THE INFLUENCE OF DUAL CYP17 EXPRESSION ON ADRENAL STEROIDOGENESIS IN THE SOUTH AFRICAN ANGORA GOAT

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Dissertation presented for the
Degree of Doctor of Philosophy (Biochemistry)
in the Faculty of Science

at
Stellenbosch University

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December 2008

DECLARATION

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Date: Desember, 2008

SUMMARY

This study describes:

- the cloning and sequencing of cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17), 3β-hydroxysteroid dehydrogenase (3βHSD) and cytochrome b_5 from the South African Angora goat;

- the identification of two CYP17 genes encoding two unique CYP17 isoforms in the Angora goat;

- the development of a UPLC-APCI-LC method for the separation and quantification of seven adrenal steroids;

- the characterisation of the enzymatic activity of the two Angora CYP17 isoforms expressed in non-steroidogenic COS-1 cells. The K_m and V-values for the metabolism of pregnenolone and progesterone were determined;

- the development of a rapid and accurate real-time PCR genotyping test for CYP17 in Angora goats. Three unique genotypes were identified;

- the determination of blood cortisol levels upon the stimulation of the HPA-axis by intravenous insulin injection in the three Angora goat genotypes.

OPSOMMING

Hierdie studie beskryf:

- die kloneering en nukleotiedvolgordebepaling van sitochroom P450 17α-hidroksilase/17,20 liase (CYP17), 3β-hidroksisteroïed dehidrogenase (3βHSD) en sitochroom b_5 van die Suid-Afrikaanse Angorabok;

- die identifisering van twee CYP17-gene in die Suid-Afrikaanse Angorabok wat elkeen vir ‘n unieke CYP17-isoform kodeer;

- die ontwikkeling van ‘n UPLC-APCI-LC-metode vir die skeiding en kwantifisering van sewe steroïede wat in die bynier geproduseer word;

- die ensiematiese karakterisering van die twee Angorabok-CYP17-isoforme in COS-1-selle. Die K_m- en *V*-waardes vir pregnenoloon- en progesteroonmetabolisme deur die twee isoforme is bepaal;

- genotipering van Angorabokke met die ontwikkeling van ‘n nuwe genotype-toets wat op intydse PKR gebaseer is. Drie unike genotipes is geïdentifiseer;

- die bepaling van kortisol vlakke in die serum van Angorabokke van die drie verskillende genotipes na die toediening van insulien.

In memory of Wolfgang Eberhart Storbeck

ACKNOWLEDGEMENTS

I hereby wish to express my sincerest gratitude to the following persons and institutions:

Prof P Swart for his leadership, support and for allowing me to make this project my own.

Dr AC Swart for her continual support, encouragement and guidance.

Dr MA Snyman for her support of this project and for providing us with Angora goat blood samples.

Ralie Louw for her support and technical assistance.

Dr M Stander and **Désirée Prevoo** for technical assistance with the LC-MS.

Carel van Heerden and **Gloudi Agenbag** for valuable discussion and technical assistance.

Prof LC Hoffman, **Francois van de Vyver** and **Danie Bekker** for technical assistance with the collection of blood and tissue samples.

Tino Herselman and **the staff** at the Jansenville experimental farm for their help with the insulin induced stress experiment.

Dr A Louw for her help with the statistics.

Robin Thomas and **Cary Blackburn** for introducing me to real-time PCR.

Mohair SA, the **NRF**, **University of Stellenbosch** and the **Wilhelm Frank Bursary Fund** for financial support.

All persons at the Department of Biochemistry who made my work there enjoyable.

My mom, **Sue,** for her love, support and encouragement.

My other parents, **Mike** and **Zita**, for their love and support.

My wife, **Patricia**, for her continual love, support and belief in me.

Almighty God, for allowing me to study His creation at this level.

