, it is capped, spliced, cleaved and polyadenylated prior to being exported to the cytoplasm (McKee and Silver, 2007). In the cytoplasm, it can be transcribed, stored and transcribed later or degraded (Shyu et al., 2008). Clearly, there are many steps which can be

disrupted and could lead to reduced transcription of protein. One such mechanism that has received increasing attention in recent years is the action of microRNA. MicroRNAs are short (around 21 base pairs) strands of RNA that have been cleaved from longer (around 70 base pairs) hairpin structured molecules. They have imperfect complementarity for a target sequence and controversy remains as to their precise mechanism of action. Current consensus suggests that there is likely to be more than one mechanism of action and that microRNAs are able to inhibit translation as well as cause degradation of mRNA (Jackson and Standart, 2007). Another mechanism of RNA interference (RNAi) that causes degradation of mRNA is siRNA; these are also short strands of RNA but are derived from longer double stranded RNA precursors. Given that qRT-PCR detects amplified fragments of mRNA, it will not distinguish between degraded and intact mRNA, and thus RNAi may be an explanation for why increase in *CYP11B1* mRNA is not associated with increased cortisol in the experiments described above.

Alternatively, there are a number of reasons why the protein may not catalyse the conversion of 11-deoxycortisol to cortisol efficiently. Alterations in the gene sequence could affect the function of the protein while not altering its rate of production. However, both the *CYP11B2* and B1 gene in the H295R cell line have been sequenced in our laboratory and no mutations were detected. *CYP11B1* along with *CYP11B2* and CYP11A1 are cytochrome P450 enzymes and require electron transfer from the NADPH/adrenodoxin/adrenodoxin reductase (Miller, 2005) system (see Section 1.2.5). Changes in this electron transfer system may impact on enzyme activity; indeed co-transfecting adrenodoxin with the cytochrome P450 enzyme CYP11A1 increases enzyme activity (Zuber et al., 1988) and there is evidence that the availability of adrenodoxin and adrenodoxin reductase has rate limiting effects on the activity of *CYP11B1* (Cao et al., 2000; Hakki et al., 2008). It is interesting to note that adrenodoxin expression is increased with exposure to dibutyryl cAMP but not to the same extent as the effect observed on expression of *CYP11B1* (Sewer and Waterman, 2002). The availability of electron donors may be responsible for the blunted production of cortisol in the face of substantially increased expression of *CYP11B1*. However; it is not clear why a similar phenomenon is not seen with *CYP11B2* which depends on the same system for reducing equivalents. There is some evidence using

bovine *CYP11B1* that truncated forms of adrenodoxin can specifically increase aldosterone production without increasing cortisol production (Cao and Bernhardt, 1999), but this has not been tested for human *CYP11B1* and *CYP11B2*. Alternatively, there could be competition for reducing equivalents between *CYP11B1*, *CYP11B2* and CYP11A1.

Finally, protein-protein interactions may also have an effect that could be specific to *CYP11B1* or *CYP11B2* beyond competition for NADPH. Bovine CYP11A1 increases 11ß-hydroxylation and reduces 18-hydroxylation and 18-oxidation leading, to increased cortisol and reduced aldosterone when co-transfected with bovine adrenodoxin and *CYP11B1* in COS-1 cells (Cao and Bernhardt, 1999). It has been proposed that CYP11A1 induces conformational change of the CYP11B1 enzyme, leading to altered function (Lisurek and Bernhardt, 2004). However it should be said that the only evidence that exists for this is in a bovine model system and human *CYP11B1* and B2 co-transfected with bovine adrenodoxin did not show the same relationship (Cao and Bernhardt, 1999).

4.6 Conclusion

These studies of the H295R cell line have confirmed and extended previous work. The H295R cell line remains the best and most widely used model for adrenal cell function and its ability to produce steroid from all three biochemical pathways and respond to stimulation makes their utility unquestionable. However, these data raise some interesting and useful points for their further use. The changes in gene expression and steroid production over time in culture have been previously acknowledged but not comprehensively described and it has not previously been well recognised that the rate and pattern of this change across the steroid pathway can be diverse. In addition, by examining both the mRNA expression as well as the indices of steroid conversion, is it apparent that there are other rate limiting steps in the production of the end points of mineralocorticoid and glucocorticoid synthesis than the levels of enzyme expression of the terminal steps in steroidogenesis. How comparable these *in vitro* findings are to the *in vivo* systems is not yet known, particularly as the H295R cell collectively expresses components of the glomerulosa, fasiculata and reticularis, in contrast to the distinct functional zonation seen in mammals. However, as the major human adrenal cell line, it is the most informative model

at our disposal currently. Further studies could provide greater insight into the intricate sequence of events leading to human mineralocorticoid and glucocorticoid production.

In vitro studies of CYP11B2 transcription

5. *In vitro* studies of CYP11B2 transcription

5.1 Introduction

As there is virtually no storage of steroid hormones within the adrenal gland, increased synthesis of products from cholesterol in response to trophins is the main mechanism for increasing circulating steroid hormone levels. Thus, regulation of transcription is the pivotal point of homeostatic control of steroidogenesis. This chapter aims to investigate the functional effects of polymorphic change in the promoter region of *CYP11B2* on gene transcription.

Transcriptional regulation of *CYP11B2* has been studied in some detail and has been previously described in depth (see Section 1.2.8). However, to recap briefly, both angiotensin II and potassium produce an increase in transcription of *CYP11B2* beginning with increase intracellular calcium. Angiotensin II achieves this by binding to the Type 1 angiotensin receptor (AT1) which is coupled to a heterotrimeric G-protein (Gq) (Higuchi et al., 2007). This causes activation of phospholipidase C and consequent rise in diacylglycerol (DAG) and inositol trisphosphate (IP3) (Neves et al., 2002) and activation of protein kinase C (PKC). Increased extracellular potassium initiates a depolarisation of the glomerulosa cell membrane. Both processes culminate in an increased intracellular calcium flux which activates calmodulin (CaM), in turn activating CaM protein kinases to regulate *CYP11B2* transcription (Clyne et al., 1996). Adrenocorticotrophin stimulating hormone (ACTH) acutely increases aldosterone production directly via cAMP (Bassett et al., 2004a).

Several regulatory elements in the 5' flanking region of the gene have been localised (Clyne et al., 1997), proteins that bind to them have been identified (Bassett et al., 2004b), and the influence of post translational modification of transcription factors e.g. phosphorylation (Nogueira and Rainey, 2010), have been described. The acknowledged sites of transcription factor binding in the *CYP11B2* promoter region are illustrated in Chapter 1 Figure 1.17 and were first described by Clyne *et al* (Clyne et al., 1997) but the effects of polymorphic variation at the sites under investigation in this work on transcription are not known. The positions of the known regulatory sites in relation to these polymorphisms are displayed in Figure 5-1.

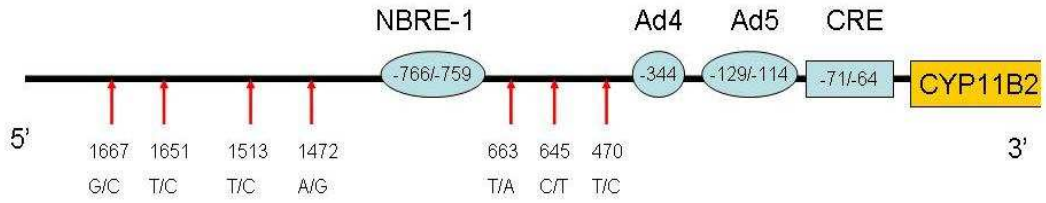


Figure 5-1 Schematic diagram of promoter region of CYP11B2.

CREB and ATF1 bind to the CRE and this binding is increased after phosphorylation by CamKinases as a result of Angiotensin II receptor activation. Ad5 and NBRE-1 bind the transcription factors NURR1 and NGFIB and these are increased in response to angiotensin II. The -344 polymorphism is shown as Ad4. However, deletion of this site has no effect on transcriptional activity. The red arrows demonstrate the polymorphisms described in chapter 3 in relation to these known transcription factor binding sites.

In order to investigate the functional effects of the polymorphic variants in *CYP11B2* promoter region, a number of approaches were undertaken. A bioinformatics search strategy was used to prioritise the polymorphisms to be studied in more detail, reporter gene assays undertaken to assess any effect of allelic variation on transcriptional activity and transcription factor binding studies to begin to identify proteins that may play a role.

5.2 Aims

To investigate functional effects of polymorphic variation in the promoter region of *CYP11B2* by:

1. Identifying putative transcription factor binding at the sites of polymorphic variation using a bioinformatic database (Transfac® Professional) and compare polymorphic variants in order to prioritise further *in vitro* studies.

2. Establishing if promoter activity is altered by single nucleotide polymorphic variation at candidate site within the promoter region of *CYP11B2* using an *in vitro* reporter gene system

3. Investigating alterations in transcription factor binding with contrasting alleles in candidate polymorphic sites using electromobility shift assay system.

5.3 Bioinformatics

5.3.1 Methods

To investigate whether the polymorphisms altered putative transcription factor binding sites the DNA sequences flanking the SNPs were entered into the Transfac® Professional V 10.1 database. This is a commercial, subscription only database which contains experimental data extracted from peer reviewed journals to provide a searchable database for transcription factor binding sequences. Within Transfac® Professional is Match, a web-based tool which identifies transcription factor binding sites in DNA sequences by weight matrix search (Matys et al., 2006). Matrices are constructed using both experimentally confirmed transcription factor binding sequences and data extrapolated from biological studies to produce a measurement of the relative likelihood of a transcription factor binding to a particular sequence. The frequency that a particular nucleotide appears at a certain position in a binding sequence is recorded and consensus binding sequence is produced for the matrix. The five most highly conserved bases are called the “core”. Thresholds can be set for “core” and “matix” matching with 1.0 corresponding to 100% similarity. If the sequence passes the threshold for core similarity it is then aligned for matrix similarity. If this threshold is also passed, the transcription factor is included in the output.

Searches were undertaken between Tuesday 23/10/07 and Monday 12/11/07. Thresholds were set at 0.75 for core binding and 0.70 for matrix binding. Only vertebrate matrices were analysed. The input sequences are shown in Table 5-1.

SNP POSITION 5'	SNP POSITION EXON 1	SEQUENCE
357 G/C (331-380)	-1667	>ccgaggtgggcagatcacctgaggtcaggagttcgagact >ccgaggtgggcagatcacctgacgtcaggagttcgagact
373 T/C (351-400)	-1651	>cctgaggtcaggagttcgagactagcctggccaacacggt >cctgaggtcaggagttcgagaccagcctggccaacacggt
511 C/T (491-530)	-1513	>tgaacccgggagacagaggtcgcagtgagccgagatcaca >tgaacccgggagacagaggttgcagtgagccgagatcaca
552 G/A (531-570)	-1472	>ccattgcactctagcctgggcgacagagtgagactctgtc >ccattgcactctagcctgggcaacagagtgagactctgtc
1361 A/T (1341-1380)	-663	>ctggggcaggagggatgagcaggcagagcacaggttttct >ctggggcaggagggatgagctggcagagcacaggttttct
1379 C/T (1361-1400)	-645	>aggcagagcacaggttttcttttcctcttttaagacagt >aggcagagcacaggttttttttcctcttttaagacagt
1554 C/T (1533-1573)	-470	>tcacatggaaccagtgcgctcctgtggtggagggtgtacc >tcacatggaaccagtgcgctcttgtggtggagggtgtacc

Table 5-1 Flanking sequence entered in bioinformatics search for putative transcription factor binding around sites of polymorphic variation

Output files from Transfac® Professional searches were converted to Microsoft Excel files and the results for each sequence containing contrasting alleles at the site of polymorphic variation were compared.

5.3.2 Results

All SNPs were associated with variation in predicted transcription factor binding. In total, over 900 alterations in bindings were introduced across the seven polymorphisms. Most suggested an altered binding affinity for one allele compared to the other. However, there were 131 incidences of where transcription factor binding was predicted to be introduced on the basis of a single base change and was not predicted to be present in the presence of the alternate allele. These results for each SNP are shown in Table 5-2, Table 5-3, Table 5-4, Table 5-5, Table 5-6, Table 5-7, and Table 5-8.

357 (-1667) C		357 (-1667) G	
ATF1 (Nogueira and Rainey, 2010; Clyne et al., 1997; Bassett et al., 2004a; Bassett et al., 2000)	activating transcription factor 1	RUNX1	runt-related transcription factor 1
ATF6	activating transcription factor 6	AREB6	zinc finger E-box binding homeobox 1
C/EBP	CCAAT/enhancer binding protein	COUP-TF (Bassett et al., 2004a; Wang et al., 1989; Clyne et al., 1997)	nuclear receptor subfamily 2, group F
Myc	v-myc myelocytomatosis viral oncogene homolog	deltaEF1	zinc finger E-box binding homeobox 1
E4F1	E4F transcription factor 1	GR	Glucocorticoid receptor
ER	Oestrogen receptor	HEN1	nescient helix loop helix 1
HIF1	Hypoxia inducible factor 1	LXR direct repeat	nuclear receptor subfamily 1, group H, member 2
Pax-2	paired box 2	RUNX2	runt-related transcription factor 2
SREBP-1	sterol regulatory element binding transcription factor 1	PEBP	phosphatidylethanolamine binding protein 1
v-ErbA	Also known as thyroid hormone receptor, alpha		
v-Jun	Jun protoconcogene, also known as AP-1		
XBP-1	X-box binding protein 1		

Table 5-2 Transcription factors predicted to bind to only the C allele or the T allele at position 373 (-1667) of CYP11B2.

Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

373 (-1651) C		373 (-1651) T	
AP-4	activating enhancer binding protein 4	AP-3	activating enhancer binding protein 3
AR	Androgen receptor	AREB6	zinc finger E-box binding homeobox 1
ER	Oestrogen receptor	Pax-2	Paired box 2
GR	Glucocorticoid receptor	Pitx2	paired-like homeodomain 2
Pax-6	Paired box 6	SF1 (Ye et al., 2009; Bassett et al., 2002; Clyne et al., 1997)	Steroidogenic factor 1
PR	Progesterone receptor	SMAD	SMAD family member
Zic3	Zic family member 3 (odd-paired homolog)	SREBP	sterol regulatory element binding transcription factor
		VDR (Romero et al., 2007; Romero et al., 2010)	Vitamin D receptor

Table 5-3 Transcription factors predicted to bind to only the C allele or the T allele at position 357 (-1651) of CYP11B2.

Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

510 (-1513) C		510 (-1513) T	
COUP-TF (Bassett et al., 2004b; Clyne et al., 1997; Wang et al., 1989)	nuclear receptor subfamily 2, group F	C/EBP	CCAAT/enhancer binding protein
Egr (Nogueira et al., 2007)	Early Growth response	Gfi1	growth factor independent 1 transcription repressor
GR	Glucocorticoid receptor	HIC1	hypermethylated in cancer 1
HNF4	Hepatocyte nuclear factor 4	HMG IY (Romero et al., 2010; Romero et al., 2007)	high mobility group AT-hook 1
RORalpha1	RAR-related orphan receptor A	HOXA7	homeobox A7
SMAD	SMAD family member	MYB	v-myb myeloblastosis viral oncogene homolog
		Nkx2-5	NK2 transcription factor related, locus 5
		Pax-3	Paired box 3
		RFX	regulatory factor X, 1
		SOX10	SRY (sex determining region Y)-box 10

Table 5-4 Transcription factors predicted to bind to only the C allele or the T allele at position 510 (-1513) of CYP11B2.

Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

551 (-1472) G		551 (-1472) A	
AP-2	activating enhancer binding protein 2	AP-4	Activating enhancer binding protein 4
ATF (Bassett et al., 2000; Sirianni et al., 2010; Clyne et al., 1997)	activating transcription factor	C/EBP	CCAAT/enhancer binding protein
CREB (Clyne et al., 1997)	cAMP responsive element binding protein	FOXO1	forkhead box O1
Egr (Nogueira et al., 2007)	Early Growth response	FOXO4	forkhead box O4
MEIS1	Meis homeobox 1, myeloid ecotropic viral integration site 1 homolog	HIC1	hypermethylated in cancer 1
MZF1	myeloid zinc finger 1	HNF3	Hepatocyte nuclear factor 3
EGR4	early growth response 4	IRF-1	interferon regulatory factor 1
SMAD3	SMAD family member 3	LEF1	lymphoid enhancer-binding factor 1
WT1	Wilms tumor 1	MYB	v-myb myeloblastosis viral oncogene homolog
YY1	YY1 transcription factor	RBP-Jkappa	recombination signal binding protein for immunoglobulin kappa J region
		RFX1	regulatory factor X, 1
		SOX10	SRY (sex determining region Y)-box 10

Table 5-5 Transcription factors predicted to bind to only the G allele or the A allele at position 551 (-1472) of CYP11B2.

Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

1361 (-663) A		1361 (-663) T	
AhR	aryl hydrocarbon receptor	AP-4	activating enhancer binding protein 4
HIC1	hypermethylated in cancer 1	C/EBP	CCAAT/enhancer binding protein
HSF1	heat shock transcription factor 1	DBP	D site of albumin promoter (albumin D-box) binding protein
KAISO	zinc finger and BTB domain-containing protein 33	E2A	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog	HNF4	Hepatocyte nuclear factor 4
Msx-1	msh homeobox 1	MEIS1	Meis homeobox 1, myeloid ecotropic viral integration site 1 homolog
NERF1a	E74-like factor 2 (ets domain transcription factor)	NF-1	neurofibromin 1
p53	tumor protein p53	RFX	regulatory factor X, 1
HIC1	hypermethylated in cancer 1	RORalpha1	RAR-related orphan receptor A
SMAD	SMAD family member	TGIF	TGFB-induced factor homeobox 1
VDR (Romero et al., 2007; Romero et al., 2010)	Vitamin D receptor	YY1	YY1 transcription factor

Table 5-6 Transcription factors predicted to bind to only the A allele or the T allele at position 1361 (-663) of CYP11B2.

Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

1379 (-645) T		1379 (-645) C	
AR	Androgen receptor	BLIMP1	PR domain containing 1, with ZNF domain
FAC1	bromodomain PHD finger transcription factor	C/EBP	CCAAT/enhancer binding protein
FOXD3	forkhead box D3	Elf-1	E74-like factor 1 (ets domain transcription factor)
FOXJ2	forkhead box J2	GATA-1	GATA binding protein 1 (globin transcription factor 1)
FOXO1	forkhead box 01	GATA-2	GATA binding protein 2 (globin transcription factor 2)
FOXO3A	forkhead box 03A	HSF	Heat Shock factor
FOXO4	forkhead box 04	ICSBP	interferon regulatory factor 8
HNF3alpha	Hepatocyte nuclear factor 3 alpha	NF-AT	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein
HNF3beta	Hepatocyte nuclear factor 3 beta	NIT2	nitrilase family, member 2
TBP	TATA box binding protein		
ZBRK1	zinc finger protein 350		

Table 5-7 Transcription factors predicted to bind to only the T allele or the C allele at position 1379 (-645) of CYP11B2.

Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

1554 (-470) C		1554 (-470) T	
AP-2	activating enhancer binding protein 2	AP-3	activating enhancer binding protein 4
AP-4	activating enhancer binding protein 4	C/EBP	CCAAT/enhancer binding protein
AREB6	zinc finger E-box binding homeobox 1	FAC1	bromodomain PHD finger transcription factor
AREB6	zinc finger E-box binding homeobox 1	HNF3	Hepatocyte nuclear factor 3
E2A	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	LRH1	nuclear receptor subfamily 5, group A, member
Elk-1	ELK1, member of ETS oncogene family	MRF-2	Modulator recognition factor 2
Eve	SH3 domain containing 19	Nkx2-5	NK2 transcription factor related, locus 5
LEF1	lymphoid enhancer-binding factor 1	p53	tumor protein p53
p54	non-POU domain containing, octamer-binding	SOX10	SRY (sex determining region Y)-box 10
Sp1	Sp1 transcription factor	SOX9	SRY (sex determining region Y)-box 9
Spz1	spermatogenic leucine zipper 1	TBP	TATA box binding protein
USF	upstream transcription factor, c-fos interacting		
Eve	SH3 domain containing 19		

Table 5-8 Transcription factors predicted to bind to only the T allele or the C allele at position 1554 (-470) of CYP11B2.

Candidate transcription factors identified above were further investigated with literature search to explore the possibility that they may be involved in the regulation of aldosterone synthase. Transcription factors implicated in the literature in the transcriptional regulation of aldosterone synthase are highlighted.

The role of ATF1 and CREB has been previously investigated with regard to the regulation of *CYP11B2*. As described in Chapter 1.2.8, a CRE site at position -64 is known to be crucial for basal expression of aldosterone synthase. COUP-TF has been identified as binding to oligonucleotide spanning -129/114 (known as the Ad5 element) (Bassett et al., 2004b; Wang et al., 1989; Clyne et al., 1997). SF-1 is involved in the regulation of a number of steroidogenic enzymes and its role in the transcriptional regulation of aldosterone synthase is controversial; the extensively investigated polymorphism at -344 is a putative SF-1 site with the C allele binding more avidly than the T allele. However, SF-1 at the -344 site does not appear to influence transcription of the gene (Bassett et al., 2002; Ye et al., 2009). Finally, recent data has been published examining angiotensin II responsive genes and suggested that Vitamin D receptor and HMG1Y and angiotensin II responsive and may play a role in regulating aldosterone synthase (Romero et al., 2010; Romero et al., 2007).

5.3.3 Discussion

Numerous transcription factor binding sites are predicted to be introduced or abolished with the substitution of a single nucleotide change. In the first instance, a literature search was undertaken to identify transcription factors known to be involved in the regulation of aldosterone synthase as a means to prioritise further in vitro investigation of functional effects of SNPs. The SNPs and relevant transcription factors are summarised in Table 5-9.

Position	Allele	Predicted binding Sequence	Transcription factor
357	C G	tGACGTcagga gcagatcacctgaGGTCAggagt	ATF-1 COUP-TF
373	T	tagCCTGGc tagcctggccAACAC	SF-1 VDR
510 510	C T	ggagacagaGGTCG agGTTGC	COUP-TF HMG1Y
551	G	gggCGACAg	CREB
1361	A	ggaggGATGAgcaggcagagc	VDR

Table 5-9 Transcription factors implicated in the regulation of aldosterone synthase.

Putative binding sites relative to single nucleotide polymorphisms are given. Nucleotide sequence deemed to be "core" five nucleotides are in upper case.

However, there are a number of points that should be made regarding the published literature. Initial information regarding possible transcription factor binding sites were identified by reporter gene deletion assays and DNAse I footprint studies of the bovine CYP11B gene (Takayama et al., 1994; Kirita et al., 1990). This raises obvious issues regarding the validity of animal models with regard to human steroidogenesis, as previously mentioned in Chapter 4. There is only one bovine CYP11B gene and its transcript codes for a protein with both glucocorticoid and mineralocorticoid functions. As a result, the regulation of this gene may not be comparable to the human *CYP11B2*, or indeed the human *CYP11B1*. Later work was carried out by Clyne et al (Clyne et al., 1997) used the H295R cell model, extensively described in Chapter 4. Some of the results observed in the bovine model were indeed confirmed in these investigations. However, promoter deletion assays, by removing relatively large sections of the promoter in an iterative process, may remove positive and negative regulating regions simultaneously, obscuring any individual effect. Further, it has been suggested that the binding and action of transcription factors can vary depending on the three dimensional structure of the DNA, the conformation of which can be altered by protein: DNA interactions up or down stream of the binding site of interest. Thus, promoter deletion assays may provide incomplete data regarding sites involved in gene regulation. Recently published data has looked at the effect on aldosterone production of H295R cells of over expression

of genes previously shown to respond to angiotensin II (Romero et al., 2007). These included HMGA1 and VDR, both of which increased aldosterone production in response to angiotensin but not under basal conditions (Romero et al., 2010). These and other transcription factors may be involved in the regulation of the gene and clearly require further study however, the genotype dependant difference may not be angiotensin II mediated and may in fact be under basal conditions.

The parameters of the bioinformatics search were deliberately permissive and as a result, are likely to have generated a significant proportion of false positive results. In addition, the Transfac® Professional database is based only on experiments that have been undertaken and the extrapolation of published results, therefore it is difficult to quantify the false negative rate for this type of investigation. Transfac® Professional, by necessity, makes certain assumptions in order to compile the database and create search results. These assumptions may also be a source of error. For example, as mentioned above, the 5 most highly conserved bases are designated the "core" binding site and the logarithm that is followed requires a user determined threshold to be passed regarding alignment with the binding site. However, this may not accurately reflect the biological binding process as not all transcription factors have a core binding sequence of just five nucleotides. In addition, transcription factors often form homo or heterodimers before binding to DNA and the three dimensional structure is central to the specificity of binding. Further, the three dimensional structure of the DNA strand is also crucial and this may be affected by up or down stream protein:DNA interactions (Sarai and Kono, 2005). The results described above must be interpreted with this in mind. Whilst the *in silico* investigation should not be seen as definitive evidence of biological interactions it raises a number of further lines of enquiry and certainly provides a rational for ongoing investigations around the functional effect of SNPs in this region.

One of the objectives of the bioinformatics search was to prioritise which SNP to proceed with for further in vitro studies. On the basis that the SF-1 is a transcription factor most heavily implicated in the regulation of steroidogenic genes, albeit with a less clear role in the transcriptional regulation of *CYP11B2*, it was decided that further work would continue with the SNP which putatively introduced a new SF-1 site (-1651).

5.4 Reporter gene assays

Reporter gene assays can be used to study the function of promoter regions of a gene of interest. The promoter region is cloned into a plasmid, upstream of a "reporter gene", in this case firefly luciferase. The plasmid is then transfected into a cell line along with a control plasmid, this allows for normalisation of results to account for variables like transfection efficiency or variation in cell handling/ survival. The cell line, which should be chosen to contain the necessary signal transduction pathways and transcription factors necessary to induce transcription, is cultured under controlled conditions. A strong promoter will produce more luciferase mRNA than a weak promoter and this is translated into protein which can be detected using a simple luciferase assay.

5.4.1 Methods

Based on the results of the bioinformatics search, reporter constructs of the 2 polymorphic variants at position -1651 were designed (Figure 5-2).

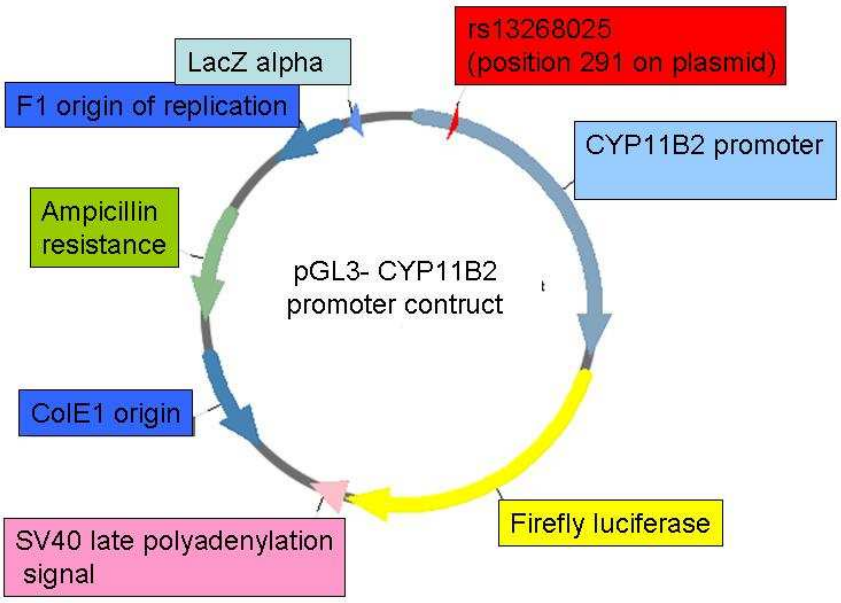


Figure 5-2 pGL3-CYP11B2 promoter construct.

1880 bp of the CYP11B2 promoter was cloned into the pGL3 plasmid containing a luciferase reporter gene. Features on the plasmid are origin of replication (Col E1 origin) for replication of plasma in bacteria, viral late polyadenylation signal (SV40 late polyA), Firefly luciferase gene (Firefly luciferase), 1.8kb section of the CYP11B2 promoter (CYPB2 promoter), LacZ alpha gene (LacZ alpha) for blue-white screening and the F1 origin of replication sequence (F1ori). The site of polymorphic variation (rs13268025 T/C) of interest is shown in red.

The wild type construct was used as a template and the allele at -1651 was mutated from T to C (Eurofins MWG Operon, Ebersberg, Germany) (Section 2.3.1). Plasmids were prepared and sequence confirmed as described in section 2.2.2-2.3.5. (Plasmid sequence is shown in Appendix 7.9). Transient transfection was carried out as described (section 2.3.6) and a renilla luciferase containing control plasmid pGL4.73, (Promega, Wisconsin, USA) was co-transfected at a ratio of 50:1, to enable adjustment for transfection efficiency. Control wells containing untransfected cells, *Renilla* luciferase plasmid alone (pGL4.73), firefly luciferase plasmid alone (pGL4.10) as well as the empty vector (pGL3 basic) with renilla luciferase plasmid (pGL4.73) were also included in each

experiment. Transfected H295R cells were incubated under basal conditions, and with the addition of agonists of aldosterone production: angiotensin II ($1x10^{-7}$M), Bu_2cAMP (1mM) and potassium (22mM). After 24 hrs, cells were lysed and assayed for firefly and *Renilla* luciferase using Dual Luciferase assay (Promega, Wisconsin, USA) (section 2.3.7). The Dual-Luciferase Reporter (DLR) Assay System allows the activities of firefly and *Renilla* luciferases to be measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a "glow-type" luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated by adding Stop & Glo Reagent to the same tube. As both measurements can be made from the same sample of lysate, there is a reduction in variation and simplification of the experimental procedure over other methods (e.g. ß-galactosidase). Six biological replicates were included in each experiment and this was repeated in 3 independent experiments.

5.4.2 Results

The two plasmids, identical with the exception of a single base change at position -1651 (T/C) were compared. Transfection efficiency is influenced by the number and density of cells in each well and in order to control for this variable, constructs were co-transfected with pGL4.73, a plasmid which produces renilla luciferase. The results are expressed as firefly luciferase activity (relative light units)/ renilla luciferase activity (relative light units). There were six biological replicates in each experiment and the experiment was repeated three times. A graph showing representative results is displayed in Figure 5-3.

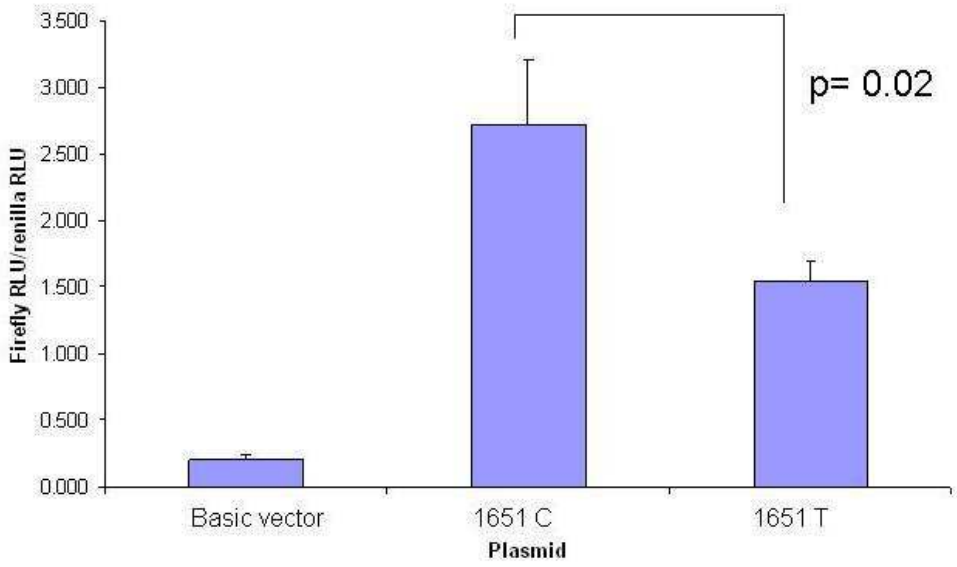


Figure 5-3 Basal expression of -1651 C and T constructs.

H295R cells were transfected with luciferase reporter constructs. Basic plasmid contains the pGL3 empty vector only. WT CC plasmid is TT at position -1651 and 1651C plasmid is CC at position -1651. Results are displayed as relative light units (RLU) of firefly luciferase relative to RLU renilla luciferase. Data expressed as mean plus standard deviation of the mean, t-test. Representative graph of 3 independent experiments, each with n=6.

These results demonstrate that the C allele (1651 C basal) has an approximately 80% increased transcriptional activity compared to the T allele (WT CC basal) under basal conditions. This is statistically significant with a p value of 0.02. The transcriptional activity of the empty vector (Basic Basal) is shown to demonstrate that the transcriptional activity arises as a result of the insertion of 1.8kb of the *CYP11B2* promoter and not the vector backbone.

The response of the reporter constructs to stimulation with trophins of aldosterone was assessed and results are shown in Figure 5-4.

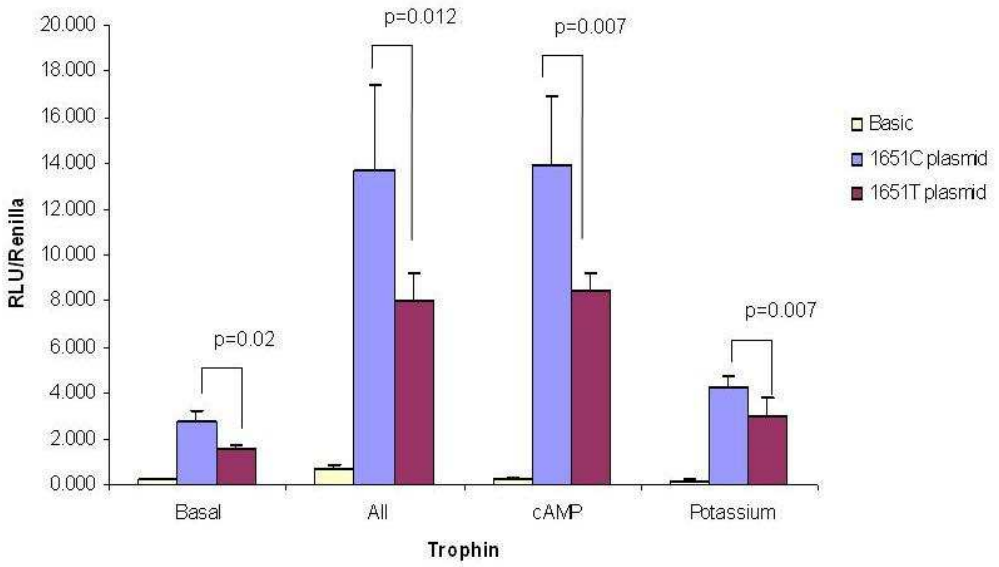


Figure 5-4 Basal and stimulated expression of -1651 C and T constructs.

H295R cells were transfected with luciferase reporter constructs. Basic plasmid contains the pGL3 empty vector only. WT CC plasmid is TT at position -1651 and 1651C plasmid is CC at position -1651. Reporter constructs were studies under basal conditions and in response to angiotensin II ($1x10^{-7}$M), Bu_2cAMP (1mM) and potassium (22mM). Results are displayed as relative light units (RLU) of firefly luciferase relative to RLU renilla luciferase. Data expressed as mean plus standard deviation of the mean, t-test. Representative graph of 3 independent experiments, each with n=6.

The difference in transcriptional activity between the constructs carrying the T and C allele at position -1651 is present under all conditions and particularly exaggerated with stimulation with angiotensin II and Bu_2cAMP. Figure 5-5 displays this data as fold change and this clearly demonstrates that the change in transcriptional activity in response to stimulation with all trophins does not differ between the constructs with the T or C allele. It does not appear, therefore that the response to trophins is altered by the allele at -1651 but rather there is an increased basal transcription associated with the C allele at position -1651 which is exaggerated when transcription is increased by stimulation with trophins.

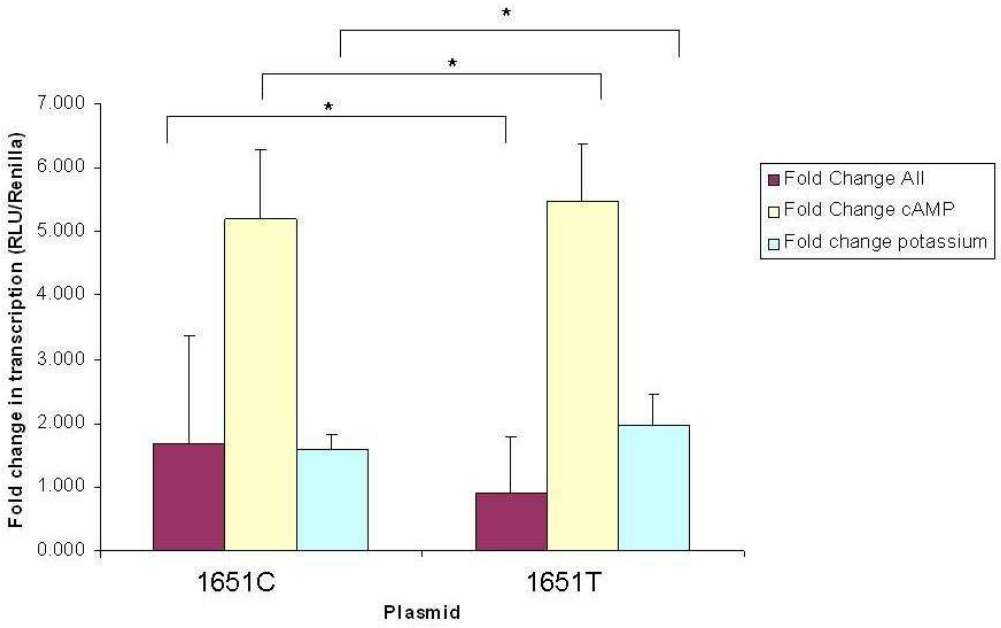


Figure 5-5 Fold change in transcriptional activity of of -1651 C and T constructs.

Graph shows comparison of C allele (1651C plasmid) and T allele at -1651 (WT CC plasmid) in response to stimulation with angiotensin II ($1x10^{-7}$M), Bu_2cAMP (1mM) and potassium (22mM). Data expressed as mean and plus standard deviation of the mean, t test. * indicates no statistically significant change.

5.4.3 Discussion

The reporter gene assays described above are useful biological tools to assess transcriptional activity in an in vitro system. However, there are a number of considerations when interpreting the results. Transfection can vary considerably from well to well, both as a result of variation in cell number and density as well as a result of cell death due to toxicity from the transfectant. The H295R cells proved to be difficult to transfect and optimisation was a lengthy process. Toxicity and cell death remained a problem and as a result, 6 biological replicates were included in each experiment to ensure that adequate numbers of representative wells were available for analysis at the end of the experiment. The use of the dual luciferase assay where both renilla and firefly luciferase were measured on one sample of lysate meant that this number of biological replicates was feasible. As a result of the lengthy optimisation procedure, the large number of biological replicates and the use of a robust method for controlling for transfection efficiency, the variability in transcriptional efficiency under basal conditions were minimised. The results were reproducible

and convincing. However, the response to stimulation was more variable and greater variability as can be observed in the wider error bars for these experiments. There may be an alteration in response to trophins (in particular in response to angiotensin II) which is obscured as a result of the limitations of the experimental model. However, this is not supported by the data presented above.

In summary, it has been demonstrated that a single base change at position -1651 in the promoter region of the *CYP11B2* gene leads to an alteration in transcriptional activity under basal conditions but does not induce a greater responsiveness to trophins. This is consistent to some extent with the *in vivo* results in Chapter 6 which demonstrated that subjects who were homozygote for the T allele at position -344 (corresponds to the C allele at position -1651) had a higher excretion of tetrahydroaldosterone that those who were homozygote for the C allele at -344, but that there was no demonstrable difference in response to trophins. Clearly the complexities of the in vivo system are very poorly reproduced in the reporter gene assays described above; nevertheless the consistency of the results gives encouragement for further investigation.

5.5 Electromobility shift assay

Having established that there is allelic dependant increase in transcriptional activity at the -1651 site, further work was undertaken to establish the mechanism. The electromobility shift assay, or gel retardation assay, is based on the principle that a complex of double stranded oligonucleotide and protein will move more slowly through a polyacrylamide gel under an electric current than free double stranded oligonucleotide (Garner and Revzin, 1981) (Figure 5-6). Free oligonucleotides and oligonucleotide: protein complexes can be detected if they are end labelled with $\gamma^{32}P$. As H295R cells have been demonstrated to produce aldosterone they must also produce the nuclear proteins necessary to drive aldosterone synthase transcription, therefore nuclear extracts from H295R cells were hybridised with oligonucleotides containing contrasting alleles at the site of polymorphic variation (-1651 base pairs upstream of the transcription start site). The hypothesis that there will be a difference in the bands seen on a polyacrylamide gel in the oligonucleotide containing the C allele at -1651 compared to the T allele at -1651 was tested in the following experiments.

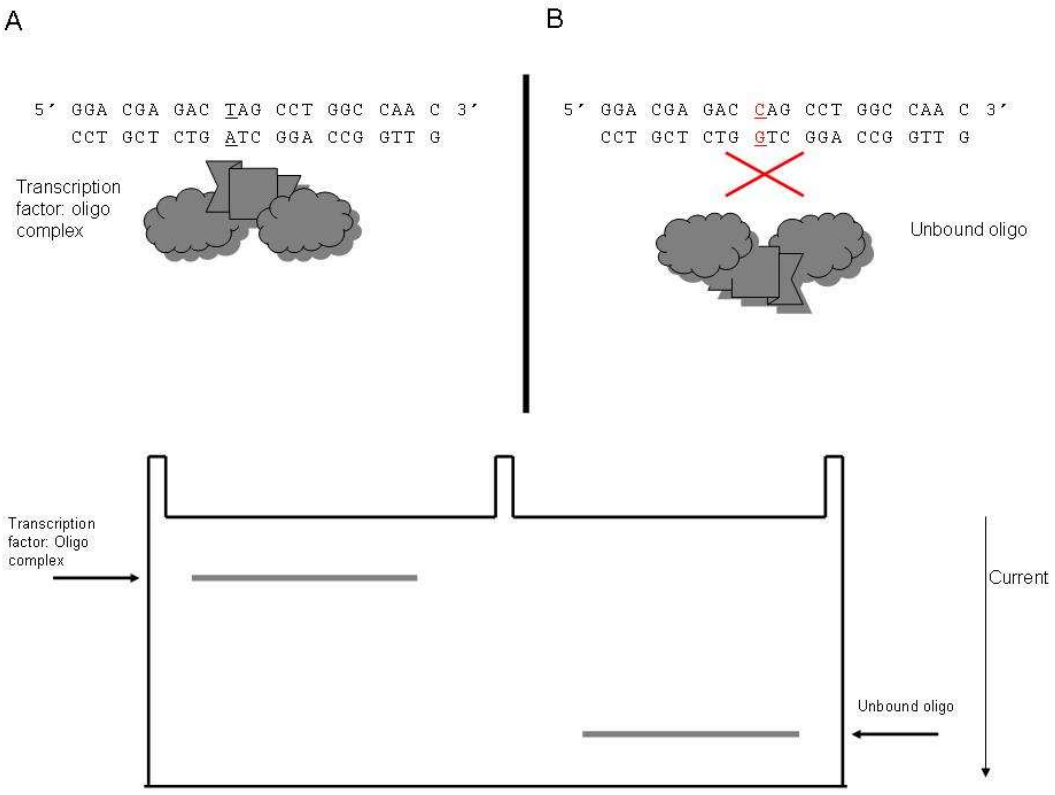


Figure 5-6 Principles of the electromobility shift assay.

The oligonucleotide containing the T allele at -1651 is shown in panel A and the oligonucleotide containing the C allele in panel B. If the single base change obliterates a binding site for a transcription factor the free probe in panel B will move more rapidly through the gel than the probe bound to transcription factor.

5.5.1 Methods

Cells were lysed and cellular compartments fractionated as described in section 2.4.1, according to a method based on Dignam et al (Dignam et al., 1983). Oligonucleotides containing contrasting alleles at the site of polymorphic variation were commercially obtained (Eurofins MWG Operon, Ebersberg, Germany). Oligonucleotides were end labelled with γ^{32} P as described in section 2.4.3.

Oligonucleotides were incubated with nuclear extracts containing transcription factors from H295R cells obtained under 3 different sodium chloride conditions as well as with and without sodium orthovanadate, a phosphotase inhibitor. Culture conditions for the H295R cells are described in Chapter 2 and the cells were lysed without stimulation at passage number 22.

5.5.2 Results

The results of the electromobility shift assay are shown in Figure 5-7. Firstly, it can be seen that the phosphorylation state of the proteins significantly affects binding. Bands of the lowest molecular weight (marked arrow 1) are consistent regardless of the presence or absence of sodium vanadate. However, the remainder of the complexes are significantly dependant on the phosphorylation state. This is particularly striking with regard to the complexes marked arrow 3 which are not apparent under any sodium chloride concentration in the presence of sodium vanadate.

The sodium chloride concentration of the extraction buffer also has a strong effect on binding. This is best seen in the highest molecular weight complexes (arrow marked 2) where in the presence and absence of sodium vanadate, the strongest binding is seen with the extraction buffer of 250mM sodium chloride. The most consistent difference between the T and C allele, in the presence and absence of sodium vanadate and across all sodium chloride concentrations can be seen in the complexes of the lowest molecular weight (arrow marked 1). Strong protein- DNA complexes of a low molecular weight can be seen in all lanes with probes containing T allele with only very weak binding seen in the lanes with C allele probes.

The relevance of the variation in binding with phosphorylation and in the context of varying concentrations of sodium chloride is not clear. However, the main aim was to assess if there was a difference in DNA: protein binding between oligonucleotides containing the T and the C allele and this has been clearly demonstrated. It is somewhat unexpected that greater binding is seen in the T allele as the plasmid carrying this allele demonstrated less transcriptional activity and it is possible that the protein bound to the T allele acts as a transcriptional repressor in this context.

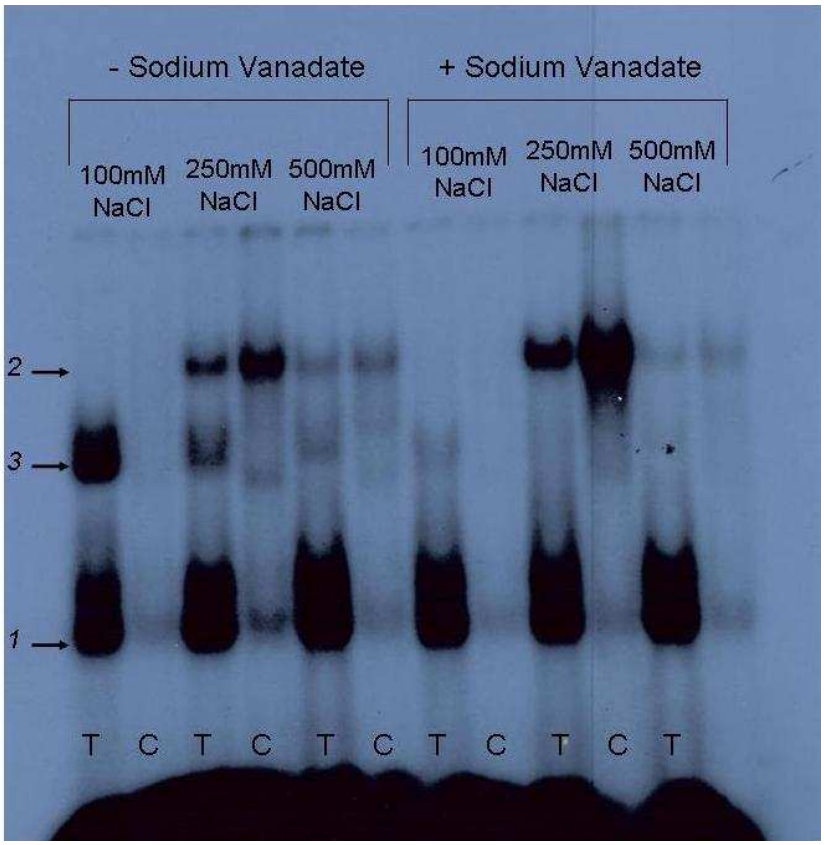


Figure 5-7 Electomobility shift assay.