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ABBREVIATIONS

3βHSD	3β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
ACAT1	acyl-CoA:cholesterol acyltransferase 1
ACTH	adrenocorticotropic hormone
Adx	adrenodoxin
AdxR	adrenodoxin reductase
Apo E	apolipoprotein E
AR	androgen receptor
CAH	congenital adrenal hyperplasia
CE	cholesterol esters
CBG	cortisol binding globulin
COUP-TF	chicken ovalbumin promoter-transcription factor
CPR	cytochrome P450 oxidoreductase
CRG	corticotropin releasing hormone
CRS	cAMP regulatory sequence
CYP11A1	cytochrome P450 side-chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17	cytochrome P450 17α-hydroxylase/17,20 lyase
CYP21	cytochrome P450 21-hydroxylase
DBD	DNA binding domain
DHEA	dehydroepiandosterone
FAD	flavinadenine dinucleotide
FMN	flavinmononucleotide
FSH	follicle stimulating hormone
GH	growth hormone
GHRH	growth hormone releasing hormone
GIH	growth hormone-inhibiting hormone
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid-response element
HDL	high density lipoprotein
HPA axis	hypothalamic-pituitary-adrenal axis
HSD	hydroxysteroid dehydrogenases
HSL	hormone sensitive lipase
LBD	ligand binding domain
LDL	low density lipoprotein
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MKP	mitogen-activated protein kinase phosphatase
MR	mineralocorticoid receptor

NF-1	nuclear factor-1
nGRE	negative GRE
NHR	nuclear hormone receptor
p37	37-kDa StAR
p30	30-kDa StAR
PAP7	PBR-associated protein 7
PBR	peripheral benzodiazepine receptor
PIH	prolactin-inhibiting hormone
PKA	cAMP dependent protein kinase
POMC	pro-opiomelanocortin
pp30	phosphorylated p30
pp37	phosphorylated p37
PP2A	protein phosphatase 2A
PP4	protein phosphatase 4
PR	proline rich sequence
PSF	poly-pyrimidine tract-binding protein-associated splicing factor
RE	response element
ROCK	Rho-associated, coiled-coil containing protein kinase
Sf-1	steroidogenic factor-1
SR-BI	scavenger receptor class B, type I receptor
StAR	Steroid Acute Regulatory Protein
TRH	thyrotropin releasing hormone
TSH	thyrotrophin

CHAPTER 1

INTRODUCTION

South African Angora goats (*Capra hircus*) are the most efficient fibre producing, but least hardy, small stock breed in Southern Africa. South Africa produces approximately 4 million kg mohair annually, of which more than 95% is exported, supplying approximately 55% of the global demand for mohair. Furthermore, South African mohair is currently recognised as being of the best quality in the world. However, the industry is hampered by the severe loss of young, newly shorn Angora goats, which occur during cold spells.

Wentzel et al. (1979) has shown that the primary cause of stock loss during cold spells is due to an energy deficiency resulting from a decrease in blood glucose levels, which causes a drop in body temperature and subnormal heart function. In mammals, physiological stress stimulates the release of glucocorticoids from the adrenal cortex, via the hypothalamic-pituitary-adrenal (HPA) axis, which favours glucose production at the expense of glycolysis (Munch, 1971).

Fourie (1984) demonstrated that Angora goats could not cope with wet and windy cold conditions for as long as the more hardy Boer goats could, even when supplementary feeding practices were employed. Fourie (1984) concluded that the Angora goat does not have the metabolic capacity to produce sufficient heat, a problem that is further compounded by the weak insulation of the short hair found in shorn goats. Cronje (1992) later demonstrated that Angora does have a lower blood glucose concentration and a slower response of glucose synthesis rate to dietary energy increments,

than Boer goat does, providing further evidence of the inability of the Angora goat to mobilise glucose precursors.

Van Rensburg (1971) had previously suggested that selection for high mohair production indirectly resulted in reduced adrenal function and, as a result, reduced cortisol levels, as a negative relationship between plasma cortisol levels and hair production was identified. Herselman (1990) confirmed that the high hair production in Angora goats resulted in the total energy metabolism being less effective when compared to other goats, and suggested that hair production might be at the expense of other physiological functions. Herselman and van Loggerenberg (1995) subsequently investigated cortisol production in a number of small ruminant breeds with varying potentials for fibre production. While intravenous insulin injection caused a drop in blood glucose concentration in all breeds with a resulting increase in the plasma cortisol concentration, the peak plasma cortisol concentration was three to five times lower in the Angora when compared to the other breeds. Similarly, the response in plasma cortisol levels to intravenous corticotropin releasing hormone (CRH) was three to four times lower in the Angora than in the other breeds. This study concluded that a form of hypocortisolism contributes significantly to the disorders in carbohydrate metabolism observed in the Angora.

Engelbrecht et al. (2000) subsequently investigated the site of cortisol production, the adrenal cortex. In a comparative study, the adrenal response of Angora goats, Boer goats and Merino sheep to insulin-induced stress as well as adrenocorticotropic hormone (ACTH) and CRH stimulation were investigated. Insulin induced a hypoglycaemic condition in all three species.