γ32P end labelled oligonucleotide probes, spanning polymorphism at position 373 (-1651) were hybridised with nuclear extract from H295R cells extracted under varying salt conditions with and without the presence of sodium orthovanadate (a tyrosine phosphatase inhibitor). Complexes were run on 6% gel and film exposed for 4 days at -80℃.

5.5.3 Discussion

The in vitro model system of reporter gene assays demonstrated that a single base change of a T to a C allele at position -1651 causes increased transcriptional activity. The substitution of a T allele for a C allele at position -1651 was predicted to alter binding of numerous transcription factors *in silico* but the EMSA demonstrated that there is indeed altered protein: DNA binding in this region in an *in vitro* system. However, the identity of the proteins bound to the DNA can not be identified as it is not clear whether the bands contain only one transcription factor or a complex of numerous factors, making the molecular weight an unreliable guide.

One approach to identifying the transcription factor or factors in the protein: DNA complex is by the use of a “super-shift assay”. This involves incubating the

protein: DNA complex with an antibody to a candidate protein. If the protein has been correctly identified, the larger protein: DNA: antibody complex will move even more slowly through the gel under current and the band will be observed "shifted" higher up the gel. An antibody to SF-1 is available but based on the bioinformatics study, there were a further seven transcription factors which were predicted to bind to the T allele only and a there are a further nine that have reduced but not absent affinity for the C allele. Even if all 16 "supershift" assays were performed, given the uncertainties regarding the bioinformatics databases and the possibility of false negative results described earlier, there was significant concern that this approach could overlook the correct protein.

An alternative approach is to identify the protein by direct sequencing. This can be achieved by using a mass spectrometer to separate ions by their mass: charge ratio. The band from the EMSA gel can be isolated and the protein digested with a proteolytic enzyme (trypsin) which selectively cleaves the protein at specified amino acid sites. The resulting products from the proteolytic digestion are then separated on a liquid chromatography column and transferred to the mass spectrometer. The protein fragments are subjected to electron spray ionisation and broken down to form the individual component amino acids which are identified by the mass spectrometer. From these data the identity of the protein can be confirmed by checking the sequence tag and molecular weight against a database. This technique was felt to be more robust and reliable and was carried out by Dr W Sands (MRC Blood Pressure Group). Nuclear extracts prepared as described previously were incubated with 5'biotinylated double-stranded DNA probes and streptavidin-agarose beads. The protein-DNA complexes were separated on SDS-PAGE gel and following trypsin digestion, peptides were analysed by tandem mass spectrometry by FingerPrints Proteomics Facility College of Life Sciences, University of Dundee.

Two peptides were identified which bound to the T oligo only, apyrimidinic endonuclease (APE 1) and Heterogeneous Nuclear Ribonucleoprotein K (HNRNPK). Although neither of these is known to be involved in transcriptional regulation of *CYP11B2*, they both have known generic functions as transcriptional regulators. APE 1 has functions as a redox factor, maintaining transcription factors in an active reduced state (Evans et al., 2000), and HNRNPK interacts with RNA polymerase II transcription machinery to stimulate

transcription(Michelotti et al., 1996). The availability of a commercially available APE 1 inhibitor made it an attractive candidate for further study.

5.6 APE 1 inhibitor assay

5.6.1 Methods

Plasmids were prepared and sequence confirmed as previously described (section 2.3.2-2.3.5). The protocol for transient transfection was not altered. Transfected H295R cells were incubated under basal conditions, and with the addition of agonists of aldosterone production: angiotensin II ($1x10^{-7}$M), Bu_2cAMP (1mM) and potassium (22mM). However, in this experiment, they were also incubated with either 1μM APE 1 inhibitor E3330 (Sigma- Alderich, Missoiuri, USA) or vehicle. After 24 hrs, cells were lysed and assayed for firefly and *Renilla* luciferase as previously described (section 2.4.7). Six biological replicates were included.

5.6.2 Results

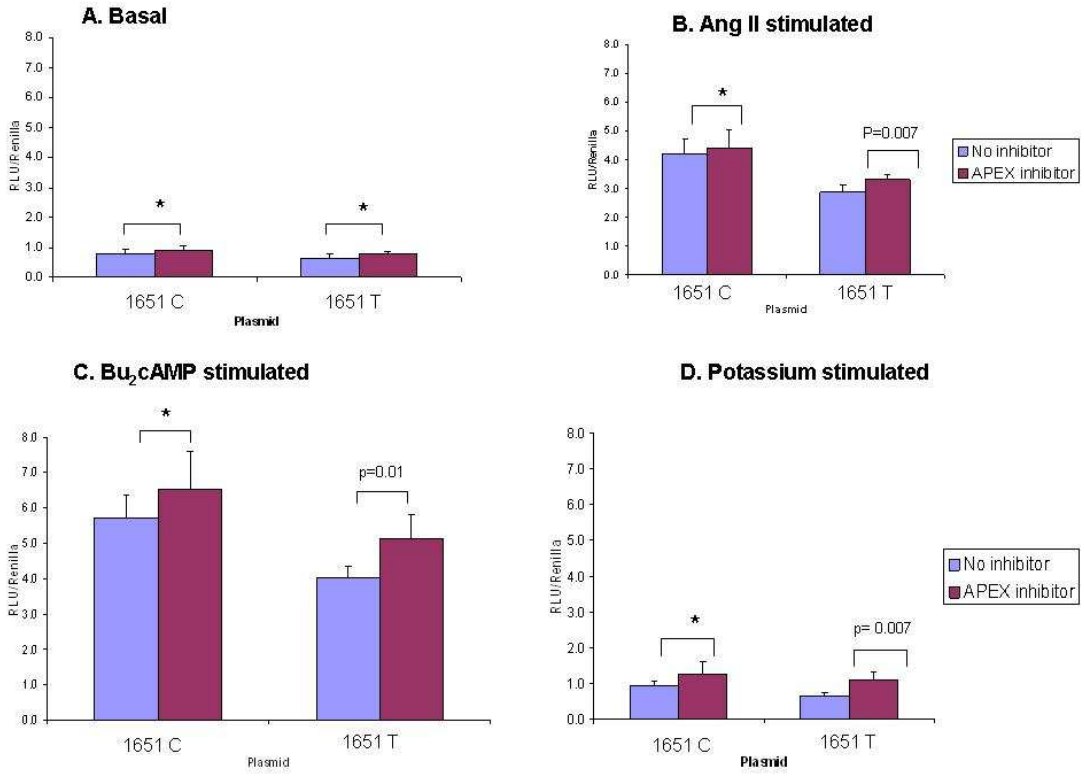


Figure 5-8 Effect of APEX-1 inhibitor on transcriptional activity of reporter constructs

Graphs show reporter constructs containing the C (1651C plasmid) and T (WT CC plasmid) allele at position -1651 under basal (Panel A), angiotensin II ($1x10^{-7}$M) (Panel B), Bu_2cAMP (1mM) (Panel C) and potassium (22mM) (Panel D). Data expressed as mean plus standard deviation of the mean, t-test. * indicates no statistically significant change. Representative graph of 3 independent experiments, each with n=6.

As previously shown in section 5.4, the plasmid with T allele at position -1651 produced less luciferase that the plasmid with C allele at -1651 under basal conditions. The addition of the APE 1 inhibitor produced a trend to increased transcriptional activity in both plasmids. There difference did not reach statistical significance under basal conditions, possibly as the transcriptional activity was too low to be able to detect a significant difference. However, under stimulated conditions (angiotensin II: Panel B, Bu_2cAMP: Panel C and potassium: Panel D) the plasmid carrying the T allele demonstrated a statistically significant increase in transcriptional activity in the presence of the APE1 inhibitor as compared to vehicle. This is consistent with the previous results and the hypothesis that the T allele binds APE 1 which functions as a

negative regulator of transcription of *CYP11B2*. Thus inhibition of this protein leads to an up regulation of transcription. The effect would be expected to be less in the C allele as no APE 1 was detected bound to the sequence containing the C allele (Section 5.5).

5.6.3 Discussion

The regulation of aldosterone production is a heritable trait (Inglis et al., 1999), yet the genetic determinants of increased aldosterone and a consequent rise in blood pressure are poorly understood, particularly at a mechanistic level. These data demonstrate a clear, plausible means by which an alteration in a single base in the promoter region of *CYP11B2* may alter transcription via allele dependent binding of a repressive transcription factor. They provide evidence that the presence of the C allele at position -1651 in the *CYP11B2* promoter is associated with decreased transcriptional activity and that the mechanism of this is likely to be mediated by the transcription factor APE 1. This hypothesis is supported by further work within our group demonstrating the association of APE1 to the promoter of *CYP11B2* in H295R cells using a chromatin immunoprecipitation assay *(personal communication, Dr W Sands)*.

APE 1 (apurinic/apyrimidinic endocunlease, also known as APEX 1, Ref-1, HAP-1) is encoded on chromosome 14 and is ubiquitously expressed. It was first described as a key enzyme in the base excision repair (BER) pathway (Levin and Demple, 1990). It responds to damage by reactive oxygen species by participating in a four step process; firstly, excision of a damaged base by a DNA glycosylase which results in the generation of an AP site due to cleavage of the N-glycosidic bond of the damaged base. APE 1 cleaves the AP site in the second step, to generate 3′ OH and 5′ deoxyribose phosphate terminus. The third reaction in the pathway involves the DNA polymerase to fill in the single nucleotide gap generated due to lesion base removal and finally the gap is sealed by the DNA ligase in the final step (Hegde et al., 2008). Essentially, APE1 functions as an “end cleaning” molecule in the repair process. It is thought that the C-terminus of the protein mediates this function and it is interesting to note that this is the most highly conserved region (Xanthoudakis et al., 1994).

However, there is evidence that APE 1 has a dual function and also plays a role as a redox co-activator in mammals (Xanthoudakis and Curran, 1992). Several transcription factors possess a redox sensitive cysteine residue which can be reduced by APE1, enhancing their DNA binding activity. Members of the CREB/ATF and AP1 family have been shown to be reduced by APE1 (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992), and these, as discussed previously, are known to bind to the CRE element in the *CYP11B2* promoter to increase transcription of aldosterone synthase. The N-terminus of the protein is thought to contain the structure necessary for the redox reactions (Xanthoudakis et al., 1994). The small molecule inhibitor of APE 1 (E3330) used in the experiments described in this chapter selectively inhibits the redox function of APE 1 (Luo et al., 2008), probably via the cysteine residue at position 65, although this remains controversial (Ordway et al., 2003; Georgiadis et al., 2008). Thus it may be that the APE 1 effect on transcriptional regulation of *CYP11B2* is mediated via a redox interaction with activating transcription factors. However, most interactions between APE and transcription factors described in the literature suggest that APE1 converts transcription factors from an oxidised to reduced state, allowing them to bind to their target promoters and switch on the transcription of genes (Tell et al., 2009) rather than functioning as a negative regulator. It is not clear therefore, how increased binding of APE1 in the *CYP11B2* T allele leads to reduced transcriptional activity. It is possible that binding of APE at the -1651 prevents it from participating in the redox reactions of CREB/ ATF -1 transcription factors, thus leading to reduced transcription but further evidence would be required to support this hypothesis. It would be interesting to repeat the reporter construct experiments described above after mutation of the CRE site; if APE 1 is acting via CREB/ATF there should be no difference between the T allele which binds APE 1 and the C allele which does not.

APE 1 has a further mode of action. It has been shown to act as a negative transcription factor in its own right and this has been demonstrated in the context of its regulation of the parathyroid hormone gene (PTH) (Okazaki et al., 1994), and the process by which APE 1 appears to regulate its own expression (Kuninger et al., 2002). In these circumstances APE 1 binds to the negative calcium response elements (nCaRE) in the promoters of these genes and the

sequence of the nCaRE site is demonstrated below along with the sequence within the *CYP11B2* promoter for comparison.

GGA CGA GAC ***T***AG CCT GGC	CYP11B2 T allele
GGA CGA GAC ***C***AG CCT GGC	CYP11B2 C allele
TTT TGA GAC AGA GTT TCA	nCaRE-B
TTT TGA GAC AGG GTC TCA	nCaRE-A

It seems that APE 1 is incapable of binding alone and must form heterodimers with other proteins. In the case of the PTH gene, it dimerised with the Ku antigen p70 and p86 to nCaRE-A (Chung et al., 1996), and with heterogeneous nuclear ribonucleoprotein L (hnRNP-L) in the APE gene itself (Kuninger et al., 2002). It is interesting that the complex that was analysed in the experiments described above identified not only APE 1 but also hnRNP-K in the association with the T allele but not the C allele. Thus, the mechanism by which APE 1 regulates the expression of aldosterone synthase may be analogous to these examples.

A potential mechanistic link between the regulation of the PTH gene and aldosterone is particular interest given the long recognised but poorly understood relationship between hyperparathyroidism and hypertension (Yu et al., 2010; Hedback et al., 1990). It could be speculated that if APE 1 was down regulated in some way in patients with hyperparathyroidism, leading to the development of parathyroid adenomas, they may also develop hypertension as a result of the lack of inhibition of aldosterone synthase. Bearing in mind that the excess of cardiovascular death appears to persist in hyperparathyroid patients even after a surgical “cure” (Hedback et al., 1991; Hedback et al., 1990), this hypothesis, while highly speculative, may be worthy of further investigation.

Attempt have been made to generate an APE 1 null mouse however these animals have been found to die *in utero* (Xanthoudakis et al., 1996). Animals heterozygous for the APE 1 allele survive to adulthood. It is particularly interesting to note that the $APE^{+/-}$ mouse is hypertensive (Jeon et al., 2004), although to date, there has been no exploration of their renin angiotensin

system and the blood pressure phenomenon is thought to be mediated at least in part by endothelial nitric oxide production and increased vascular tone. It would be interesting to investigate whether the $APE^{+/-}$ mouse has evidence of up-regulated aldosterone production and measuring plasma renin, aldosterone and adrenal *CYP11B2* expression would help to elucidate the potential link.

Clearly the issues specific to the investigation of steroidogenesis regarding animal models that were discussed at greater length in chapter 4 would remain problematic. It is therefore pertinent to note that a case control study examining the association of polymorphic variation in the human APE 1 gene and hypertension has suggested that it may be a susceptibility gene for high blood pressure (Naganuma et al., 2010). Although there are methodological flaws in this report and importantly, it remains a single association study that has not thus far been replicated in another population, it contributes to the body of evidence to suggest that the role of APE 1 in hypertension deserves further analysis.

5.7 Conclusion

The studies described above suggest a plausible molecular mechanism to explain the association between genetic variation in the aldosterone synthase gene and hypertension. By providing evidence that a single nucleotide change can alter transcriptional activity and subsequent identification of possible protein: DNA complexes, they suggest a novel hypothesis of regulation of aldosterone synthase transcription. A number of exiting avenues for future investigation and clarification of the role of APE1 in the regulation of aldosterone synthase and subsequent development of hypertension have been raised. In addition, numerous questions remain regarding the mechanism by which APE 1 may exert its effect either alone or in combination with other proteins. Nevertheless, the evidence presented here makes a compelling case for further study in this area.

Phenotypic consequences of variation in the CYP11B2 promoter region on corticosteroid production in normal volunteers

6. Phenotypic consequences of variation in the CYP11B2 promoter region on corticosteroid production in normal volunteers

6.1 Introduction

Variation in the promoter region of *CYP11B2*, most commonly stratified by genotype at position -344, is associated with hypertension and a relatively higher aldosterone to renin ratio (ARR)(Sookoian et al., 2007; Lim et al., 2002); however, this relationship between genotype and biochemical phenotype is not consistent. A more reliable finding is a phenotype of reduced 11ß-hydroxylase activity (Davies et al., 2001; Freel et al., 2008; Inglis et al., 1999; Keavney et al., 2005; Freel et al., 2007). A possible mechanism for this has been identified in functional polymorphisms in the promoter of *CYP11B1*, which are in linkage disequilibrium with the polymorphism at -344 in *CYP11B2*, and are associated with reduced transcriptional activity *in vitro* (Barr et al., 2007). While this could be a plausible mechanism to explain the phenotype of inefficient 11ß-hydroxylation, the mechanism behind the development of hypertension and an elevated ARR is not apparent. One hypothesis is that the reduced 11ß-hydroxylation leads to a subtle, long term rise in ACTH drive to the adrenal cortex which maintains cortisol at appropriate levels. This increased ACTH drive leads to hyperplasia of the adrenal gland and increased synthetic capacity for corticosteroids either at basal levels or in response to trophins. An alternative hypothesis is that the functional variations in the *CYP11B1* promoter is in linkage disequilibrium with further functional variations at the promoter region of the *CYP11B2* gene, as identified in the previous chapter. These may lead to increased transcriptional efficiency of aldosterone synthase and increased aldosterone production, and the digenic phenomenon where the phenotype depends on functional change at more than one point across the locus. Given that there is little robust evidence to suggest an absolute genotype-dependant difference in the end products of the corticosteroid pathway (aldosterone is not elevated but the ARR is; cortisol is not altered but the ratio of 11deoxycortisol to cortisol is higher), it seems likely that the difference is associated with altered enzyme efficiency and responsiveness to stimulation or suppression. These relationships are currently poorly understood.

The effects of environmental factors are likely to be important in the development of the phenotypic consequences of the genotype. In particular, the effect of dietary sodium intake may be a crucial factor in the development of the ultimate physiology. For example, the strain of Dahl hypertensive rat which has 5 mutations in the in 11ß-hydroxylase gene (Cicila et al., 2001), develops hypertension only in response to a high salt diet. Similarly, an animal model of increased aldosterone synthase activity develops a phenotype of hypertension and relative mineralocorticoid excess only under high salt conditions (Makhanova et al., 2008).

The studies set out in this chapter aim to explore these issues further in a study of the regulation of corticosteroid production in normal volunteers.

6.2 Aims

To examine the aldosterone production of a group of normal volunteers

1. Under standard salt intake in response to stimulation of their (RAAS) renin-angiotensin-aldosterone system by angiotensin II and adrenocorticotrophin stimulating hormone

2. Under conditions designed to suppress the RAAS (high salt intake) and in response to stimulation of their (RAAS) renin-angiotensin-aldosterone system by angiotensin II and adrenocorticotrophin stimulating hormone

To examine the effect of genetic variation at the *CYP11B1/2* locus on these responses.

6.3 Methods

6.3.1 Study volunteers and protocol

Normal volunteers were recruited by advertisement in local media as well as around the university campus. In order to recruit across a range of ages, the university's retired staff was also contacted via e mail. Volunteers were required to be in good health and between the ages of 18-70 at the time of recruitment and not on any antihypertensive or steroid containing medication. Full exclusion criteria are listed in Table 2-3. It was calculated that 60 volunteers would be

needed to achieve 80% power to detect a difference in plasma concentration of aldosterone of 20% in response to trophins of aldosterone production, with α of 0.05.

Full details of the study protocol are described in Chapter 2.5.2 and Appendix 7.5. In brief, the design was that of a double blind cross over study. The subjects were asked to adhere to a low salt diet for 5 days and were given either salt tablets (aim of total salt intake of 200mmol/day) or placebo (aim of total salt intake of 80mmol/day). On day 3 they began a 24hr urine collection and on day 4 they attended the Clinical Research Facility. Intravenous cannulation was performed and, after 30 minutes recumbent rest, blood was drawn for steroid measurements. ACTH was administered at a rate of 1ng/kg/min and after 30 minutes the infusion was stopped and further blood sampling was performed. After a further 30 minutes rest (60 mins from baseline), a third blood sample was obtained. The volunteers returned on day 5 for the same protocol at which time, the infusate was angiotensin II, administered at a rate of 3ng/kg/min. Blood pressure was monitored at 10 minute intervals throughout both infusions. At least one week for a wash out period was allowed before volunteers crossed over to the salt/ placebo arm of the study.

6.3.2 Genotyping and Haplotype analysis

Subjects were genotyped across 8 polymorphisms in *CYP11B2* promoter region as well as the Intron 2 conversion and two polymorphisms in the *CYP11B1* promoter (1889G/T and -1859 A/G). Details of the DNA extraction, PCR and sequencing reactions, as well as haplotype analysis, are given in Chapter 2.5.7 and 2.5.8.

6.3.3 Corticosteroid phenotype measurements

24 hour urine samples were collected in plain containers and the volume measured before being aliquoted and stored at -20°C without preservatives. Steroid metabolites were measured by gas chromatography using the method of Shackleton (Shackleton, 1993) with minor modifications described in Chapter 2.5.4. Blood samples for plasma steroids analysis was drawn in lithium heparin tubes and separated by centrifugation at 4°C and snap frozen at -80°C. Plasma steroids were extracted from 3ml plasma using Chem Elute cartridges (Varian, CA, USA) and eluted with dichloromethane. The eluates were evaporated to

dryness under nitrogen and reconstituted in 60ul acetylonitrile. Aliquots (20ul) were injected into a Polaris 5 micron, 150mm x2mm C-18-A reversed phase HPLC column. Identification and quantification were accomplished by tandem mass spectrometry using a Varian 1200L mass spectrophotometer with a triple quadropole detector. The internal standard was 16ßmethylprednisalone.

6.3.4 Renin measurements

Blood was drawn in EDTA tubes and plasma separated by centrifugation at room temperature for 15mins at x3000g and snap frozen at -80°C. Care was taken to ensure the samples did not thaw until renin measurements were undertaken in order to avoid cryoactivation. Plasma renin concentration was measured by means of a Diasorin Liaison® immunochemiluminometric analyser (DiaSorin Ltd, Wokingham, Berkshire, UK) (Dorrian et al., 2010).

6.3.5 Urinary electrolytes

Urinary sodium was measured in the 24 hr urine sample by the Department of Biochemistry (Gartnavel General Hospital, Glasgow, UK) using an ion selective electrode.

6.3.6 Data analysis

Biochemical data that were not normally distributed were log transformed (log $_{10}$) to enable the option of the use of parametric hypothesis tests. Statistical analysis was carried out by Minitab 12.21 and Graph Pad Prism 4. Haploview v4.2 software was used to analyse genotype frequencies for Hardy Weinberg equilibrium and to calculate the haplotype structure.

6.4 Results

6.4.1 Demographic characteristics

The demographic characteristics of the volunteers are shown in Table 6-1, stratified according to their genotype at the -344 locus. The numbers of men and women were equal and the gender ratio did not vary significantly across the genotype groups. Similarly, age, weight and blood pressure were not statistically different between the genotyped groups, although there was a trend to younger age in the -344 CC group.

Demographic characteristics -344 Polymorphism	All subjects N=60	-344 CC Subjects N=13	-344 TC Subjects N=33	-344 TT Subjects N=14	*p*
*Age, y	51 (32- 67)	36 (30-58)	57 (30-65)	57 (49-69)	*NS*
Gender, Male Female	 27 33	 6 7	 15 18	 6 8	*NS*
*Weight, kg	70 (61 -76)	65 (57-85)	71 (62-76)	71 (68-77)	*NS*
†SBP, mmHg	127 (+/- 13.91)	121.46 (+/- 14.07)	128.85 (+/-14.33)	127.29 (+/- 12.22)	*NS*
†DBP, mmHg	76 (+/- 10.26)	74.85 (+/- 8.38)	74.76 (+/- 11.51)	80.43 (+/- 7.66)	*NS*

Table 6-1 Demographic information on all study subjects.

Subjects divided according to -344C/T. *Median and IQ range, Kruksal-Wallis. †Mean and StDev, ANOVA.

6.4.2 Genotype and Haplotype data

The polymorphisms in the promoter region of *CYP11B2*, as well as in the intron 2 region and the promoter region of *CYP11B1* were sequenced and the results are shown in Table 6-2. The genotyping success rate was high (between 95-100%) and all SNPs were in Hardy-Weinberg equilibrium.

#	Name	Position	Obs. Het	Pred. Het	HWE p	% Genotyped	MAF	Alleles
1	B1-1859	143963455	0.52	0.48	0.84	100	0.408	A:G
2	B1-1889	143963486	0.48	0.48	1	100	0.392	G:T
3	B2_IC	143997133	0.50	0.48	1	100	0.400	A:C
4	B2 -344	143999600	0.55	0.50	0.65	100	0.492	T:C
5	B2 -470	143999726	0.55	0.50	0.65	100	0.492	C:T
6	B2 -645	143999901	0.39	0.35	0.74	98.3	0.229	C:T
7	B2 -663	143999919	0.54	0.50	0.74	98.3	0.492	T:A
8	B2 -1472	144000727	0.58	0.50	0.38	95	0.482	A:G
9	B2 -1513	144000786	0.56	0.50	0.53	95	0.474	T:C
10	B2 -1651	144000906	0.56	0.50	0.53	95	0.474	T:C
11	B2 -1667	144000922	0.56	0.50	0.53	95	0.474	G:C

Table 6-2 Characteristics of polymorphisms in CYP11B2 and CYP11B1.

Position in base pairs derived from NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP) Genome build 37.1, Group term GRCh37. Obs.Het, observed hetrozygosity; Pred.Het, predicted hetrozygosity; HWE, Hardy Weinberg Equilibrium p value; MAF, minor allele frequency.

Haplotypes were generated with Haploview V4.2 and the linkage disequilibrium plot and haplotype frequencies are shown in Figure 6-1. The high degree of linkage disequilibrium and the haplotype frequencies is consistent with that found in Chapter 3.

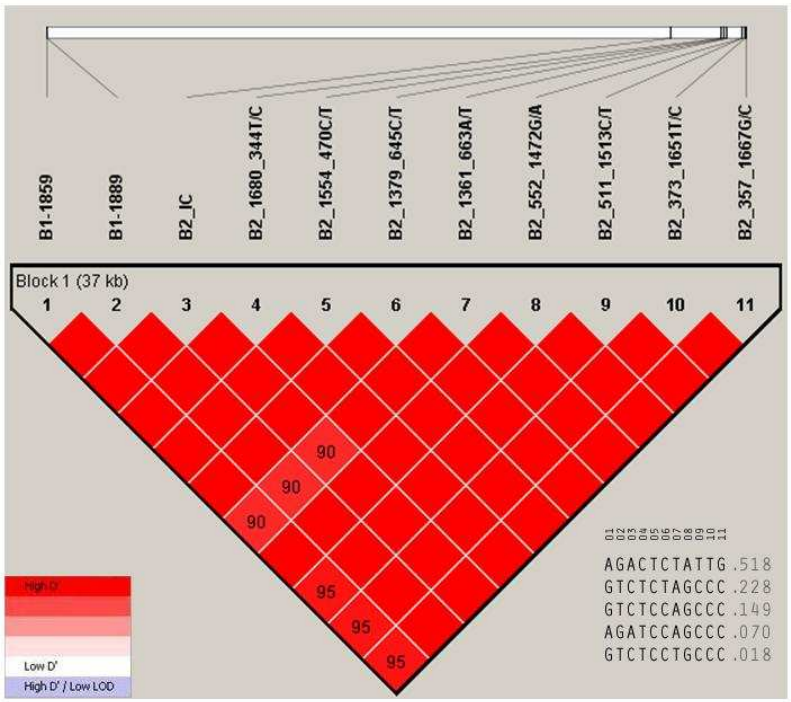


Figure 6-1 Haplotype frequencies and Linkage disequilibrium plot of 60 normal volunteer subjects.

Values shown are R^2 values.

From these results, it can be seen that in this population the polymorphisms in the promoter region are in such strong linkage disequilibrium that any individual SNP can be used as a proxy for several other SNPS with the exception of the polymorphism at position -645 and the polymorphism at -663 of *CYP11B2*. However, the minor allele frequency of these haplotypes is low. Therefore, the phenotypic data presented in this chapter will be stratified according to the allele present at position -344 in the *CYP11B2* promoter as a proxy for the other 5 SNPS (-470, -1472, -1513, -1651 and -1667) that are in 100% linkage disequilibrium.

6.4.3 Standard salt and high salt study phase

The urinary sodium collections demonstrate a clear difference in the mean urinary sodium excretion between the sodium loading phase and the standard sodium phase, although the range of urinary sodium was wide in both phases of

the study. As expected, plasma aldosterone and renin concentrations are lower under high salt conditions than under standard salt intakes. While the difference between tetrahydroaldosterone excretion rate in the high and low salt arms was not statistically different, the trend was for excretion rate under high salt conditions to be lower than under standard salt conditions. The 24 hour urine collections were commenced on day 3 and completed by day 4; this may have been too early for the subjects to have achieved salt balance.

	High salt	Standard salt	*p*
Urinary Na mmol/24 Hr	200.73 (+/-66.7)	97.99 (+/- 40.35)	*<0.001*
***Plasma aldosterone ug/ml**	1.93 (+/-3.17)	3.66 (+/-3.11)	*<0.001*
Tetrahydroaldosterone ug/ 24 Hr	51.85 (+/-20.53)	55.05 (+/-20.14)	*NS*
***Plasma renin concentration mIU/l**	8.00 (+/-1.95)	15.23 (+/-1.99)	*<0.001*

Table 6-3 Biochemical effect of high salt and standard salt conditions.

Measurements made under high salt and standard salt conditions for all subjects shown as mean and standard deviation. Data marked * were $\log_{10}$ transformed prior to paired t-test.

6.4.4 Measurement of aldosterone production stratified by genotype

The hypotheisis that aldosterone production varies in association with variation in the *CYP11B2* promoter was examined by measuring plasma aldosterone concentration, plasma aldosterone to renin ratios and 24hr urinary excretion of aldosterone metabolites. As demonstrated in 6.4.2, given the high LD at the *CYP11B1* and *CYP11B2* locus, the -344 polymorphism can be used as an effective proxy for the 5 other SNPs in the promoter region. The plasma aldosterone and aldosterone to renin ratios measurements under standard salt conditions are illustrated in Figure 6-2 and Figure 6-3.

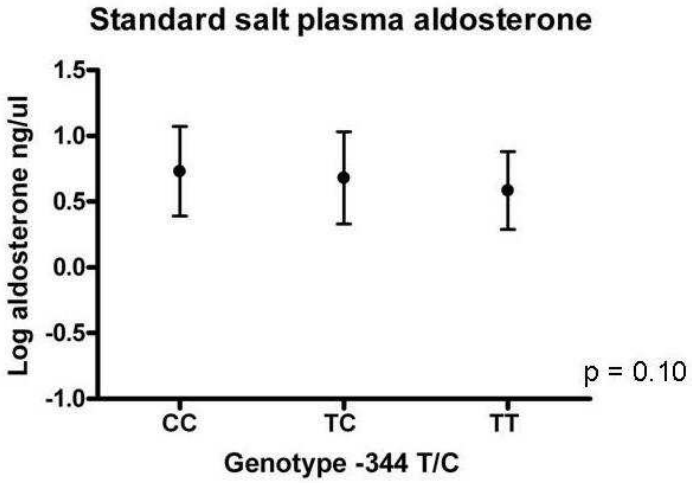


Figure 6-2 Log_{10} plasma aldosterone concentration under standard salt conditions.
Data displayed as mean and standard deviation, ANOVA.

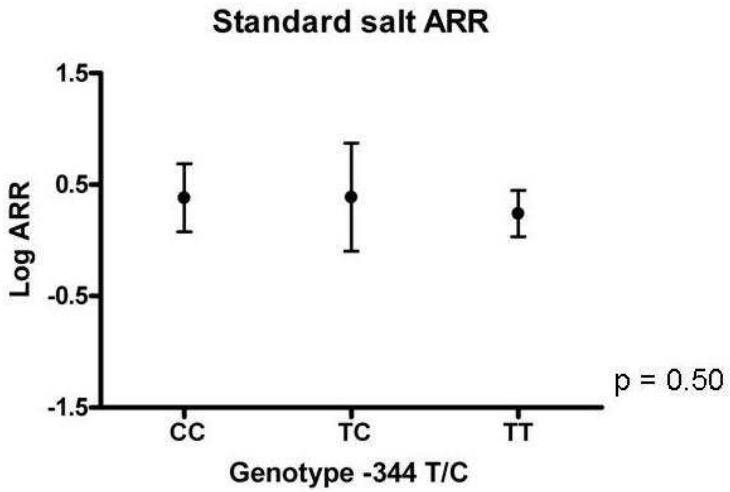


Figure 6-3 Log $_{10}$ plasma aldosterone concentration: plasma renin concentration ratio standard salt conditions.
Data displayed as mean and standard deviation, ANOVA.

It can be seen that there is no significant difference between the plasma aldosterone levels or the aldosterone/ renin ratio according to genotype at the *CYP11B2* promoter. The 24hr urinary excretion of tetrahydroaldosterone is displayed according to genotype in Figure 6-4. There is a genotype-dependent increase in THAldo excretion which rises in a "dose-response" manner.

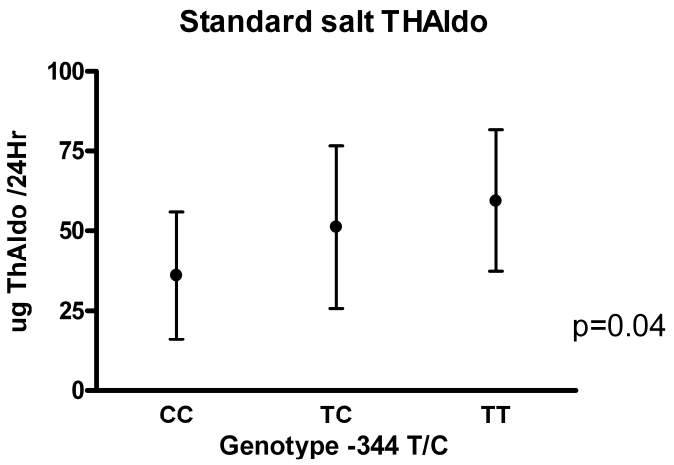


Figure 6-4 Urinary 24 Hr THAldo excretion under and standard salt conditions. Data displayed as mean and standard deviation, ANOVA

The 24hr urinary excretion rate is a more robust measurement of aldosterone production given that it integrates aldosterone production over a longer period of time than a single plasma measurement. While attempts were made to standardise the conditions under which the plasma aldosterone concentration was sampled with regards to posture, the level may have been affected by other factors, e.g. diurnal rhythm.

6.4.5 Effect of salt loading on aldosterone

The effect of salt on suppression of aldosterone was examined in both plasma and urine to test the hypothesis that variation in the *CYP11B2* promoter may affect suppressibility of aldosterone in response to salt. Change in urinary tetrahydroaldosterone (THAldo) excretion rate in response to salt suppression is displayed in Figure 6-5 and change in plasma aldosterone in response to high salt intake is displayed in Figure 1-6.

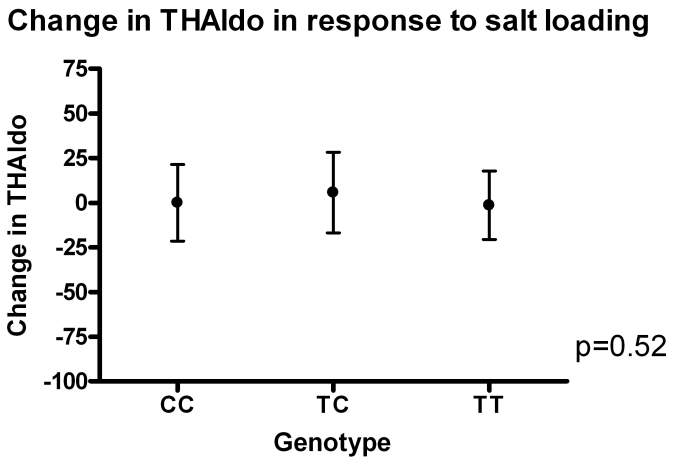


Figure 6-5 Change in urinary tetrahydroaldosterone excretion in response to salt loading. Data displayed as mean and standard deviation, ANOVA.

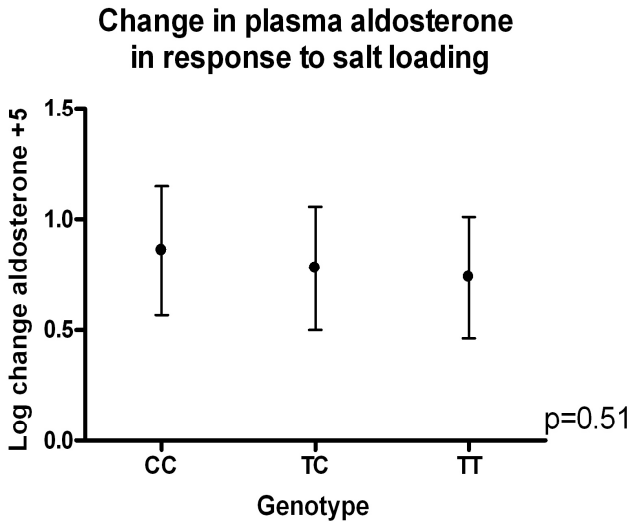


Figure 6-6 Change in plasma aldosterone concentration in response to salt loading. Data displayed as mean and standard deviation of Log_{10} transformed data, ANOVA.

Neither parameter displayed a genotype dependant difference in the suppression of aldosterone levels in response to salt. The change in plasma and urinary THAldo excretion in response to salt suppression is small across all genotypes and as such, the study may not have been large enough to detect a significant difference.

6.4.6 Effect of stimulation of the CYP11B2 pathway by trophins of aldosterone

The hypothesis that variation in the promoter of the *CYP11B2* gene may be associated with altered response to trophins of aldosterone was also tested. The response of plasma aldosterone to angiotensin II under standard salt and high salt conditions are shown in Figure 6-7.

Neither raw data, nor log transformed data were normally distributed which excluded analysis by ANOVA; therefore non parametric testing was employed (Kruskal Wallis test). As expected, there was a marked increase in plasma aldosterone measurements following the administration of angiotensin II (30 mins) which then fell over time (60 mins), likely to be due to increased expression of *CYP11B2* (Spyroglou et al., 2009). There is no observable difference in response to angiotensin II between individuals carrying contrasting alleles in the *CYP11B2* promoter, either under salt loaded or standard salt conditions. The rise in aldosterone in response to angiotensin II was blunted under high salt conditions but there was no gene dependant effect, as assessed by comparison of the difference in response to angiotensin II (baseline and 30 min) under high and low salt conditions (Table 6-4) (see Matthews et al (Matthews et al., 1990) for a discussion of the use of summary statistic in this context).

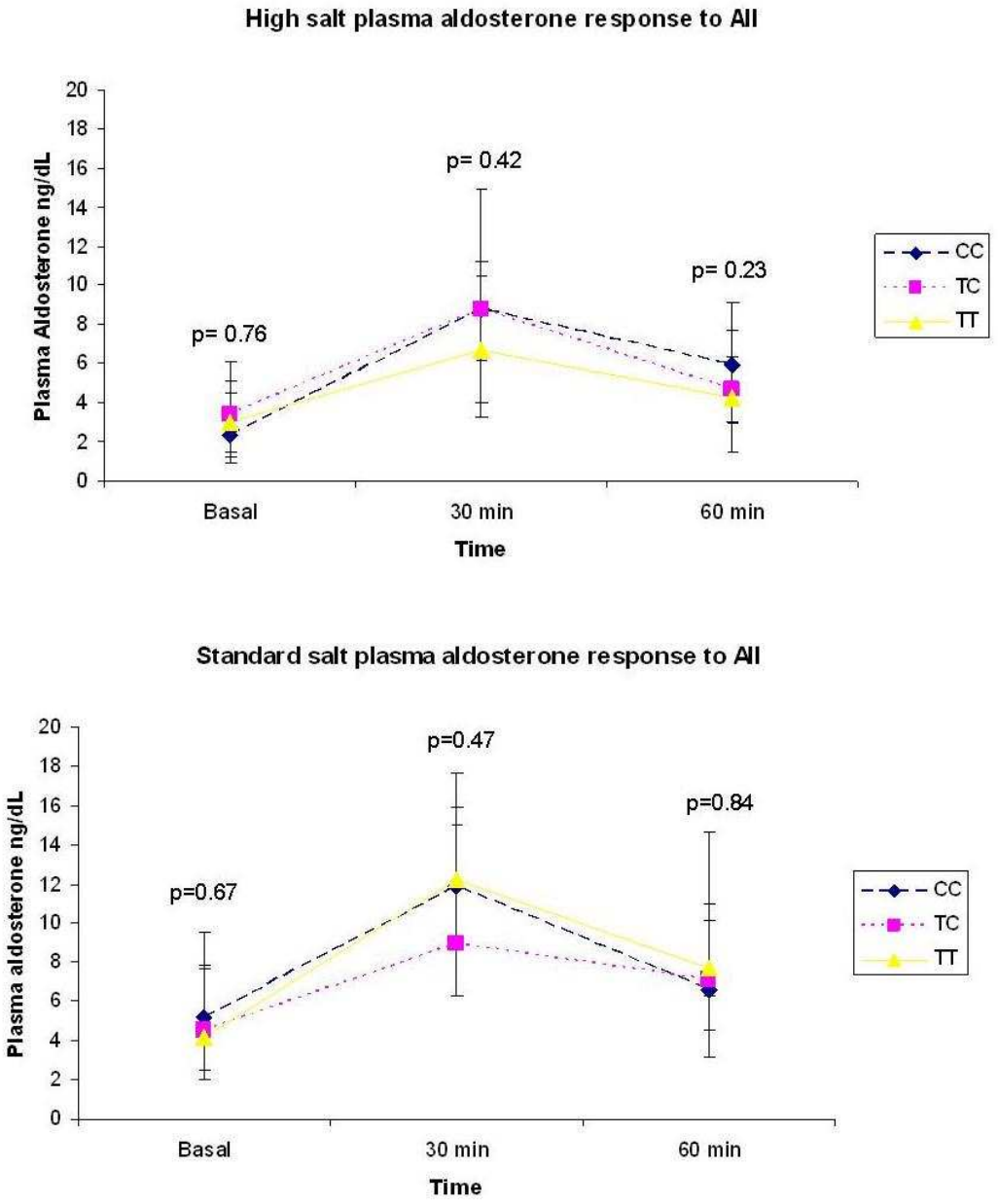


Figure 6-7 Response to angiotensin II (3ng/kg/min) under high and standard salt conditions.

Due to the non-normal distribution of the data, graphs are displayed as median and inter-quartile range. Hypothesis tests (Kruskal Wallis) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

GENOTYPE CYP11B2 -344	MEAN DIFFERENCE IN RISE IN PLASMA ALDOSTERONE	P VALUE
CC	8.38	0.11
TC	5.62	
TT	5.56	

Table 6-4 Difference in rise in plasma aldosterone following angiotensin II stimulation between high salt and low salt conditions.

Hypothesis test (AVOVA) was performed comparing response for each genotype.

The response of plasma aldosterone to adrenocorticotrophin stimulating hormone (ACTH) under standard salt and high salt conditions is shown in Figure 6-8. Data were similarly non-normally distributed and the same non-parametric testing was used for data analysis. Aldosterone was stimulated by the administration of ACTH, although to a lesser extent than with angiotensin II. As with angiotensin II stimulation, there was no genotype dependant difference between plasma aldosterone concentrations following ACTH stimulation either immediately following ACTH administration at 30 minutes or after a further 30 minute period.

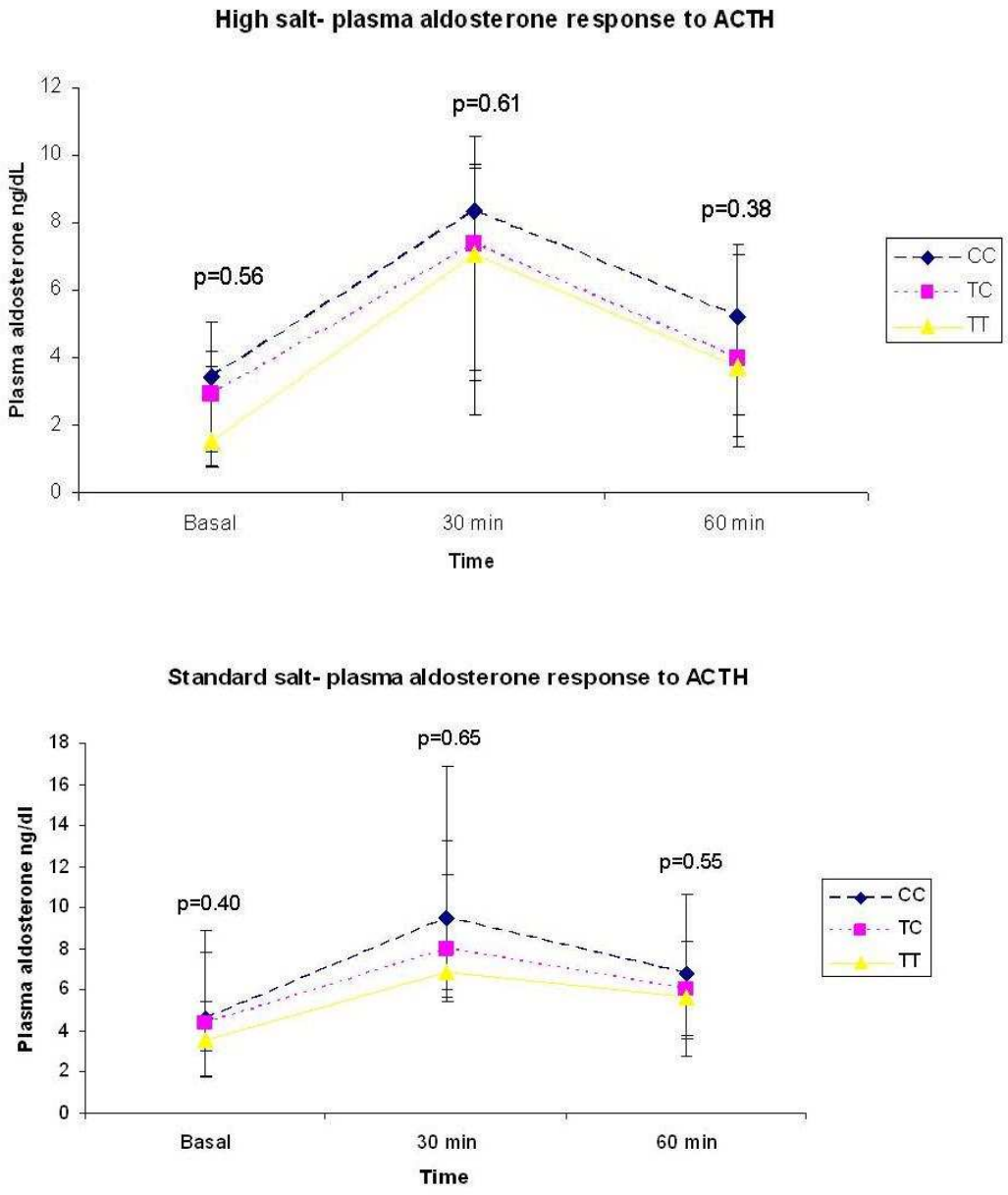


Figure 6-8 Response to ACTH (1ng/kg/min) under high and standard salt conditions.