Plasma cortisol levels increased significantly in both the Boer goat and sheep, but not in the Angora, confirming the hypocortisolism reported by previous studies (Van Rensburg, 1971; Herselman and Pieterse, 1992; Herselman and van Loggerenberg, 1995). Sheep CRH was unable to elicit a response in either of the two goat species, while ACTH stimulation resulted in an increased plasma cortisol concentration in the three species indicating that the HPA axis was functional in all three species. The response was, however, strongest in the Merino sheep and weakest in the Angora goat, indicating that the Angora adrenal may have a reduced ability to produce cortisol.

Engelbrecht and Swart (2000) subsequently used subcellular fractions (microsomes and mitochondria) to investigate and compare adrenal steroidogenesis in the same three species investigated above. In the microsomal preparations pregnenolone was used as a substrate and the production of glucocorticosteroid precursors (deoxycorticosterone and deoxycortisol) and androgens, (dehydroepiandosterone (DHEA) and androstenedione) were compared. Significantly less glucocorticosteroid precursors (36%) were produced by the Angora goat than the Boer goat (79%) and Merino sheep (82%). In contrast, the Angora goat produced significantly more 17-hydoxypregnenolone and DHEA (35%) than did the other two species (Boer goat 9%, Merino sheep 0%). Unfortunately, 17-hydroxypregnenolone and DHEA was not quantified individually during this study. In the case of progesterone metabolism, the Angora produced significantly more deoxycorticosterone and significantly less deoxycortisol than the other species, while androstenedione and 17-hydroxyprogesterone production was less than 5% in all three species.

The differences in steroid output by the microsomal preparations from the three species suggested that there was a difference in activity of one or more of the steroidogenic enzymes. The activity of specific enzymes in the adrenal steroidogenic pathway were subsequently studied by the selective addition of cofactors. Only a single enzyme, cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) demonstrated a significant difference in activity between the species. CYP17 in the Angora goat adrenal microsomes converted pregnenolone to DHEA significantly faster than in the other two species. Engelbrecht and Swart (2000) concluded that the preference exhibited by Angora CYP17 for the Δ^5-steroid pathway during adrenal steroidogenesis would likely result in an increased production of adrenal androgens *in vivo*, resulting in a decrease in the production of glucocorticoids when compared to the other species.

Slabbert (2003) suggested that there may be at least two alleles for CYP17 in the Angora goat gene pool, as some Angora breeders have reported that their breeding stocks contain goats that are not as susceptible to environmental stress as others. Subsequent RNA isolation, cDNA preparation and sequencing confirmed the presence of two CYP17 isoforms in the South African Angora population. Four nucleotide differences were identified which included a change in the recognition site for the restriction enzyme ACS I. A restriction based genotyping method was subsequently developed for CYP17 and 83 goats were genotyped. Twenty four goats were homozygous for CYP17 without the ACS I site, 59 were heterozygous and no goats homozygous for CYP17 with the ACS I site were detected. However, due to

the sample size and limited phenotypical data, no correlation could be made between the CYP17 genotype and the tolerance of goats to cold stress.

This thesis will therefore address the role of the two identified CYP17 isoforms in cold stress related deaths in the South African Angora goat population and will clearly demonstrate that CYP17 is the primary cause of the observed hypocortisolism.

Chapter 2 presents an overview of the physiological response to cold stress, with specific reference to the HPA axis. The components of the HPA axis, in particular the adrenal cortex, are addressed in detail. Though the mineralocorticoids and adrenal androgens are mentioned, this chapter focuses on the physiological importance of the glucocorticoids in response to stress. The catalytic properties and mechanism of action of the enzymes catalysing the biosynthesis of these hormones, the cytochromes P450 and hydroxysteroid dehydrogenase enzymes are discussed. In addition, the regulation of steroidogenesis, in particular glucocorticoid production is included, highlighting the importance of the availability of cholesterol and the role played by the Steroid Acute Regulatory Protein (StAR).

In adrenal steroidogenesis, CYP17 catalyses two distinct mixed-function oxidase reactions. This dual activity of CYP17 places it at a key branch point in the biosynthesis of mineralocorticoids, glucocorticoids and adrenal androgens. Chapter 3 focuses on the importance of this enzyme in the steroidogenic pathway. Aspects of the catalytic activity of this key enzyme, together with its expression, regulation and physiological importance as well as the differential regulation of the hydroxylase and lyase activity of CYP17 are covered.

In chapter 4, an overview of the history of the Angora goat and its introduction into South Africa is given, concentrating on the stress related problems encountered by the mohair industry in South Africa and the resulting research. In particular research leading to the identification of the condition of hypocortisolism is discussed. In addition, a summary is given of the research that has implicated CYP17 as the possible cause of the observed hypocortisolism.

As mentioned previously Slabbert (2003) identified two CYP17 isoforms in the South African Angora goat. Angora CYP17, 3βHSD and cytochrome b_5 were successfully cloned. The two CYP17 isoforms, which differ by three amino acid residues, were named ACS+ and ACS- based on the presence or absence of the ACS I restriction site, respectively. Two of the substitutions, A6G and V213I were conservative, while a single non-conservative substitution, P41L, was found in the highly conservative proline rich sequence (PR) which is critical for the correct folding of all cytochromes P450 (Yamazaki et al., 1993; Kusano et al., 2001a; Kusano et al., 2001b). In this study the CYP17 isoforms were expressed in COS-1 cells and assayed for activity. While the 17α-hydroxylase activity was similar for both constructs, ACS- demonstrated a significantly increased lyase activity towards 17-hydroxypregnenolone in both the presence and absence of cytochrome b_5. Site-directed mutagenesis revealed that the difference in 17,20-lyase activity was primarily due to the non-conservative P41L substitution, which was proposed to alter the three-dimensional structure of the enzyme.

In the adrenal, CYP17 and 3βHSD compete for the same substrates, with the ratio and the substrate specificities of these two enzymes determining

the steroidogenic output of the adrenal cortex. Therefore the effect of the difference in CYP17 activity was investigated by coexpressing each CYP17 isoform with 3βHSD in COS-1 cells using pregnenolone as substrate. However, such an experiment would produce a complex mixture of steroids, seven in total, and no suitable analytical method was available for the quantification of these steroids. A new UPLC based mass spectrometry method was therefore developed for this application. The development and validation of this method was submitted to Analytical Biochemistry and the published article is presented in chapter 5.

The development of the UPLC-APCI-MS method permitted the analysis of the steroid metabolites produced in the metabolism of pregnenolone in COS-1 cells expressing both CYP17 and 3βHSD. Cells expressing ACS- and 3βHSD produced significantly more adrenal androgens and less glucocorticoid precursors than cells expressing ACS+ and 3βHSD. The inclusion of cytochrome b_5 in the cotransfections resulted in an increased difference in the steroid profiles of PREG metabolism, with CYP17 ACS- expressing cells predominantly producing adrenal androgens (≈68%), while glucocorticoid precursor production was predominant in CYP17 ACS+ expressing cells (≈71%). The difference in androgen production in both the presence and absence of cytochrome b_5 was attributed to the greater 17,20-lyase activity of CYP17 ACS-, which resulted in a greater flux through the Δ^5 pathway, and a concomitant decrease in glucocorticoid precursors. This provided evidence that the ACS- isoform was responsible for the observed hypocortisolism. The cloning, characterisation and expression of the two

CYP17 isoforms was submitted to Drug Metabolism Reviews and the published article is presented in chapter 6.

Genotyping using a restriction digest assay had not identified goats homozygous for ACS+. A more accurate real time PCR based genotyping assay was subsequently developed and 576 Angora goats from two separate populations were genotyped. While the ACS+/ACS+ genotype remained undetected, heterozygous samples could, however, be divided into two distinct groups, H_e and H_u, based on their melting profiles. A third genotype of goats homozygous for ACS- was named H_o. The distorted peak area observed in the H_u group was subsequently investigated by performing relative copy number determinations for each of the three putative genotypes using quantitative real-time PCR. The results revealed that the two CYP17 isoforms were not two alleles of the same gene, but two separate genes. The H_e genotype had two copies of both genes, while the H_u genotype had two copies of ACS-, but only one copy of ACS+. The H_o genotype had only two copies of ACS-. This explained why the ACS+ homozygote was never detected, as ACS- is always present with ACS+. It was proposed that crossing H_o and H_e goats would yield the intermediate genotype H_u, which receives both ACS- and ACS+ from its H_e parent, but only ACS- from the H_o parent and therefore has an ACS-:ACS+ ratio of 2:1, which corresponds to the data obtained by genotyping and copy number determinations.

An *in vivo* assay for cortisol production was subsequently performed in order to establish the physiological effect of the three novel genotypes. While intravenous insulin injection resulted in a similar decrease in plasma glucose levels of the three groups, the amplitude of the response in cortisol production

was significantly greater in the H_e group than in the H_o group. The cortisol response in the H_u group was greater than in the H_o group, but not significantly different from either the H_o or the H_e groups. This data confirmed that goats with the ACS+ were able to produce more cortisol and confirmed that CYP17 is the primary cause of hypocortisolism in the South African Angora goat. The development of the real time PCR genotyping method, relative copy number determinations and *in vivo* cortisol assay were submitted to FEBS journal and the published article is presented in chapter 7.