Due to the non-normal distribution of the data, graphs are displayed as median and inter-quartile range, Hypothesis tests (Kruskal Wallis) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

The ratio of the plasma concentration of a compound to that of its precursor may be a better index of the efficiency of enzyme activity. Aldosterone is synthesised from deoxycorticosterone (DOC) by three separate “hydroxylations” (see chapter 1.2.4), catalysed by aldosterone synthase in the adrenal glomerulosa. The response of this index, (DOC:Aldo) to angiotensin II, a specific inducer of the CYP11B2 pathway is worthy of examination. However,

interpretation of this index is compromised by the fact that DOC is also a product of the zona fasciculata, which secretes higher quantities than does the zona glomerulosa. The basal DOC:Aldo ratios are shown in Figure 6-9.

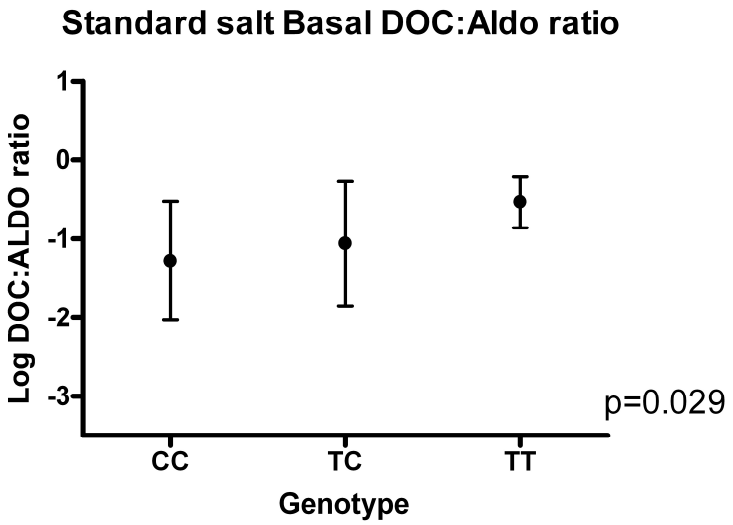


Figure 6-9 Log_{10} Deoxycorticosterone:aldosterone (DOC:Aldo) ratio under standard salt conditions.

Graphs displayed as mean and standard deviation, ANOVA.

Under standard salt conditions there is a genotype-dependant effect in DOC: Aldo ratio. The DOC: Aldo ratio is higher with TT>TC>CC (p=0.029). This could suggest that TT individuals have less aldosterone synthase activity and are less efficient at converting DOC into aldosterone. However, it could also be a consequence of less efficient 11ß-hydroxylase activity, which also uses DOC as a substrate. The effect on DOC: Aldo of stimulation of the CYP11B2 pathway with angiotensin II is shown in Figure 6-10.

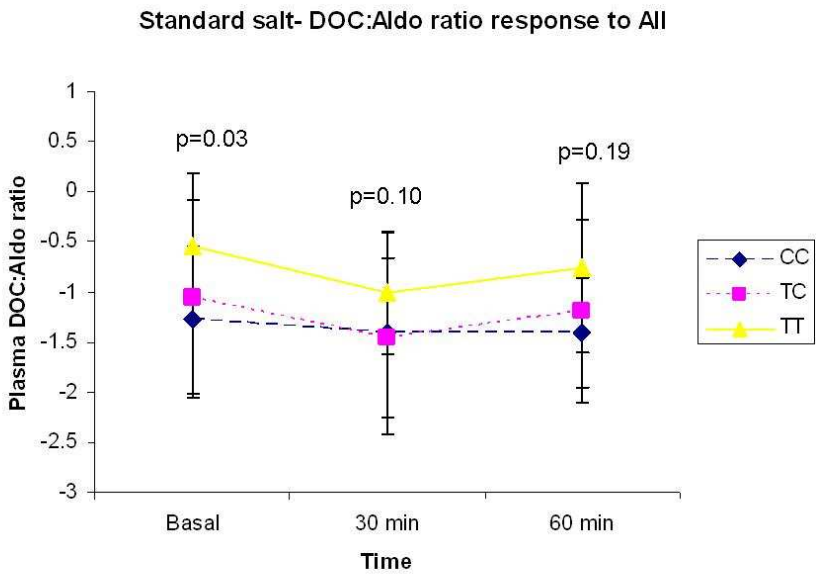


Figure 6-10 Deoxycorticosterone: Aldosterone (DOC:Aldo) ratio, response to angiotensin II (3ng/kg/min) under standard salt conditions.

Data are displayed as mean and standard deviation. Hypothesis tests (ANOVA) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

Following administration of angiotensin II the DOC: Aldo ratio would be expected to fall due to stimulation of *CYP11B2* transcription and increased aldosterone synthase activity. The difference between individuals of different genotypes seen under standard salt conditions loses statistical significance following administration of angiotensin II. It seems likely that the increased DOC: Aldo ratio observed at baseline arises as a result of inefficient 11ß-hydroxylase activity rather than aldosterone synthase activity. However, it is interesting to note that while the DOC: Aldo ratio falls in response to AII in TT individuals, indicating a clear response to AII and increased aldosterone synthase activity, the response in CC individuals is less marked. The change between DOC: Aldo ratios in TT Vs CC individuals is not statistically significant but the trend raises the possibility that TT individuals are more sensitive to the effects of AII.

6.4.7 Effect of stimulation of the CYP11B1 pathway by trophins of aldosterone

Whilst previous evidence has suggested that 11ß-hydroxylase activity is less efficient in individuals who carry the T allele at position -344 in the promoter region of *CYP11B2* than those carrying the C allele, there is no evidence of any absolute difference in the level of cortisol produced. The cortisol measurements at baseline for study participants stratified according to genotype are shown in Figure 6-11 which confirms these previous findings with no genotype dependant difference in plasma cortisol concentration.

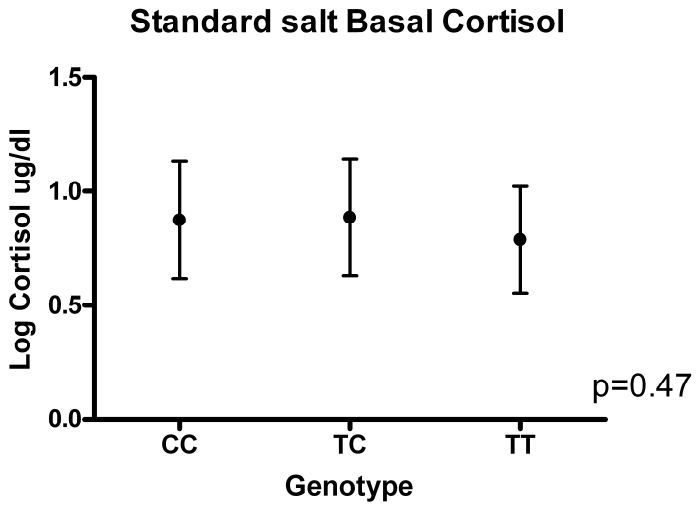


Figure 6-11 Log $_{10}$ Basal cortisol under standard salt conditions.
Data displayed as mean, standard deviation, ANOVA.

The ratio of 11-deoxycortisol (S) to cortisol (F) can be used as a measure of 11ß-hydroxylase efficiency. This was measured at baseline and in response to stimulation of the CYP11B1 pathway with ACTH to test the hypothesis that carriers of the -344 TT polymorphism in *CYP11B2* display a phenotype of relative CYP11B1 inefficiency. S:F ratios stratified according to genotype are displayed in Figure 6-12 while the response of the S:F ratio to stimulation of the CYP11B1 pathway by ACTH is shown in Figure 6-13.

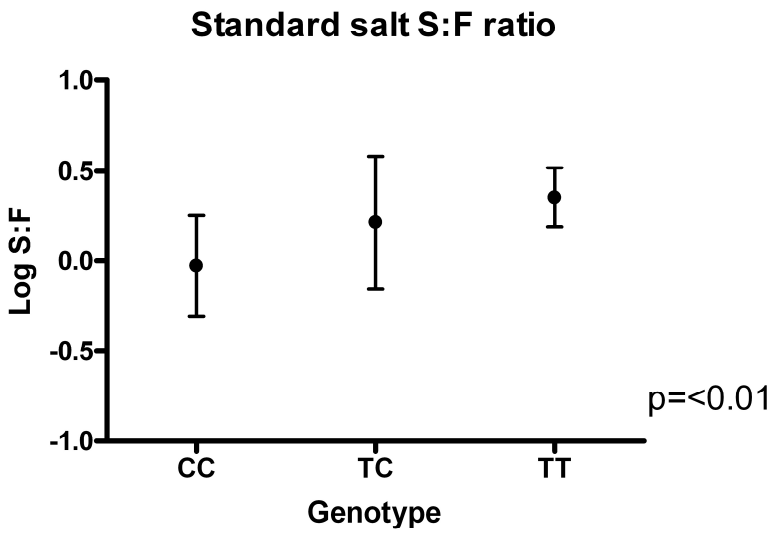


Figure 6-12 Log $_{10}$ Basal 11-deoxycortisol (S): cortisol (F) under standard salt conditions.

Data displayed as mean, standard deviation, ANOVA.

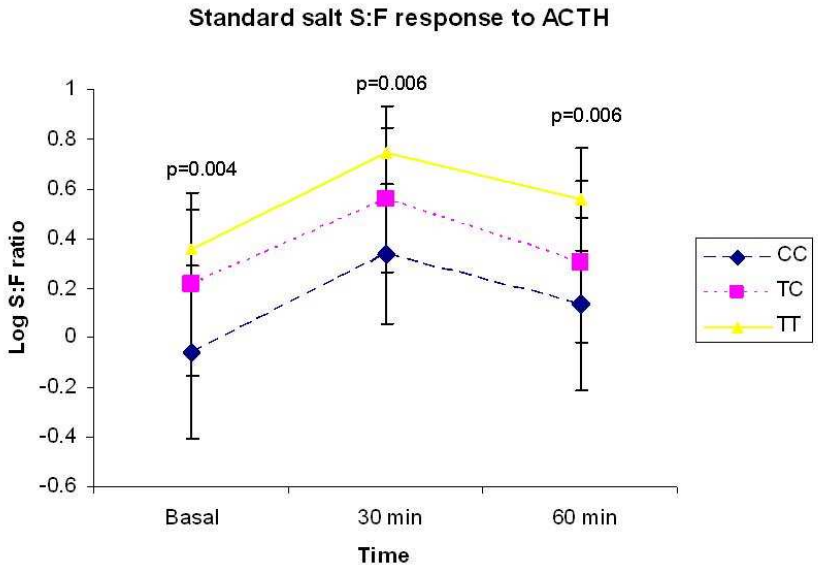


Figure 6-13 Log $_{10}$ 11-deoxycortisol (S): cortisol (F), response to ACTH (1ng/kg/min) under standard salt conditions.

Data displayed as mean, standard deviation. Hypothesis tests (ANOVA) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

There is less efficient 11ß-hydroxylation in individuals carrying the T allele at position -344 in the *CYP11B2* promoter than those carrying the C allele at baseline. Stimulation with ACTH causes S:F to rise, i.e. there is relatively more precursor (11-deoxycortisol) relative to its product, cortisol. However, the genotype-dependant difference remains relatively constant at baseline, immediately following ACTH (30 minutes) and after a further 30 minutes.

As mentioned above, DOC is a substrate for both aldosterone synthase and 11ß-hydroxylase, thus, both can generate corticosterone (B). However, in the glomerulosa, aldosterone synthase can also perform an 18-oxidation (to produce aldosterone) and 18-hydroxylation (to produce 18-hydroxycorticosterone). As the fasciculata is a higher capacity system than the glomerulosa, it is likely that the ratio of DOC: B relates more to 11ß-hydroxylase activity than aldosterone synthase activity. DOC: B ratios response to ACTH is shown in Figure 6-14 and in order to discern whether this is a *CYP11B1* or *CYP11B2* effect, the response to angiotensin II, a specific trophin of *CYP11B2* is also displayed Figure 6-15.

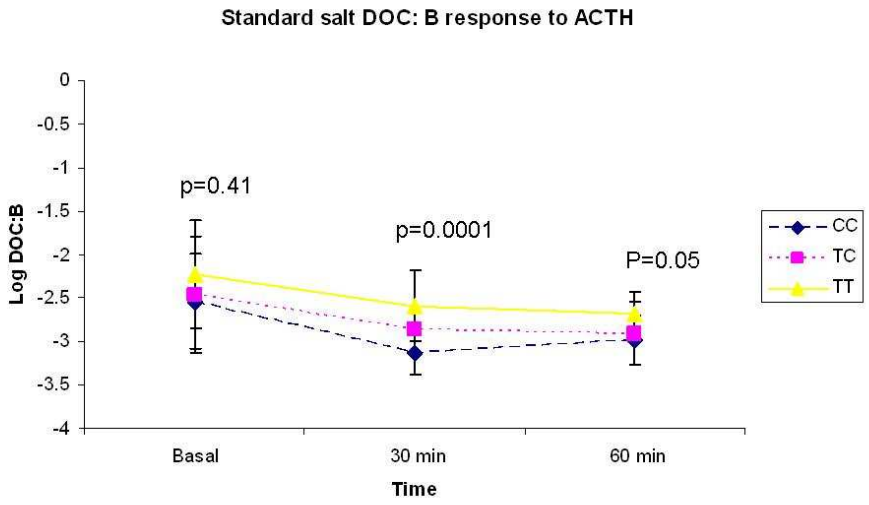


Figure 6-14 Log $_{10}$ Deoxycorticosterone (DOC): corticosterone (B) ratio response to ACTH (1ng/kg/min) under standard salt conditions.

Data displayed as mean, standard deviation. Hypothesis tests (ANOVA) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

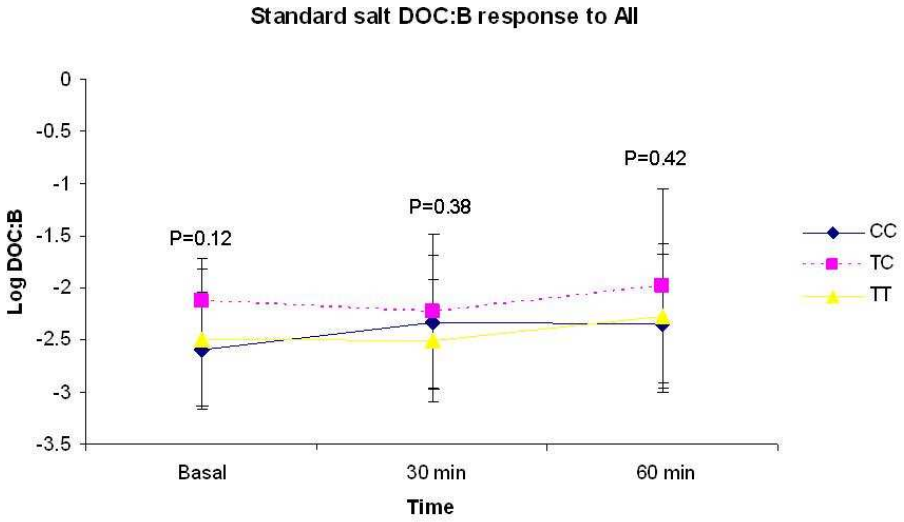


Figure 6-15 Log $_{10}$ Deoxycorticosterone (DOC): corticosterone (B) ratio response to Angiotensin II (3ng/kg/min) under standard salt conditions.

Data displayed as mean, standard deviation. Hypothesis tests (ANOVA) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

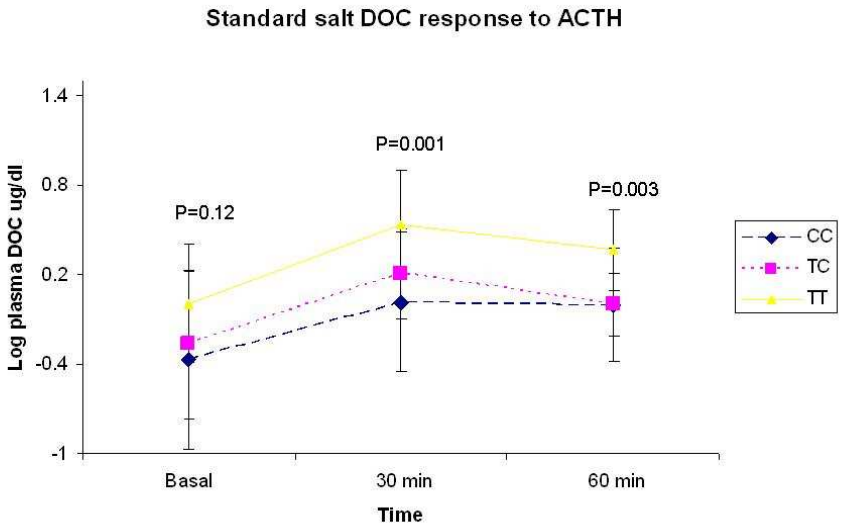


Figure 6-16 Log $_{10}$ Deoxycorticosterone (DOC) ACTH (1ng/kg/min) under standard salt conditions.

Data displayed as mean, standard deviation. Hypothesis tests (ANOVA) were performed comparing genotypes at each time point (Basal, 30 min and 60 min).

Administration of ACTH reveals a genotype-dependant difference in DOC: B ratios with TT individuals demonstrating less efficient conversion of precursor (DOC) to product (B) at 30 minutes than TC or CC individuals ($P<0.0001$). The ratio is driven by a higher DOC in TT individuals Figure 6-16. As this is not observed on stimulating the CYP11B2 pathway with angiotensin II, it can be concluded that this is likely to be an effect of less efficient 11β-hydroxylation. There are no statistically significant differences between genotype groups on stimulation with angiotensin II.

6.5 Discussion

Polymorphic variation in the *CYP11B2* promoter region at position -344 has previously been shown to be associated with reduced 11β-hydroxylation (Davies et al., 2001; Freel et al., 2008; Inglis et al., 1999; Keavney et al., 2005; Freel et al., 2007) and the data above confirm these findings in a normal population. The association with indices of aldosterone production have been more controversial, and clarification of this issue was one of the main aims of this study. 24 Hr

urinary THAldo excretion was significantly higher in TT genotyped individuals in this study, confirming that they are exposed to higher levels of aldosterone than CC individuals. Although plasma measurements showed no genotype dependant difference, the longer duration of sampling makes the urine collection a more robust measurement of aldosterone production than plasma concentration measurement which may be more sensitive to confounding factors.

There was no genotype-dependant difference in the extent of suppression of aldosterone levels by salt loading. However, the study methodology may have been a limiting factor here, particularly given the greater reliance on urinary measurements to detect differences in aldosterone production bearing in mind that the difference between THAldo excretion under high salt and standard salt conditions was not statistically significant. Subjects commenced the 24 hour urine collection on day 3 and the 24 hour urine collection in the salt loading phase may have been more useful if subjects were given longer to achieve sodium balance. Further, the plasma aldosterone to renin ratio has been more reliably associated with -344 polymorphism (Lim et al., 2002) but in this study, the method chosen to measure plasma renin was plasma renin concentration (PRC), using a chemiluminescent method, rather than plasma renin activity (PRA). The chemiluminescent method has the advantage of low cost and high throughput capacity, but is less specific especially at the lower end of the normal range than PRA (Dorrian et al., 2010). This would have been particularly relevant for measurements during the high salt phase of the study and may be a possible explanation for why there was no detectable difference in ARR between the genotyped groups.

The relationship between aldosterone production and genetic variation in the *CYP11B2* promoter was further explored and the hypothesis that individuals with TT genotype are more sensitive to trophins of aldosterone that CC individuals was tested. No evidence to support this hypothesis was found with regard to plasma aldosterone concentration response to either angiotensin II or ACTH. It has previously been suggested that chronic subtle increase in ACTH as a consequence of inefficient 11β-hydroxylation lead to increased synthetic capacity of the glomerulosa resulting in increased sensitivity to trophins. As the age range of the study participants was wide (in particular of the TT individuals who had a mean age of 57 years, StDev 49-69 years), there is no reason to

believe that the negative result is due to inadequate time to develop this phenotype. However, the dose of ACTH used (1ng/kg/min) may have provoked a maximal adrenal response (Arvat et al., 2000) and in order to detect a genotype dependant difference, a much lower, sub-maximal dose may be necessary.

Although there were no genotype dependant differences in absolute aldosterone levels in response to stimulation, interesting trends were observed in the ratios of product to precursor which can be used as a marker of enzyme efficiency. In particular, the contrasting response of DOC:Aldo ratio following stimulation of the *CYP11B1* and *CYP11B2* pathways between TT and CC individuals raises intriguing questions. Bearing in mind the ambiguities of this ratio as a marker of aldosterone synthase activity as well as the relatively small magnitude of change of the DOC:Aldo ratio means that these results should be interpreted with caution. In addition, the study is significantly underpowered for this measurement. Although these results should not be over interpreted, the difference in response to stimulation may be worthy of further study in larger numbers.

An alternative explanation for the lack of a consistent association between aldosterone and variation in *CYP11B2* could be the existence of another compound with mineralocorticoid properties, leading to lower renin without an elevated aldosterone. This can be seen in the syndrome of apparent mineralocorticoid excess where deficiency of 11ßhydroxysteroid dehydrogenase type 2 (11ßHSD2) allows cortisol to occupy the mineralocorticoid receptor illicitly (Stewart et al., 1996). In the situation of relative 11ß-hydroxylase deficiency, a relative excess of DOC could function as a mineralocorticoid, freed from the regulation of the RAS system. In this study, plasma DOC concentration is higher in TT individuals although there is no significant difference in THDOC excretion rate. However, although DOC is present in roughly equivalent concentrations to aldosterone and although the mineralocorticoid activity of the compound is similar (Porter and Edelman, 1964), the affinity for the MR is significantly less than aldosterone (Baxter et al., 1976). Thus the physiological relevance of these genotype dependant differences is questionable, although it is possible that the increased DOC in TT individuals in response to increased ACTH in combination with the basal increase in aldosterone seen in the same individuals could lead to greater activation of the mineralocorticoid receptor. It

would be useful to have more accurate measurement total body sodium in order to have a better understanding of total mineralocorticoid activity.

How could these studies be taken further to explore whether the corticosteroid phenotype is a result of functional changes in *CYP11B1* alone or in combination with functional variation in *CYP11B2*? ACTH measurements may provide additional evidence to support the hypothesis of chronic, low grade increased ACTH drive, but the limits of assay sensitivity and sampling difficulties are likely to limit this approach. Increased ACTH drive to the adrenals should also result in increased adrenal androgens; indeed earlier work has suggested an association between variation in the *CYP11B1*/2 locus and adrenal androgen production (Imrie et al., 2006). However, this study population may not be the most suitable for this analysis given the inclusion of both men and women of a range of ages. Inhibitors of CYP11B1 and CYP11B2 are available but are promiscuous and it is not possible to replicate with pharmacological agents the clinical hypothesis of decreased CYP11B1 activity with preserved or increased CYP11B2 activity. Patients with increased ACTH as a result of Cushing's disease may provide useful insights in terms of whether they are more responsive to trophins of the CYP11B2 pathway. But this extreme situation is quite dissimilar to the subtle, long term increase that is being proposed as a mechanism in this situation. An alternative situation where ACTH drive is increased may be the use of 11 beta hydroxysteroid dehydrogenase (11ßHSD1) inhibitors. 11 ßHSD1 converts cortisone to cortisol and inhibition of this enzyme has been shown to lead to increased ACTH and adrenal volume in animal models (Kotelevtsev et al., 1997). A recent study on the use of 11 ßHSD1 inhibitors in humans with type 2 diabetes confirms a dose dependant elevation in ACTH level (Rosenstock et al., 2010). Basal cortisol levels did not alter and adrenal androgens were unchanged, however it would interesting to observe the effect of this increased ACTH drive on long term mineralocorticoid production under basal conditions as well as in dynamic studies.

In conclusion, the studies outlined above have confirmed and extended current knowledge regarding the relationship between the *CYP11B1*/2 locus and ultimate phenotype. They have confirmed the relationship between *CYP11B2* polymorphisms and relative aldosterone excess and provided further evidence to support the phenotype of relative 11ß-hydroxylase deficiency. No evidence was

found to support the notion of an increased response to trophins of aldosterone production in this study. Further questions have been raised; in particular the role of intermediate corticosteroids should be further examined in order to discern whether they could play any meaningful role in the pathophysiology of hypertension.

7. General Discussion

The investigations presented in this thesis were designed to explore the hypothesis that there are functional polymorphisms in the aldosterone synthase promoter region that are co-inherited with the much studied site of variation at -344 (rs1799998) of CYP11B2, and that these functional SNPs in the promoter region of CYP11B2 contribute significantly to the phenotype of hypertension and an elevated aldosterone to renin ratio. Starting with a descriptive study of the pattern of linkage disequilibrium in this locus, followed by the validation of an in vitro model system and identification of a functional variation at -1651 (rs13268025) of CYP11B2, the data presented here support this original hypothesis.

It could be argued that the *in vitro* studies presented in Chapter 5 are limited in that only one SNP from the haplotype structure was investigated in an artificial context i.e., the normal pattern linkage disequilibrium would suggest that this a T at -1651 would be inherited along with a C at -344 as well as a predictable pattern of variation at a number of other sites. In examining the effects of an individual SNP out of the context of the haplotype block, the possible functional effects (potentially of opposite effect) may be overlooked. Thus, in combination, the cumulative effect of the haplotype may be neutral. However, there is validity in the experimental approach undertaken here. In particular, it should be borne in mind that the data from the normal volunteers in Chapter 6 demonstrates a genotype-dependant effect on THAldo excretion (in the context of the entire promoter haplotype). This confirms that the cumulative effect of the SNPs in the haplotype is not neutral. In addition, the influence of rare alleles and haplotypes in the population determinants of blood pressure should be borne in mind; the individual effects on single polymorphic variation may exert a bigger physiological impact, albeit in a small number of individuals. However certainly, there has been no data presented here to suggest that the SNP at -1651 of CYP11B2 is the only functional variation and further assessment of the remaining SNPs is warranted.

One aspect of the hypothesis was not supported in the data from chapter 6. That is, that polymorphic variation in the aldosterone synthase promoter region leads to altered responsiveness to trophins of aldosterone production or suppression

under high salt intake. There were no genotype-dependant differences observed in any of these phenomena in the data presented. The hypothesis is therefore not supported by the results. There are a number of possible reasons for this. The experimental design could have made it difficult to observe a significant effect. For example, as mentioned in the discussion section in Chapter 6, the period of salt loading may not have been long enough for the subjects to achieve salt balance. While a longer period of dietary restriction would be difficult in terms of subject recruitment and compliance, it may be that this is required to discern any genotype dependant difference. Other mechanisms of salt excretion (sweat, gastrointestinal losses) were not controlled for. While these may be minor in comparison to the effect of renal mechanisms of salt handling, they were unquantified in this study. On the other hand, there was evidence that the experimental design was sufficient to observe physiological changes in response to salt intake. For example, there was a clear difference observed between the high salt and standard salt phase of the study in terms of responsiveness to angiotensin II. Specifically, under lower salt intake, subjects had a greater rise in plasma aldosterone concentration in response to angiotensin II as compared to the change in plasma aldosterone concentration under salt loading conditions. This demonstrates that the experimental design was robust enough for these changes to be observed. It is certainly clear that any genotype differences are likely to be small and may even be obscured by technical variability, thus requiring a significant increase in the number of individuals studied to be able to discern.

In addition, the participants of the experiments described here were all normotensive and covered a wide range of ages. It may be that older, hypertensive individuals would be most likely to demonstrate these responses than younger participants, having had more time to develop the genotype-phenotype relationship. Or it may be that in selecting older, normotensive subjects, they may have contributed to a “survivor bias” and therefore less likely to display the phenotype of increased responsiveness to trophins or less aldosterone suppression in response to salt loading, possible due to undetermined compensatory, protective mechanisms. In practice, this hypothesis would be difficult to test; older hypertensive subjects are likely to be taking antihypertensive medications which will make interpretation of

biochemical measurements difficult and changing or stopping medications for the purposes of research studies would be ethically questionable.

Despite the difficulties of delineating the phenotype in terms of response to suppression and stimulation of aldosterone, it seems clear that the hypothesis that there is an allele specific effect on transcription mediated by APE1 is strongly supported by these studies and should be further investigated. The mechanism by which APE1 exerts its effects has not been addressed here although a number of possibilities have been discussed. Further experiments would be necessary to explore this. Firstly, whether APE1 acts by activating or inactivating other transcription factors known to be involved in the regulation of aldosterone synthase, could be tested by mutating the known transcription factor binding sites and comparing a plasmid with -1651 T and C with and without APE1 inhibitor or SiRNA. Secondly, the role of HNRNPK remains unclear. Given the previous reports that APE1 heterodimerises with members of the HNRNP family, the hypothesis that it is also involved in the regulation of aldosterone synthase is attractive. Again, some simple experiments could increase understanding here, for example SiRNA knock down of HNRNPK in combination reporter gene assays using plasmids containing the T and C alleles at -1651. Finally, looking back again at the shift assay gel from chapter 5 (Figure 5-7), it would be worth investigating the identity of the other DNA: protein complexes, given the numerous complexes that can be observed. It may be that APE1 is only one of a number of transcriptional proteins that bind at this site.

The APE1 deficient mouse is hypertensive (Jeon et al., 2004) and provides an exciting opportunity to explore the relevance of my hypothesis *in vivo*. The first stage in exploiting this system would be to assess the renin-angiotensin-aldosterone system. If the mouse displayed increased aldosterone levels (in combination with reduced renin) or increased transcription of aldosterone synthase compared to its wild type littermates, this would provide compelling evidence to support the role of APE1 in the regulation of CYP11B2.

Is this relationship of clinical utility? Given that APE1 is a multifunctional protein with roles in fundamental processes regulating DNA repair, preventing mutations and oncogenesis as well as redox regulation of numerous transcription factors with disparate function, the idea of this as a focused therapeutic target does not

seem promising, due to the huge scope for off target actions and side-effects. One possibility could be to find a means of targeting the adrenal gland specifically. The possibility of using viral vectors for this purpose has been of much interest but remains a theoretical strategy in the laboratory rather than a practical technique in the clinic. A greater understanding of the role of APE1 may reveal other steps in the regulatory process which could be targeted in order to inhibit aldosterone synthase transcription, particularly if, as mentioned above, APE1 acts as part of a complex of regulatory proteins. There is currently much interest in developing a specific aldosterone synthase inhibitor but progress is hindered by the sequence homology between aldosterone synthase and 11ß-hydroxylase, (LaSala et al., 2009). Targeting aldosterone synthase by an alternative mechanism, for example by manipulating the transcriptional machinery, may result in less off-target effects.

Another possible area of interest is pharmacogenomics or stratified medicine, developing techniques to better select pharmacological therapies specific for individuals, tailored to their genetic predispositions. There has been no evidence to date to suggest that variation in the CYP11B1/B2 locus is associated with a differential response to pharmacotherapy. Indeed, there is mounting evidence that MR blockade is a useful strategy in an ever increasing range of clinical indications, regardless of genotype. However, there remains controversy as to whether this is as a result of blocking MR activation by aldosterone or preventing activation by another ligand (e.g. Cortisol in non-epithelial sites lacking 11ßHSD). A better and more specific inhibitor of aldosterone synthase would better answer this question, and it may be that individuals carrying polymorphic variation in CYP11B2 leading to greater enzyme transcription respond better to therapy than those without such a genetic pattern.

This thesis began with an overview of investigations to date in the field of genetic determinants of hypertension, and presented data resulting from two contrasting methods of enquiry, namely the hypothesis-driven candidate gene approach and, more recently, the unbiased genome wide association studies. However, despite large investments of time as well as economic resources, the genes responsible for the large, heritable component of blood pressure variation are unknown and the genetic mechanisms underpinning essential hypertension remain elusive. Much specultation has been made in the literature regarding the

next leap in technology and potential advances possible with next generation sequencing with massive parallel sequencing platforms and the prospect of detecting rare variants with larger physiological effects. However, what is often overlooked is the crucial element of phenotyping of subjects. For example, salt sensitivity, response to angiotensin II, age of onset, etc.

In this thesis I have explored the significant advances that have been made in understanding the pathophysiology of hypertension using well developed, robust hypotheses and careful phenotyping. As discussed in Chapter 1, many of these advances have originated in the study of monogenic conditions in individuals, and some have lead to insights into mechanisms that influence the blood pressure of populations, such as the analysis by Ji et al of the Framingham cohort and frequency of rare alleles in genes associated with Gordon's, Liddle's and Bartter's syndrome (Ji et al., 2008). A similar success can be observed in the recent discovery of the KCNJ5 mutation in a proportion of aldosterone producing adrenal adenomas (Choi et al., 2011), which arose from careful examination of a series of patients with familial hyperaldosteronism (Geller et al., 2008) but may be involved in the development of this important, curable cause of hyperaldosteronism and hypertension. The success of future work examining the role of aldosterone synthase in hypertension (as well as progress in many other area of hypertension research) will depend on similar careful clinical studies with high fidelity phenotyping and a clear hypothesis. The idea that hypertension is a homogeneous entity and that it is reasonable to study "hypertensive" subjects without further description is in some ways analogous to the idea that all patients with a fever are suffering from identical pathophysiological processes and will be cured by paracetamol. While it is tempting to pursue ever larger screens of genetic analysis, including more samples, and covering a greater number of sites of variation, it seems unlikely that progress will be made until studies taking greater account of factors like age of onset, gender and salt status are undertaken. Knowledge of the roles of epigenetic modification including methylation, histone modification and the actions of microRNA remain rudimentary and a greater understanding of the transcriptional process is needed. Until greater attention is paid to the spectrum of pathophysiological mechanisms involved, including epigenetic phenomenon and environmental factors, and greater attention paid to the consequent

intermediate phenotypes, progress will be limited, despite advancing technology and resources.

In conclusion, as a result of a combined approach, utilising laboratory techniques and in vivo observations, I have demonstrated a plausible mechanism behind the phenotype of hypertension with an elevated aldosterone to renin ratio and the associated genetic motifs in aldosterone synthase/ 11β hydroxylase. I have presented data which identify a role for APE1 in the regulation of aldosterone synthase gene transcription. Further investigation is warranted to clarify the mechanisms involved and explore the potential for therapeutic intervention.

8. Appendix

8.1 Monogenic disorders affecting blood pressure

DISORDER	GENE	BLOOD PRESSURE	CLINICAL FEATURES
Liddle's Syndrome	SCNN1B, SCNN1G	High	Gain of function of ENaC, leading to increased sodium reabsorption, low renin, low aldosterone, hypokalaemia and metabolic alkalosis.
Gitelman's syndrome	SCL12A3	Low	Constitutative activity of thiazide sensitive channel (distal convoluted tubule) causing hypokalemic alkalosis with hypocalciuria and hypomagnesemia
Bartters' syndrome	SCL12A1, ROMK, CLCNKB	Low	Defect in sodium chloride channel in thick ascending limb, causing hypokalemic alkalosis, hypercalcuria, normal serum magnesium
Pseudo-hypo-aldosteronism type 1 (autosomal dominant)	NR3C2	Low	Resistance of the mineralocorticoid receptor. Increased sodium loss and hyperkalaemic acidosis despite aldosterone and renin levels. Neonatal salt wasting but adults may be asymptomatic. Mutation L810 associated with hypertension exacerbated by pregnancy as progersterone able to activate mutant mineralocorticoid receptor.
Pseudo-hypo-aldosteronism type 1 (autosomal recessive)	SCNN1A, SCNN1B, SCNN1G	Low	Loss of function of ENaC leading to increased sodium loss from urine, sweat, stool, and saliva despite increased aldosterone and renin levels with hyperkalaemia.
Gordon's syndrome (Pseudo-hypo-aldosteronism type 2)	WNK1, WNK4	High	Increased activity of Na-Cl co-transporter in distal nephron causing increased sodium retention and hyperkalaemia
Syndrome of apparent mineralocorticoid excess	HSD11B2	High	Lack of conversion of cortisol to cortisone, leading to activation of the MR by cortisol. Low aldosterone and suppressed renin.

Glucocorticoid remediable hyper-aldosteronism	CYP11B1/ CYP11B2	High	Ectopic expression of aldosterone synthase under the control of ACTH, leading to elevated aldosterone, low renin.
21α-hydroxylase deficiency	CYP21A1	Low	Loss of function of 21α hydroxylation leads to lack of conversion of 17OHP 11-deoxycortisol, and progesterone to DOC. Reduced cortisol and aldosterone leads to increased ACTH drive causing hyperandrogonism.
11ß- hydroxylase deficiency	CYP11B1		Loss of function of 11ß hydroxylation leads to lack of conversion of 11deoxycortisol to corticosterone and cortisol. Increased precursors lead to increased blood pressure
Aldosterone synthase deficiency	CYP11B2	Low	Loss of function of aldosterone synthase, leading to a lack of aldosterone production, increased renin, reduced sodium reabsorption, hyperkalaemia and metabolic alkalosis
17α-hydroxylase deficiency	CYP17A1	High	Loss of function of 17α hydroxylation leads to lack of conversion of progesterone to 17OHP. Loss of 17, 20 lyase function leads to lack of conversion of 17OHP and 17OHPreg to DHEA and androstenedione.
3ß-hydroxysteroid dehydrogenase deficiency	HSD3B2	Low	Variable clinical presentation. Reduced aldosterone and cortisol production can result in salt wasting. Variable degrees of abnormal androgen production.

8.2 Nucleotide sequence of human CYP11B2 5' untranslated region.

Contructed from Entrez Nucleotide database in 2002 (http://www.ncbi.nlm.nih.gov/gene , Accession number D10170) Location of primers and SNPs highlighted in green.

```
1    Gaattctgca tcctgtgaaa ttatccttca aaagtgaaca
41   taaatatttt ctcaggtaaa taaaaattga ggggattcgc
       B2PROMF2 ⟶
81   tgccaataga actgacttgc cagaaatgtt ttttaaaagt
121  tctgcagaga gaaagaaaat gatacaggtc agcaaccctg
161  agctacataa agaaaggaag agcatttcag aaggaatcag
201  taaagagaaa atgaagtctt ttatttttc ttaatcttaa
241  ttgatctaag agtttgctaa aacaaaacaa caacaataaa
281  aataggccgg gcgcggtgac tcaccacacc tgtaatccca
321  gcactttggg aggccgaggt gggcagatca cctgaggtca
361  ggagttcgag actagcctgg ccaacacggt gaaaccccgt
401  ctctactaaa aatacaaaaa ttagctgggc gtggtggtgg
                                    ⟵ B2Pro494
441  cacatgcctg taatcccagc tacttgggag gctgaggcgg
481  gagaattgct tgaacccggg agacagaggt cgcagtgagc
521  cgagatcaca ccattgcact ctagcctggg cgacagagtg
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681  gatgcaaggg atgggtgagt ggaattaaaa atatcttatt
721  atttatttat tttgagatgg agtcttgctt tgctgcccag
           B2 5'783 ⟵
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881  aattttgtat ttttagtaga gacagggttt tgccatgttg
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961  tgcatcagcc tcccaaagtg ctgggatgac agacatgagc
1001 cactatgccc agcctaagaa tatctgatga ttataaagtg
1041 cttgcattac ctctgaagct gtatagtgtt atatgaaggt
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1121 tagggcaacc actaaagaag tgagacccag cctctagaaa
1161 aaaaaaaaaa aaaggaaatt agctatcaag ccacgaaaag
1201 aaatggagga accttaaacg catattacta actgagatac
1241 gtcactttga aaaggctaca aacggtgtca ttccaactat
1281 acaacatttt ggaaaaggcc aaagcatggt gatgataaaa
1321 aagatcggag atgtcaggga ctggggcagg agggatgagc
1361 aggcagagca caggttttct tttcctcttt ttaagacagt
1401 gaaaatactc ctaggatcct gcaaggaggg atacaaatta
1441 catacatttg tcaaaaccca cagcatgttg accaccagga
1481 ggagacccca tgtgactcca ggaccctggt tgataacaac
1521 gtatcgagat tcctcacatg gaaccagtgc gctcctgtgg

                              SF1 F ⟶
1561 tggagggtgt acctgtgtca gggcaggggg tacgtggaca
1601 ttttctgcag tttttgatca attttgcaat gaactaaatc
```

```
1641 tgtggtataa aaataaagtc tattaaaaga atccaaggct
1681 ccctctcatc tcacgataag ataaagtccc catccatttt
1721 actcctctca gccctggaga aaggagaggc caggtcccac

                                        ← SF1_R
1761 caccttccac cagcatggac ccccagtcca gaccccacgc
1801 ctttttctcag catcctcaga ccagcaggac ttgcagcaat
1841 ggggaattag gcacctcact tctccttcat ctacctttgg
1881 ctgggggcct ccagccttga ccttcgctct gagagtctca
1921 ggcaggtcca gagccagttc tcccatgacg tgatatgttt
                                     B2-R1 ←
1961 ccagagcagg ttcctgggtg agataaaagg atttgggctg
2001 aacagggtgg agggagcatt gga
```

8.3 Consensus sequence for CYP11B1 and CYP11B2 5’ untranslated region and exon 1

```
           1
130
  b1b1b1b1  TCCTTC-GCA TCCCTTGTAA GTTGGATTCC TAAGTAT--- TTTATTCTCT TTGAAGCAAT TGTGAAT--- GGGAGTTCAC TCATGATTTG GCTCTCTGTT TGTCTGTTAA GTGTGTATAA GAATGCTTGT
  b2b2b2b2  GAATTCTGCA TCCTGTGAAA TTATCCTTCA AAAGTGAACA TAAATATTTT CTCAGGTAAA TAAAAATTGA GGGGATTCGC TGCCAATAGA ACTGACT--- TGCCAGAAAT GTTT-TTTAA AAGTTCTGCA
 Consensus  gaaTTC.GCA TCCcgTGaAA gTagcaTTCa aAAGTaa... TaaATacTcT cTcAaGcAAa TaaaAAT... GGGaaTTCaC TcacaATaga aCTcaCT... TGcCaGaaAa GTgT.TaTAA aAaTgCTgca

           131
260
  b1b1b1b1  GATTTTTGTA CGTTGATTTT GTATCCTGAG ACTTTGCTGA AGTTGCTTAT CAGCTTAAGG AG-ATTTTGG GCTGAGACAA TGGGGTTTTC TAGATATACA ATCATGTCGT CTGCAAACAG AGACAATTTG
  b2b2b2b2  GAGAGAAAGA AAATGATACA GGT--CAGCA ACCCTG---- AGCTACATAA AGAAAGGAAG AGCATTTCAG AAGGAATCAG TAAAGAGAAA ATGAAGT-CT TTTATTTTTT CTTAATCTTA ATTGATCTAA
 Consensus  GAgagaaagA aaaTGATaca Gga..CaGaa ACccTG.... AGcTaCaTAa aaaaagaAaG AG.ATTTcaG aagGAaaCAa TaaaGagaaa aaGAaaT.Ca aTcATgTcgT CTgaAaacaa AgacAactaa

           261                                                                                                                                          390
  b1b1b1b1  ACTTCCTCTT TTCCTAATTG AATACCCTTT ATTTCCTTCT CCTGCC-TAA TTGCCCTGGC CAGAACTTCC AACACTATGT TGAATAGGAG TGG------- ---TGAGAGA GGGCATTCCT GTCTTGTACC
  b2b2b2b2  GAGTTTGCTA AAACAAAACA ACAACAATAA AAATAGGCCG GGCGCGGTGA CTCACCACAC CTGTAATCCC AGCACTTTGG GAGGCCGAGG TGGGCAGATC ACCTGAGGTC AGGAGTTCGA GACTAG--CC
 Consensus  aagTccgCTa aaaCaAAaca AaaACaaTaa AaaTacgcCg cccGCc.TaA cTcaCCacaC CaGaAaTcCC AaCACTaTGg gaaacaGaaG TGG.....  ...TGAGaga aGGaaTTCca GaCTaG..CC

           391                                                                                                                                          520
  b1b1b1b1  AGTTTTCAAA GGGAATGCTT CCAGTTTTTG ACCATTCAGT ATGATATTGG CTGTGGGTTT GCCATAGATA GCTCTTATTA TTTTGAGATA CGTCCCATCA A-TACCTAAT TTATTGAGAG TTTTTAGCAT
  b2b2b2b2  TGGCCAACAC GGTGAAAC-- CCCGTCTCT- ACTAAAAATA CAAAAATTAG CTGGGCGT-- ---GGTGGTG GCACATGCCT GTAA------ --TCCCAGCT ACTTGGGAGG CTGAGGCGGG AGAATTGCTT
 Consensus  aGgccaaaAa GGgaAaaC.. CCaGTcTcT. ACcAaaaAga aaaAaATTaG CTGgGcGT.. ...agaGaTa GCaCaTacca gTaa...... ..TCCCAgCa A.TaccgAag cTaagGaGaG agaaTaGCaT

           521                                                                                                                                          650
  b1b1b1b1  GAAGGTTGTT GAATTTTGTC AAAG---GCC TTTTCTGCAT CTATTGAGAT AATCATGTGG TTTTTGTCTT TGGTTCTGTT TATATGCTGG ATTAC--ATT TATTGATTTG CATATATTGA ACCAGCCTTG
  b2b2b2b2  GAACCCGGGA GACAGAGGTC GCAGTGAGCC GAGATCACAC CATTGCACTC TAGCCTG-GG CGACAGAGTG AGACTCTGTC TCAAAAATAA ATAAATAAAT AAATAAATAA ATAATAAAAA TAAATAAATA
 Consensus  GAAcccgGga GAaagagGTC aaAG...GCC gagaccaCAc CaaTgcAcac aAgCaTG.GG cgacaGacTg aGacTCTGTc TaaAaaaTaa ATaAa..AaT aAaTaAaTaa aaaATAaaaA aaaAgaaaTa

           651                                                                                                                                          780
  b1b1b1b1  CATCCCAGGG ATGAAGCCCA CTTGATCATG GT---GGATA AGCTTTTTGA TGTGCTGCTG -GATTC--GG TTTGCCAGTA TTTTATTGAG GATTTTTGCA TCAATGTTCA TCAAGGATAT TGGTCTAAAA
  b2b2b2b2  AAAGCCAGAA A-GTGTATTT GATGATCATA GTTATGTATA TGTGAAATGA AGGACAGCAA TGATGCAAGG GATGGGTGAG TGGAATTAAA AATATCTTAT TATTTATTTA TTTTGAGATG GAGTCTTGCT
 Consensus  aAacCCAGaa A.Gaagacca caTGATCATa GT...GgATA aGcgaaaTGA aGgaCaGCaa .GATgC..GG gaTGccaGaa TggaATTaAa aATaTcTgaa TaaaTaTTcA TcaaGaaaag gaGTCTaaaa

           781                                                                                                                                          910
  b1b1b1b1  TTCTCTTTTT TGGTTGTGTC TCTGCCCGGC TTTGGTATCA GGATGATGCT GGCCTCATAA AATGAGTTAG GGAGGATTCC CT--CTCTTT CTATTGATTG GAATAGTTTC AGAAGGAATG GTACCAGTTC
  b2b2b2b2  TT-GCTGCCC AGGTTGGAG- --TGCAGTGG GATGATCTCA ACTCACTGCA ACCTCCGCCT CCTTGATTCA AGCATTCATC TTGACTCAGC CTGCTGAGAA GCCGAGATTA CAGGCATGCG CCACCACACC
 Consensus  TT.gCTgccc aGGTTGgag. ..TGCacgGc gaTGaTaTCA acacaaTGCa acCccCacaa aaTgaaTTaa aGaagacacC cT..CTCagc CTacTGAgaa GaagAGaTTa aaaacaaacG ccACCAcacC

           911                                                                                                                                         1040
  b1b1b1b1  CTCCTTGTAC -GTCTGGTAT AATTCGGCTG TGAAT--CCA TCTGGTCATG GACTCTTTTT GGTTGGTAAT CTATTGATTA TTGCCACAAT TTCAGATCCT GTTATTGGTC TATTCAGAGA TTCAACTTCT
  b2b2b2b2  TGGCTAATTT TGTATTTTTA GTAGAGACAG GGTTTTGCCA TGTTGGCCAG GCTGGTCTCG AACTCCTGAC CTCAGG--TG ATCCACCTGC ATCAG--CCT CCCAAAGTGC TGGGATGACA GACATGAGCC
 Consensus  cgcCTaaTac .GTaTggTaa aaagaGaCaG gGaaT..CCA TcTgGgCaaG GacgcTcTcg aacTccTaAc CTaagG..Ta aTcCaaCaac aTCAG..CCT cccAaaGggC TaggaaGAcA gaCAacagCc

           1041
1170
  b1b1b1b1  TACTGGTTTA GTCTTGGGAG AGTGTATGTG TCGAGGAATT TATCCATTTC TTCTAGATTT -TCTAGTTTA TTTGCGTAGA GGTGTTTGTA GTATTC--TC TGATGGTAGT TTGTATT-TC TGTGGGATC-
  b2b2b2b2  ACTATGCCCA GCCTAAGAAT ATCTGATGAT TATAA-AGTG CTTGCATTAC CTCTGAAGCT GTATAGTGTT ATATGAAGGT GGAGTTGGAG AGATGAGTTT TAAGCGTATA TTGCAAACTC TAGGGCAACC
 Consensus  aacagGcccA GcCTaaGaAg AgcggATGag TagAa.AaTg caTcCATTaC cTCTaaAgcT .TaTAGTgTa aTagcaaaGa GGaGTTgGaa agATga..Tc TaAgcGTAga TTGcAaa.TC TagGGcAaC.
```

```
          1171                                                                                                                1300
 b1b1b1b1 -----GGTGG TGATATCC-- CCTTTATCAT TTTTTATTGC ATC---TATT TGAT-TCTTC TCTCTTTTTT TCTTT-ATTA GTCTTGCTAG CGGTCTATCA ATTTTGTTGA -TCCTTTCAA AAAACCAGCT
 b2b2b2b2 ACTAAAGAAG TGAGACCCAG CCTCTAGAAA AAAAAAAAAA AAAGGAAATT AGCTATCAAG CCACGAAAAG AAATGGAGGA ACCTTAAACG CATATTACTA ACTGAGATAC GTCACTTTGA AAAGGCTACA
Consensus  .....aGaaG TGAgAcCC.. CCTcTAgaAa aaaaaAaaaa Aaa...aATT aGaT.TCaac cCaCgaaaag aaaTg.AggA acCTTaaaaG CagacTAccA AcTgaGaTaa .TCacTTcaA AAAacCaaCa


          1301                                                                                                                1430
 b1b1b1b1 CCTGGATTCA TTAATTTTTT GAAGGGTTTT TTGTGTCTCT ATTTCCTTCA GTT-CTGCAC TGATTTTAGT TATTTCTTGC CTTCTGCTAG TTTTGAATGT GTTTGCTCTT GCTTTTCTAG TTCTTTTAAT
 b2b2b2b2 AACGGTGTCA TTCCAACTAT ACAACATTTT GGAAAAGGCC AAAGCATGGT GATGATAAAA AGATCGGAGA TGTCA-GGGA CTGGGGCAGG AG--GGATGA GCAGGCA-GA GCACAGGTT- TTCTTTTCCT
Consensus  aacGGagTCA TTaaaacTaT aaAacaTTTT ggaaaacgCc AaagCaTgca GaT.aTaaAa aGATcggAGa TaTca.ggGa CTgcgGCaaG ag..GaATGa GcagGCa.ga GCacagcTa. TTCTTTTaaT

          1431                                                                                                                1560
 b1b1b1b1 TGTGATGTTA GGGTGTCAGT TTTGGATCTT TCCTGCTTTC TCTTGTGGGC ATTTAGTGCT ATAAATTTCC CTCTACACAC TGCTTTGAAT GTGTTCCAGA GATTCTGGTA TGCTGTGTCT TTGTTCTCGT
 b2b2b2b2 CTTTTTAAGA CAGTGAAAAT ACTCCTAGGA TCCTGCAAG- ----GAGGGA TACAAATTAC ATACATTTGT CAAAACCCAC AGCAT--GTT GACCACCAGG AGGAGACCCC ATGTGACTCC AGGACCCTGG
Consensus  cgTgaTaagA caGTGaaAaT acTccaacga TCCTGCaag. ....GaGGGa aacaAaTgac ATAaATTTcc CaaaACaCAC aGCaT..aaT GaccaCCAGa aagacaccca agcTGacTCc agGacCccGg


          1561                                                                                                                1690
 b1b1b1b1 TGGTTTCAAG AACATCTTTA TTTCTGCCTT CATTTTGTTA CGTACCCA-- ---GTAGTCA TTCAGGAG-C AGGTTGCTCA GTTTCCATGT AA---TTGAG CGGTTTTGAG TGAGTTTCTT AATCCTGAGT
 b2b2b2b2 TTGATAACAA CGTATCGAGA TTCCTCACAT GGAACCAGTG CGCTCCTGTG GTGGAGGGTG TACCTGTGTC AGG--GCAGG GGGTACGTGG ACATTTTCTG CAGTTTTTGA TCAATTTTGC AATGAACTAA
Consensus  TgGaTaaaAa aacATCgagA TTcCTcaCaT caaaccagTa CGcaCCca.. ...GaaGgca TaCagGaG.C AGG..GCaca GggTaCaTGg Aa...TTcaG CaGTTTTgaa TcAaTTTcgc AATcaacaaa


          1691                                                                                                                1820
 b1b1b1b1 TCTAGTTTGA TTGCACTAAA ATTTTTAAAA AGTAAAAAAA ATACATGTGG TTTAATACAA TTCATGCCAA CTCATTCCCT CGTTTTTTGC TATAAACCTT G---CAAGGA GATGAATAAT CCAAGGCTCT
 b2b2b2b2 ATCTGTGGTA TAAAAATAAA GTCTATTAAA AGAATCCAAG GCTCCCTCTC ATCTCACGAT AAGATAAAGT CCCCATCCAT --TTTACTCC TCTCAGCCCT GGAGAAAGGA GAGGCCAGGT CCCACCACCT
Consensus  accaGTgggA TaaaAaTAAA aTcTaTaAAA AGaAaaaAAa acaCacgcgc aTcaaaacAa aacATaaaaa CcCaaTCCaT ..TTTacTcC TaTaAaCCcT G...aAAGGA GAgGaaaaaT CCaAccacCT


          1821                                                                                                                1950
 b1b1b1b1 TGGATAAGAT AAGGGCCCCA -TCCA--TCT TGCTCCT--- ---------C TCAGCCCTGG AGGA-----G GAGGGAGAGT CCTTTTCCCC TGTCTACGCT CATGCACCCC CAATGAGTCC CTGCCTCCAG
 b2b2b2b2 TCCACCAGCA TGGACCCCCA GTCCAGACCC CACGCCTTTT CTCAGCATCC TCAGACCAGC AGGACTTGCA GCAATGGGGA ATTAGGCACC TGACTTCTC- CTTCATCTAC CTTTGGCTGG GGGCCTCCAG
Consensus  TccAcaAGaa aaGacCCCCA .TCCA..cCc caCgCCT... .........C TCAGaCCaGc AGGA.....a GaaagaGaGa acTaggCaCC TGaCTaCgC. CaTcaaCcaC CaaTGacTcc cgGCCTCCAG


          1951                                                                                                                2080
 b1b1b1b1 CCCTGACCTC TGCCCTCGGT CTCTCAGGCA GATCCAGGGC CAGTTCTCCC ATGACGTGAT CCCTCTCGAA GGCAAGGCAC CAGGCAAGAT AAAAGGATTG CAGCTGAACA GGGTGGAGGG AGCATTGGAA
 b2b2b2b2 CCTTGACCTT CGCTCTGAGA GTCTCAGGCA GGTCCAGAGC CAGTTCTCCC ATGACGTGAT ATGTTTCCAG AGCAGGTTCC TGGGTGAGAT AAAAGGATTT GGGCTGAACA GGGTGGAGGG AGCATTGGAA
Consensus  CCcTGACCTc cGCcCTcaGa cTCTCAGGCA GaTCCAGaGC CAGTTCTCCC ATGACGTGAT accTcTCcAa aGCAaGgcaC caGGcaAGAT AAAAGGATTg caGCTGAACA GGGTGGAGGG AGCATTGGAA


          2081                                                                                                                2210
 b1b1b1b1 TGGCACTCAG GGCAAAGGCA GAGGTGTGCA TGGCAGTGCC CTGGCTGTCC CTGCAAAGGG CACAGGCACT GGGCACGAGA GCCGCCCGGG TCCCCAGGAC AGTGCTGCCC TTTGAAGCCA TGCCCCGGCG
 b2b2b2b2 TGGCACTCAG GGCAAAGGCA GAGGTGTGCG TGGCAGCGCC CTGGCTGTCC CTGCAAAGGG CACGGGCACT GGGCACTAGA GCCGCTCGGG CCCCTAGGAC GGTGCTGCCG TTTGAAGCCA TGCCCCAGCA
Consensus  TGGCACTCAG GGCAAAGGCA GAGGTGTGCa TGGCAGcGCC CTGGCTGTCC CTGCAAAGGG CACaGGCACT GGGCACgAGA GCCGCcCGGG cCCCcAGGAC aGTGCTGCCc TTTGAAGCCA TGCCCCaGCa


          2211                                                                                                                2340
 b1b1b1b1 TCCAGGCAAC AGGTGGCTGA GGCTGCTGCA GATCTGGAGG GAGCAGGGTT ATGAGGACCT GCACCTGGAA GTACACCAGA CCTTCCAGGA ACTAGGGCCC ATTTTCAGGT AAAGCCCTCC CTGGCCCTCG
 b2b2b2b2 TCCAGGCAAC AGGTGGCTGA GGCTGCTGCA GATCTGGAGG GAGCAGGGTT ATGAGCACCT GCACCTGGAG ATGCACCAGA CCTTCCAGGA GCTGGGGCCC ATTTTCAGGT AAAGCCCTCC CTGGCCCTCG
Consensus  TCCAGGCAAC AGGTGGCTGA GGCTGCTGCA GATCTGGAGG GAGCAGGGTT ATGAGcACCT GCACCTGGAa aTaCACCAGA CCTTCCAGGA aCTaGGGCCC ATTTTCAGGT AAAGCCCTCC CTGGCCCTCG
```

Exon 1 highlighted.

8.4 Coefficient of variation and limits of detection for LC:MS plasma corticosteroid measurements

CORTICOSTEROID	ADDED STANDARD	CV %	PLASMA %	LIMITS OF DETECTION
18 OH corticosterone	50pg	15.1	18.7	*
Aldosterone	50pg	11.8	13.4	3.0pg
Cortisol	25ng	6.1	9.1	1.1µg
Cortisone	5ng	5.0	7.9	0.7 µg
Corticosterone	500 pg	10.9	8.2	61.3pg
11-deoxycortisol	250 pg	11.1	14.5	15.0pg
Deoxycortcosterone	50 pg	9.4	20.1	2.7pg

CV, coefficient of variation; Plasma, between batch variations. (* No value for limits of detection for 18OH corticosterone is shown due to difficulty obtaining standard.)

8.5 Patient information sheet

Patient Information Sheet

1. Study Title

"Role of Genetic variation in aldosterone synthase and hypertension."

"A study of the control of blood pressure."

2. Invitation to take part

You are invited to take part in a study looking at how blood pressure is controlled. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

3. What is the purpose of this study?

High blood pressure affects a large number of people and increases the risk of a number of medical problems (heart disease, stroke etc). Why some people have high blood pressure and others do not, is not known. The hormones that are produced by the adrenal glands (steroid hormones) affect blood pressure. One of these hormones regulates fluid and salt balance in the body and it has been suggested that variation in this gene may play a role in high blood pressure. This study aims to examine more closely how people with different genetic patterns respond to stimulation of their adrenal glands in terms of blood pressure and hormones.

4. Why have I been chosen?

You have been asked to take part because you do not have any conditions that are affected by taking too much salt (kidney problems, heart problems, high blood pressure.) We will look at approximately 60 to 80 people to get an overall picture of how different people respond.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you withdraw, information that has already been gathered will form part of the analysis. If you decide not to take part it will not affect the care you receive at any time in the future.

6. What will happen to me if I take part?

This study will involve 5 visits to the Glasgow Cardiovascular Research Centre (travel expenses will be paid). The first visit will take around 15 mins. The next visits will take 1.5-2 hours.

1) On the first visit, a blood sample (approx one tablespoon) will be taken. This will take only a few minutes. Your DNA will be analysed to see what pattern of genes you carry with regard to the steroid regulating genes. It may take a few days/weeks to get these results. We are interested in looking at specific patterns. If you carry the pattern of genes we are interested in, we will ask you to participate in the second section.

2) You will be asked to follow a diet without excess salt for 4 days. You will be given advice about this. You will be asked to take some tablets which may contain either salt supplement or placebo (A placebo is a pill which looks like the real thing but contains no active ingredient). You will not know which you have been given.

3) On the third day you will be asked to take a urine collection for 24 hours. After the third day you will be asked to visit the Glasgow Cardiovascular Research Centre where, after 30 minutes rest, another blood sample will be taken (approx one tablespoon). You will receive a drip containing a hormone that naturally occurs in the body. This will take 30 minutes. When it is complete we will take a further blood sample (one tablespoon). Your blood pressure and heart rate will be monitored during this time.

4) You will be asked to return the following day to repeat this procedure.

5) The following week the experiment will be repeated however the tablet will be switched from a salt table to placebo or vice versa.

The attached diagram helps to explain exactly what will be involved.

7. What are the possible disadvantages and risks of taking part?

The procedures described above are very safe. The main potential side effect is a **rise in your blood pressure which may be associated with a headache**. This is most likely to happen during the high salt phase with one of the infusions (a hormone called angiotensin II). Your blood pressure and pulse will be monitored throughout this period and the study will be stopped if it rises above a certain level. The effects of the hormone infusions are very short and there are no known long term effects

Blood samples will be taken. This is a safe and commonly practiced procedure however bruising may occur at the site of the needle, some people may feel faint.

You have been asked to participate as you no not have a chronic disease that may deteriorate by taking extra salt in your diet (e.g. kidney problems, heart problems, high blood pressure). **If you think you may have any of these conditions, please let the research doctor know.**

It is possible that if this study is carried out on a woman who is pregnant it could harm the unborn child. Women who could be pregnant may be asked to take a pregnancy test before the study commences. Women who could become pregnant should use effective contraception for the duration of the study. **Women who think they may have become pregnant during the duration of the study should immediately let their research doctor know.**

8. What are the possible benefits of taking part?

You will receive no direct benefit from taking part in this study. The information that is collected during this study will give us a better understanding of how high blood pressure develops and how best to treat it.

9. What if something goes wrong?

There are no special compensation arrangements if you are harmed by taking part in this research project. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure.

10. Will my taking part in this study be kept confidential?

If you consent to take part in the research your medical records may be inspected by the research doctor for purposes of analysing the results. Only government regulatory authorities and the research doctor will have access to your medical notes.

All information collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. Reports or publications resulting from the study will not contain any personal details. Your General Practitioner will be informed of your participation.

11. What will happen to the results of the research study?

The results of the research study will be stored on a computer database and are likely to be published in medical journals. Reports or publications resulting from the study will not contain any personal details. The research doctor will provide a copy of the results on request.

12. Who is organising and funding the research?

The research is being organised by the Division of Cardiovascular and Medical Science, University of Glasgow. The Medical Research Council funded Blood Pressure Research Group is the section responsible for this study.

13. Who has reviewed the study?

This study has been reviewed and approved by Glasgow West Local Research Ethics Committee which is an independent panel set up to protect your safety, rights, wellbeing and dignity.

Contact

If you have any further questions please contact

Dr Frances McManus

British Heart Foundation Glasgow Cardiovascular Research Centre

126 University Place,

G12 8TA

0141 330 1933

www.gla.ac.uk/bhfgcrc

Questionnaire

Do you have any of the following conditions? (Y/N)

High blood pressure? ☐

Heart disease (including angina, previous heart attack or heart failure)? ☐

Kidney disease? ☐

Are you pregnant of likely to become so in the next 6 months? ☐

Have you used of systemic or topical steroids within the last 6 months? ☐

Do you have a history of severe allergies? ☐

Are you on any regular medication? ☐

Please list below:

Subject Identification Number for this trial:

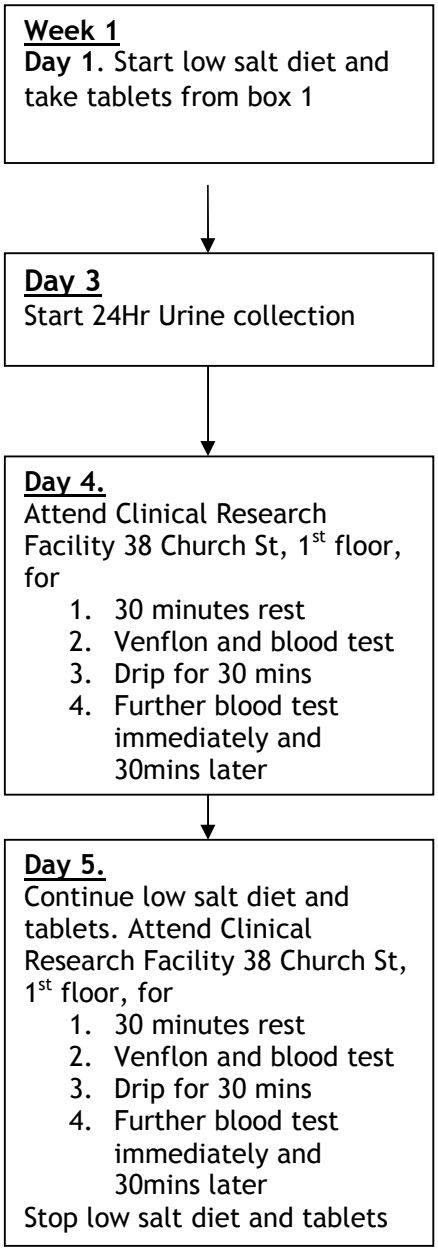
CONSENT FORM

Title of Project: Role of Genetic Variation in Aldosterone Synthase and Hypertension

Please initial box

1. I confirm that I have read and understand the information sheet dated August 2007 (version 2) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected. ☐
3. I agree to take part in the above study. ☐
4. I understand that the DNA in my blood will be analysed as part of this study ☐
5. I agree that to my GP being informed of my participation in this study ☐

_____	_____	_____
Name of subject	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent (If different from researcher)	**Date**	**Signature**
_____	_____	_____
Researcher	**Date**	**Signature**


Week 1
Day 1. Start low salt diet and take tablets from box 1
Day 3
Start 24Hr Urine collection
Day 4.
Attend Clinical Research Facility 38 Church St, 1st floor, for
1. 30 minutes rest
2. Venflon and blood test
3. Drip for 30 mins
4. Further blood test immediately and 30mins later
Day 5.
Continue low salt diet and tablets. Attend Clinical Research Facility 38 Church St, 1st floor, for
1. 30 minutes rest
2. Venflon and blood test
3. Drip for 30 mins
4. Further blood test immediately and 30mins later
Stop low salt diet and tablets

8.6 Clinical study protocol

Clinical Study Protocol: Version 2

Study Title: Role of Genetic Variation in Aldosterone Synthase in Hypertension. Physiological Studies

Chief Investigators:

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University of Glasgow,
Glasgow, G12 8TA
Tel: 0141 330 1933
e-mail: F.McManus@hotmail.com

Background: Hypertension is a common condition that contributes to the global burden of cardiovascular disease. In the majority of patients no single cause is found and its pathogenesis reflects a complex interaction of genes and environment.

It is recognised that aldosterone is an important cardiovascular hormone across a range of disorders. However, aldosterone is also recognised as an important contributor to the pathogenesis of hypertension and its consequences. Approximately 15% of patients with essential hypertension have evidence of dysregulation of aldosterone production so that there is an abnormally high ratio of aldosterone to renin (ARR). There is uncertainty about whether such patients have genuine primary aldosterone excess; in the majority of instances aldosterone is still responsive to Angiotensin II, and it is likely that the raised ARR reflects increased sensitivity of aldosterone release to its usual trophins. For this reason it is important to understand better the mechanism(s) that underlies this biochemical abnormality: the planned studies seek to investigate possible mechanisms that may contribute to the regulation of aldosterone in hypertension.

Adrenal corticosteroid secretion is strongly heritable, consistent with genetic determination, stimulating a search for candidate genes that may account for this. Attention has focussed on the late steps in adrenal steroid synthesis.

The late stages of aldosterone synthesis are catalysed by the enzyme aldosterone synthase, encoded by the gene CYP11B2, and expressed in zona glomerulosa of the adrenal cortex. This gene is located in chromosome 8 in man, and lies in close proximity to the gene CYP11B1, which encodes the enzyme 11ß-hydroxylase to catalyses the conversion of deoxycortisol to cortisol within the zona fasciculata. (Figure 1)

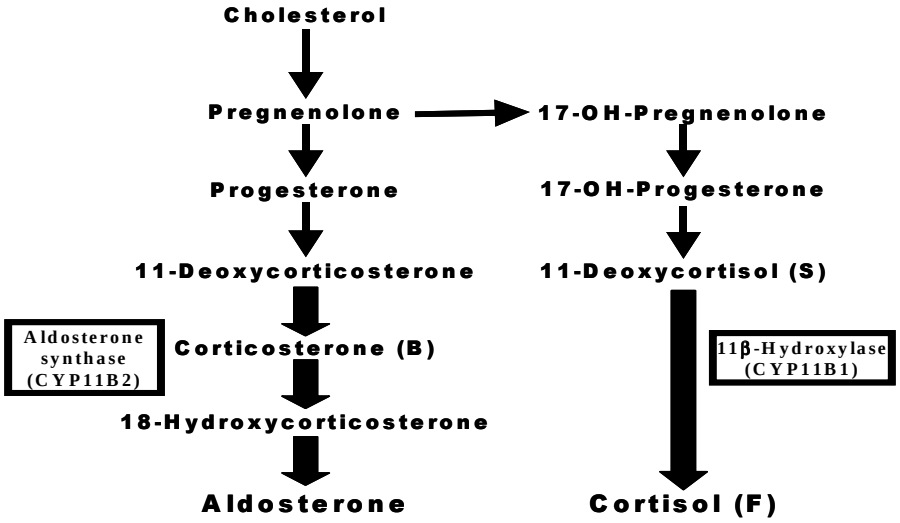


Figure 1 Corticosteroid biosynthesis in the human adrenal gland

Figure 1: Steroid synthesis pathway

These genes are plausible candidates for contributing to the population variation in aldosterone and cortisol production, and development of hypertension and cardiovascular disease.

Previous studies have identified a common single nucleotide (-344 C/T) polymorphism in CYP11B2. Studies have shown that this polymorphism is associated with hypertension, particularly with a phenotype of a raised ARR.

Recent data confirm that polymorphisms in the 5' regulatory region in CYP11B1 are in strong linkage disequilibrium with those in the regulatory regions of CYP11B2. It is not clear what the individual effects of the polymorphisms within the two separate genes are on the phenotypes of aldosterone excess and the development of hypertension, however, as in the Dahl rat, it is possible that there is an interaction between CYP11B1 and CYP11B2 (essentially, a digenic effect) that results in increased ACTH drive to the adrenal (due to changes in CYP11B1) and abnormal sensitisation of regulation of expression of CYP11B2 to agonists including potassium, ACTH and Angiotensin II, leading to the phenotype of chronically altered aldosterone regulation.

Hypothesis: Polymorphisms in the genes encoding aldosterone synthase and 11ß-hydroxylase are inherited in haplotype blocks and lead to altered production of mineralocorticoids and glucocorticoids in affected individuals.

Aims: To measure steroid response (plasma and urine) to its conventional trophin (angiotensin II) of aldosterone production in high and low salt intake in normal volunteers with contrasting haplotypes.

To measure steroid response to its unconventional trophin (ACTH) of aldosterone production in high and low salt intake in normal volunteers with contrasting haplotypes.

Study Design: The study will be carried out at the BHF Glasgow Cardiovascular Research Centre. The study duration will be 3 years. The length of participation of each subject will be 2 weeks.

Volunteers will be recruited via poster campaign and through advertisements using local media.

Screening: Subjects will be screened for participation in the study initially.

Volunteers will sign a consent form indicating their willingness to participate in screening. Consent will be taken by Dr Frances McManus. Consent forms will be stored in the site file in the Glasgow Cardiovascular Research Centre. Volunteers will be given a copy of the patient information sheet and consent form. This will be documented and filed in the project folder. Volunteers will be asked if they suffer from any medical conditions likely to deteriorate as a result of participation in this study (heart failure, hypertension, renal impairment).

Exclusion criteria are as follows

Age greater than 75 years or less than 18 years.

Cardiac failure

Renal impairment

Uncontrolled hypertension (BP > 160/90)

Treatment with beta blockers, ACE inhibitors, diuretics.

Use of systemic or topical steroids within the last 6 months

Pregnancy

Inability to comply with study protocol

History of severe allergy

Screening blood tests are as follows:

Baseline biochemistry to assess renal function (6ml gel tube).

DNA will be extracted from blood sample (5ml EDTA) and genotyped for the polymorphisms of interest.

Study: Volunteers with the polymorphisms of interest will be invited to return for the second phase of the study. Subjects will be studied during careful dietary sodium control to maintain either a high (200 mmol per day) or low (80 mmol per day) sodium intake. They will be given a diet sheet to follow which will give advice on a low salt diet (approx 4.8g per day). Salt loading will aim for an intake of 200 mmol per day (approx 12g) using sodium supplement tablets containing 600mg sodium chloride (four tabs 3 times per day).

Once in balance (after 3 days) subjects will be infused with ACTH (1ng/kg/minute for 30 mins); on the following day subjects will be given an infusion of angiotensin II (3 ng/kg/minute for 30 mins). Infusions will be made by pharmacy. Basal and stimulated plasma aldosterone levels, as well as a range of other steroids will be measured. This will require baseline blood tests before the infusion (x2 Lith Heparin and x 1 EDTA), at completion of the infusion, as well as 30 mins after completion (x2 Lith Heparin and x 1 EDTA).

In addition, 24 hr urine collections will be analysed for steroid measurements. Spot urine samples will be measured for sodium excretion.

The study will last for two weeks to allow volunteers to complete both the salt loading and salt restricted (placebo) arms.

Results will be entered onto the Clinical Record sheet by Dr Frances McManus or a research nurse. Observations (BP, HR) will be documented by the nursing staff of the clinical studies unit at the GCRC.

Risks: The risks relating to venepuncture are low and standard precautions will be taken. The participant may experience some local bruising.

The recommended daily salt intake is 6g per day. Salt loading may be associated with mild nausea however slow release tablets will be used to minimise this effect. Patients with uncontrolled hypertension, significant renal disease and heart failure may experience a small, reversible deterioration in their condition with prolonged salt loading however; volunteers will be screened for these conditions and excluded from the study.

Angiotensin II can cause the blood pressure to rise. This could cause potential harm if it was allowed to continue however the blood pressure will be monitored and the study will be stopped if it the mean arterial pressure (MAP) rises more than 20mmHg. The length of time it takes for Angiotensin II to wear off is very short.

ACTH can cause blood pressure to rise however, this effect is transient and blood pressure will be regularly monitored and stopped if MAP rises more than 20mmHg. Short term exposure to ACTH in adults has not been reported to be associated with any further complications. ACTH is commonly used in hospitals as a diagnostic tool, with no reported complications.

Adverse Events Adverse events are likely to be rare and mild given the nature of this study. All serious adverse events will be reported to the sponsor (North Glasgow NHS Trust).

Pharmacy: Pharmaceutical products will be dispensed from the pharmacy department of the Western Infirmary Glasgow/ Clinical Trials Pharmacy Unit and this has been agreed with the pharmacy department.

Placebos will be matched as closely as possible to the active substance.

Products will be manufactured to the standards of Good Medical Practice.

Labelling of the placebo and active substances will be managed by pharmacy and checked with clinicians involved.

Volunteers will be requested to return unused products to the Glasgow Cardiovascular Research Centre, where they will be passed on to pharmacy. Patients will be informed of this when the product is being dispensed.

Randomisation: Randomisation will be computer generated.

Patient Data: All patient data will be held on a password locked computer file in anonymised form. This will be stored in a locked cabinet in the Glasgow Cardiovascular Research Centre. Patient identifier data and Clinical Record Forms will be kept in a locked cabinet.

Withdrawal: Volunteers can withdraw at any time. This is explained in the patient information sheet. Information gathered up to the date of withdrawal will be used in the analysis. Once a volunteer has withdrawn they will not be invited for further blood or stimulation tests. This will be recorded in the site file and on the Clinical Record Form.

Results and Statistics: Large scale clinical studies have not been performed in this area and power calculations are hindered by this lack of information. However, we estimate that a sample size of 20 individuals per haplotype (likely to be 3 common haplotypes) will be necessary to identify a significant difference in the aldosterone response to angiotensin II, and we base this estimate on a recent study which was able to demonstrate significant differences in angiotensin responses with low salt intake using around 10 individuals in each contrasting genotype. A total of 60 individuals will be required to participate. This will require around 100 individuals to be screened.

Staff: Dr Frances McManus (Research Fellow) has attended a Good Clinical Practice course (4th April 2007 Clinical Trials Unit North Glasgow/ Glasgow University).

Dr Marie Freel (Clinical Lecturer) has experience in similar studies.

Prof J Connell has had extensive experience as a lead investigator in similar studies

Dr Eleanor Davies (Senior Lecturer) will be involved in the data analysis.

The staff in the clinical unit of the Glasgow Cardiovascular Research Centre are familiar with the principles and practices involved in Good Clinical Practice.

8.7 Clinical Records form

Clinical Record Form

Patient Study no: AFS 064

Patient name:

Address:

E mail

Telephone:

Date of Birth:

Consent form signed Yes/ No

Patient copy of consent form Yes/ No

Co-morbidities excluded Yes/ No

Slow sodium/ placebo prescription? Yes/ No

Ang II/ ACTH Prescription? Yes/ No

Infusions prescribed? Yes/ No

Initial resting blood pressure

Height (cm)

Weight (Kg)

Initial Bloods taken

Biochemistry (yellow x 1)

Result (eGFR)

DNA extraction (purple x 1)

24 Hr Urine bottle given ?

Visit 1/ 2/ 3/ 4

Date of visit:

Negative pregnancy test Yes/ No / NA

24 hr Urine collection completed? Yes/ No

Total Volume/ Weight = _____

ACTH = 1 ng/kg/min

Batch No; _____ Exp Date_____

Infusion 1 *ACTH*	**Time from start of infusion**	**Heart Rate**	**MAP**	**BP mmHg**	**Bloods taken** **Lithium Heparin (green) x 3** **EDTA (Purple) 9ml x 1**			
Blood pressure 1	- 30 min				Start Time:			
Blood pressure	0 min				Li Hep	Li Hep	Li Hep	EDTA
Blood pressure	10 min							
Blood pressure	20 min							
Blood pressure	30 min				Li Hep	Li Hep	Li Hep	EDTA
Blood pressure	60 min				Li Hep	Li Hep	Li Hep	EDTA

Infusion discontinued?

Time from start of infusion:
Bloods taken at termination of infusion Yes/ No

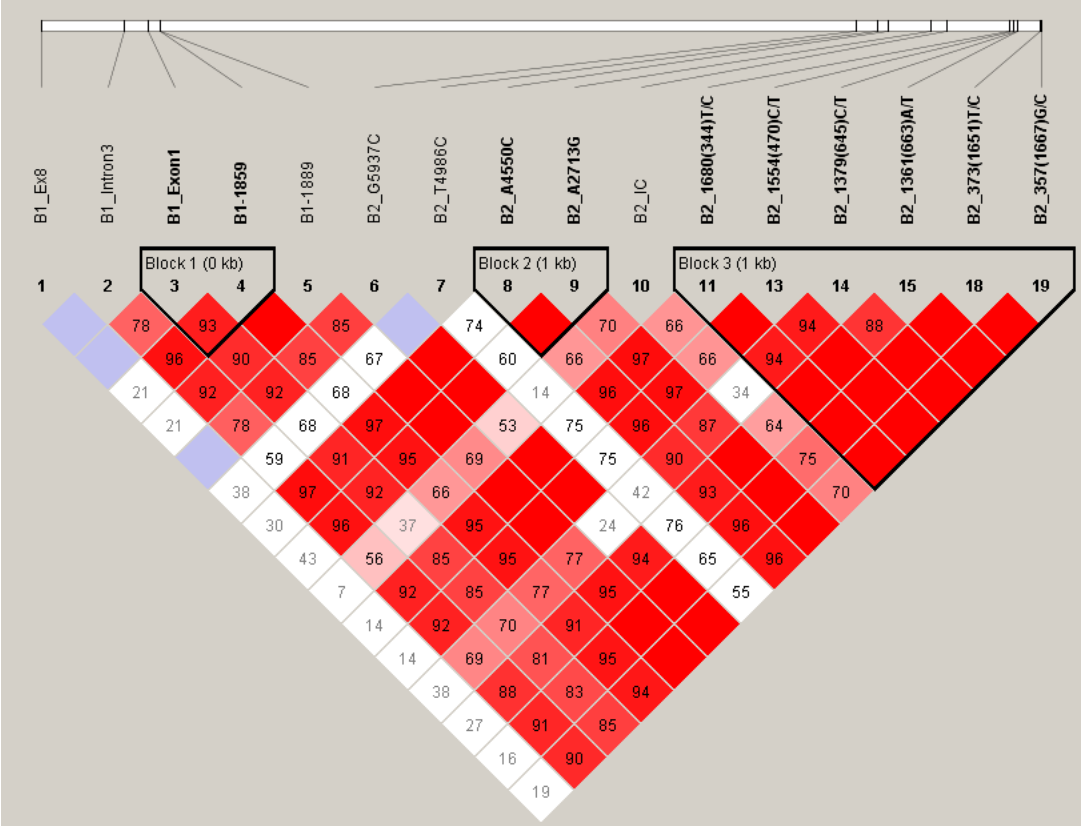
Other adverse effects?

Patient withdrawal from study?

Reason given?

Other Notes:

8.8 Linkage disequilibrium CYP11B1 and CYP11B2 locus



D' values demonstrating linkage disequilibrium across the CYP11B1 and CYP11B2 promoter region. Data pooled from genotyping described in chapter 3 and data from Imrie et al (Imrie et al., 2006) and Alvarez-Madrazo et al (Alvarez-Madrazo et al., 2009).

8.9 Table of polymorphisms

NCBI SNP database http://www.ncbi.nlm.nih.gov/projects/SNP		**Pos. from Exon1**	**Pos. from 5' end**	**Pos. on plasmid**	**ENSMBL** http://www.ensembl.org/Homo_sapiens/Gene/Sequence?g=ENSG00000179142		
Ch Position	**Group Term**				**Allele 1**	**Allele 2**	**rs number**
144000922	GRCh37	-1667	357	276	G	C	rs13254375
144000906	GRCh37	-1651	373	292	T	C	rs13268025
144000786	GRCh37	-1513	510	427	T	C	rs62524561
144000727	GRCh37	-1472	551	468	A	G	rs62524560
143999919	GRCh37	-663	1360	1269	T	A	rs28659182
143999901	GRCh37	-645	1379	1287	C	T	rs11781082
143999726	GRCh37	-470	1554	1462	T	C	rs10087214
143999600	GRCh37	-344	1680	1590	C	T	rs1799998

8.10 Sequence of pGL3-CYP11B2 plasmid

```
<Serial Cloner V2.0> -- <04 Mar 2010   14:44>
Restriction map of pGL3_pCRscript_B2proCC_march 2010.xdna
Showing restriction enzymes cutting maximum 1 time [using Serial Cloner Internal RE list as a Restriction Enzyme Library]

       SacI          NheI
       Eco53kI       BmtI       EcoRI
 KpnI          AflIII           ApoI
 Acc65I        MluI         >CdiI                                                <SapI
 |     |       |     |      |   |            • pGL3 plasmid                      |
GGTACCGAGCTCTTACGCGTGCTAGCCCATCGAATTCCTGCAGCCCGGGGGATCCGCCCGATACAGGTCAGCAACCCTGAGCTACATAAAGAAAGGAAGA  < 100
CCATGGCTCGAGAATGCGCACGATCGGGTAGCTTAAGGACGTCGGGCCCCCTAGGCGGGCTATGTCCAGTCGTTGGGACTCGATGTATTTCTTTCCTTCT
         10        20        30        40        50        60        70        80        90

GCATTTCAGAAGGAATCAGTAAAGAGAAAATGAAGTCTTTTATTTTTTCTTAATCTTAATTGATCTAAGAGTTTGCTAAAACAAAACAACAACAACAAAA  < 200
CGTAAAGTCTTCCTTAGTCATTTCTCTTTTACTTCAGAAAATAAAAAAGAATTAGAATTAACTAGATTCTCAAACGATTTTGTTTTGTTGTTGTTTT
         110       120       130       140       150       160       170       180       190

                        >TstI
                        |                                                      T • C
ATAGGCCGGGCGCGGTGACTCACCACACCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGCAGATCACCTGAGGTCAGGAGTTCGAGACTAGCCTGGC  < 300
TATCCGGCCCGCGCCACTGAGTGGTGTGGACATTAGGGTCGTGAAACCCTCCGGCTCCACCCGTCTAGTGGACTCCAGTCCTCAAGCTCTGATCGGACCG
         210       220       230       240       250       260       270       280       290


CAACACGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTAGCTGGGCGTGGTGGCACATGCCTGTAATCCCAGCTACTTGGGAGGCTGAGGCGGGAGG  < 400
GTTGTGCCACTTTGGGGCAGAGATGATTTTTATGTTTTTAATCGACCCGCACCACCGTGTACGGACATTAGGGTCGATGAACCCTCCGACTCCGCCCTCC
         310       320       330       340       350       360       370       380       390


ATTGCTTGAACCCGGGAGACAGAGGTTGCAGTGAGCCGAGATCACACCATTGCACTCTAGCCTGGGCAACAGAGTGAGACTCTGTCTCAAAAATAAATAA  < 500
TAACGAACTTGGGCCCTCTGTCTCCAACGTCACTCGGCTCTAGTGTGGTAACGTGAGATCGGACCCGTTGTCTCACTCTGAGACAGAGTTTTTATTTATT
         410       420       430       440       450       460       470       480       490


ATAAATAAATAAATAATAAAAATAAATAAATAAAAGCCAGAAAGTGTATTTGATGATCATAGTTATGTATATGTGAAATGAAGGACAGCAATGATGCAAG  < 600
TATTTATTTATTTATTATTTTTATTTATTTATTTTCGGTCTTTCACATAAACTACTAGTATCAATACATATACACTTTACTTCCTGTCGTTACTACGTTC
         510       520       530       540       550       560       570       580       590


GGATGGGTGAGTGGAATTAAAAATGTCTTATTATTTATTTATTTTGAGATGGAGTCTTGCTTTGCTGCCCAGGTTGGAGTGCAGTGGGATGATCTCAACT  < 700
CCTACCCACTCACCTTAATTTTTACAGAATAATAAATAAATAAAACTCTACCTCAGAACGAAACGACGGGTCCAACCTCACGTCACCCTACTAGAGTTGA
         610       620       630       640       650       660       670       680       690
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CACTGCAACCTCCGCCTCCTTGATTCAAGCATTCATCTTGACTCAGCCTGCTGAGAAGCCgAGATTACAGGCATGCGCCACCACACCTGGCTAATTTTGT  < 800
GTGACGTTGGAGGCGGAGGAACTAAGTTCGTAAGTAGAACTGAGTCGGACGACTCTTCGGcTCTAATGTCCGTACGCGGTGGTGTGGACCGATTAAAACA
         710       720       730       740       750       760       770       780       790


ATTTTTAGTAGAGACAGGGTTTTGCCATGTTGGCCAGGCTGGTCTCgAACTCCTGACCTCAGGTGATCCACCTGCATCAGCCTCCCAAAGTGCTGGGATG  < 900
TAAAAATCATCTCTGTCCCAAAACGGTACAACCGGTCCGACCAGAGcTTGAGGACTGGAGTCCACTAGGTGGACGTAGTCGGAGGGTTTCACGACCCTAC
         810       820       830       840       850       860       870       880       890

ACAGACATGAGCCACTATGCCCAGCCTAAGAATATCTGATGATTATAAAGTGCTTGCATTACCTCTGAAGCTGTATAGTGTTATATGAAGGTGGAGTTGG  < 1000
TGTCTGTACTCGGTGATACGGGTCGGATTCTTATAGACTACTAATATTTCACGAACGTAATGGAGACTTCGACATATCACAATATACTTCCACCTCAACC
         910       920       930       940       950       960       970       980       990


AGAGATGAGTTTTAAGCGTATATTGCAAACTCTAGGGCAACCACTAAAGAAGTGAGACCCAGCCTCTAGAAAAAAAAAAAAAAAAGGAAATTAGCTATCAA  < 1100
TCTCTACTCAAAATTCGCATATAACGTTTGAGATCCCGTTGGTGATTTCTTCACTCTGGGTCGGAGATCTTTTTTTTTTTTTTTCCTTTAATCGATAGTT
         1010      1020      1030      1040      1050      1060      1070      1080      1090


GCCACGAAAAGAAATGGAGGAACCTTAAACGCATATTACTAACTGAGATACGTCACTTTGAAAAGGCTACAAACGGTGTCATTCCAACTATACAACATTT  < 1200
CGGTGCTTTTCTTTACCTCCTTGGAATTTGCGTATAATGATTGACTCTATGCAGTGAAACTTTTCCGATGTTTGCCACAGTAAGGTTGATATGTTGTAAA
         1110      1120      1130      1140      1150      1160      1170      1180      1190


TGGAAAAGGCCAAAGCATGGTGATGATAAAAAGATCGGAGATGTCAGGGACTGGGGCAGGAGGGATGAGCTGGCAGAGCACAGGTTTTCTTTTCCTCTTT  < 1300
ACCTTTTCCGGTTTCGTACCACTACTATTTTTCTAGCCTCTACAGTCCCTGACCCCGTCCTCCCTACTCGACCGTCTCGTGTCCAAAAGAAAAGGAGAAA
         1210      1220      1230      1240      1250      1260      1270      1280      1290


TTAAGACAGTGAAAATACTCCTAGGATCCTGCAAGGAGGGATACAAATTACATACATTTGTCAAAACCCACAGCATGTTGACCACCAGGAGGAGACCCCA  < 1400
AATTCTGTCACTTTTATGAGGATCCTAGGACGTTCCTCCCTATGTTTAATGTATGTAAACAGTTTTGGGTGTCGTACAACTGGTGGTCCTCCTCTGGGGT
         1310      1320      1330      1340      1350      1360      1370      1380      1390


TGTGACTCCAGGACCCTGGTTGATAACAACGTATCGAGATTCCTCACATGGAACCAGTGCGCTTCTGTGGTGGAGGGTGTACCTGTGTCAGGGCAGGGGG  < 1500
ACACTGAGGTCCTGGGACCAACTATTGTTGCATAGCTCTAAGGAGTGTACCTTGGTCACGCGAAGACACCACCTCCCACATGGACACAGTCCCGTCCCCC
         1410      1420      1430      1440      1450      1460      1470      1480      1490


TACGTGGACATTTTCTGCAGTTTTTGATCAATTTTGCAATGAACTAAATCTGTGGTATAAAAATAAAGTCTATTAAAAGAATCCAAGGCCCCCTCTCATC  < 1600
ATGCACCTGTAAAAGACGTCAAAAACTAGTTAAAACGTTACTTGATTTAGACACCATATTTTTATTTCAGATAATTTTCTTAGGTTCCGGGGGAGAGTAG
         1510      1520      1530      1540      1550      1560      1570      1580      1590
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TCACGATAAGATAAAGTCCCCATCCATTTTACTCCTCTCAGCCCTGGAGAAAGGAGAGGCCAGGTCCCACCACCTTCCACCAGCATGGACCCCCAGTCCA  < 1700
AGTGCTATTCTATTTCAGGGGTAGGTAAAATGAGGAGAGTCGGGACCTCTTTCCTCTCCGGTCCAGGGTGGTGGAAGGTGGTCGTACCTGGGGGTCAGGT
         1610      1620      1630      1640      1650      1660      1670      1680      1690


GACCCCACGCCTTTTCTCAGCATCCTCAGACCAGCAGGACTTGCAGCAATGGGGAATTAGGCACCTGACTTCTCCTTCATCTACCTTTGGCTGGGGGCCT  < 1800
CTGGGGTGCGGAAAAGAGTCGTAGGAGTCTGGTCGTCCTGAACGTCGTTACCCCTTAATCCGTGGACTGAAGAGGAAGTAGATGGAAACCGACCCCCGGA
         1710      1720      1730      1740      1750      1760      1770      1780      1790


CCAGCCTTGACCTTCGCTCTGAGAGTCTCAGGCAGGTCCAGAGCCAGTTCTCCCATGACGTGATATGTTTCCAGAGCAGGTTCCTGGGTGAGATAAAAGG  < 1900
GGTCGGAACTGGAAGCGAGACTCTCAGAGTCCGTCCAGGTCTCGGTCAAGAGGGTACTGCACTATACAAAGGTCTCGTCCAAGGACCCACTCTATTTTCC
         1810      1820      1830      1840      1850      1860      1870      1880      1890


                              pGL3 plasmid•
ATTTGGGCTGAACAGGGTGGAGGGAGCATTGGGCTAGAGCGGCCGGGCTCGAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCC  < 2000
TAAACCCGACTTGTCCCACCTCCCTCGTAACCCGATCTCGCCGGCCCGAGCTCTAGACGCTAGATTCATTCGAACCGTAAGGCCATGACAACCATTTCGG
         1910      1920      1930      1940      1950      1960      1970      1980      1990


ACCATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGAT  < 2100
TGGTACCTTCTGCGGTTTTTGTATTTCTTTCCGGGCCGCGGTAAGATAGGCGACCTTCTACCTTGGCGACCTCTCGTTGACGTATTCCGATACTTCTCTA
         2010      2020      2030      2040      2050      2060      2070      2080      2090


ACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTCGGTTGGCAGAAGC  < 2200
TGCGGGACCAAGGACCTTGTTAACGAAAATGTCTACGTGTATAGCTCCACCTGTAGTGAATGCGACTCATGAAGCTTTACAGGCAAGCCAACCGTCTTCG
         2110      2120      2130      2140      2150      2160      2170      2180      2190


TATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTATGCCGGTGTTGGGCGCGTTATTTATCGGA  < 2300
ATACTTTGCTATACCCGACTTATGTTTAGTGTCTTAGCAGCATACGTCACTTTTGAGAGAAGTTAAGAAATACGGCCACAACCCGCGCAATAAATAGCCT
         2210      2220      2230      2240      2250      2260      2270      2280      2290


GTTGCAGTTGCGCCCGCGAAC  < 2321
CAACGTCAACGCGGGCGCTTG
         2310
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