In conclusion, chapter 8 presents an overview of the results obtained in this study. The physiological role of CYP17 in causing hypocortisolism in the South African Angora goat is discussed. In addition, a strategy to investigate the feasibility of producing more hardy Angora goats without negatively affecting mohair production qualities is presented.

CHAPTER 2

ADRENAL STEROIDOGENESIS

2.1 Introduction to stress

The term "*stress*" was first popularised by Hans Selye (1936). Selye's stress theory focused on the HPA axis, which is discussed in detail later in this chapter, as the key effector of the stress response and considered the adrenal cortex "*the organ of integration which participates in the normal and pathological physiology of virtually all tissues in the body,*" by virtue of its endocrine function (Selye, 1950). Later, Selye proposed that most of the stressful stimuli induce two types of responses: a general stress response, which is common to all stressors and involves the release of ACTH and adrenal glucocorticoids; and individual stress responses mediated by "*conditioning factors*," such as genetically determined predispositions (Selye, 1976).

Scientists have subsequently not been able to agree on the exact definition of stress. Some view stress as the situation when the HPA axis, represented mainly by elevated ACTH levels, is activated (Ganong, 1995), while others have suggested that the activation of other systems with or without an elevation in ACTH may reflect stress-induced disturbed homeostasis (Vigas, 1985; Pacák et al., 1998).

The term "*homeostasis*" was first introduced by Cannon (1929) to describe the "*coordinated physiological processes which maintain most of the steady states in the organism*." Pacák and Palkovits (2001) have defined

stress as a state of threatened homeostasis (physical or perceived threat to homeostasis), which results in the activation of an adaptive compensatory specific response in the organism to sustain homeostasis. Furthermore, they suggest that the adaptive response reflects the activation of specific central circuits which are genetically and constitutionally programmed and constantly modulated by environmental factors. Pacák and Palkovits (2001) provide evidence that specific stressors may elicit specific responses; and that different stressors may activate different brain systems by specific pathways within the central nervous system, demonstrating that not all stress is dependent on the HPA axis.

Stress can be either physical or psychological. This discussion will only deal with physical stress, in particular with cold stress. Stressors can be further divided into two groups, depending on the duration of the stressor, namely acute (single, intermittent and time-limited exposure) and chronic (intermittent and prolonged exposure) stressors (Pacák and Palkovits, 2001). The cold stress related deaths in Angora goats are primarily related to prolonged exposure to cold conditions.

2.2 The hypothalamic-pituitary-adrenal axis

The hypothalamus, anterior pituitary and adrenal cortex constitute a controlling loop known as the HPA axis (Vander et al., 2001). Interplay between these organs through the endocrine, paracrine and autocrine action of their hormones is essential in the maintenance of homeostasis in response to physiological and environmental stimuli. Stimulation of the HPA axis results

in increased hormone secretion from the three zones of the adrenal cortex, as will be discussed later in this chapter. The HPA axis is therefore vital for adrenocortical regulation, although other adrenocortical regulatory mechanisms occur, such as the reninangiotensin system and other intra-adrenal regulatory mechanisms. The hypothalamus and pituitary will be discussed in some detail here, while the third part of the HPA axis, the adrenal, will be discussed later in this chapter.

2.2.1 The hypothalamus

The hypothalamus is a complex structure of the brain forming part of the diencephalon. It is central to a complex neural network that enables it to control the homeostasis of an organism. The hypothalamus is connected to a number of different centres in the central nervous system via many different afferent and efferent neural pathways. These include afferent pathways from the brain stem, hippocampus, limbic lobe, midbrain, thalamus and the medulla; and efferent pathways to the hippocampus, limbic lobe, medial eminence, pituitary stalk and posterior pituitary (Carrasco and van der Kar, 2003). The hypothalamus receives an array of chemical and electrical stimuli allowing it to sense the homeostatic state of the organism. In response, it generates chemical and electrical signals, which in turn stimulate the anterior and posterior pituitary to release the appropriate hormones necessary for the maintenance of homeostasis. This discussion will focus only on the regulation of adrenal steroidogenesis by the hypothalamus via the anterior pituitary. A

schematic representation of the human hypothalamus and pituitary gland is shown in figure 2.1.

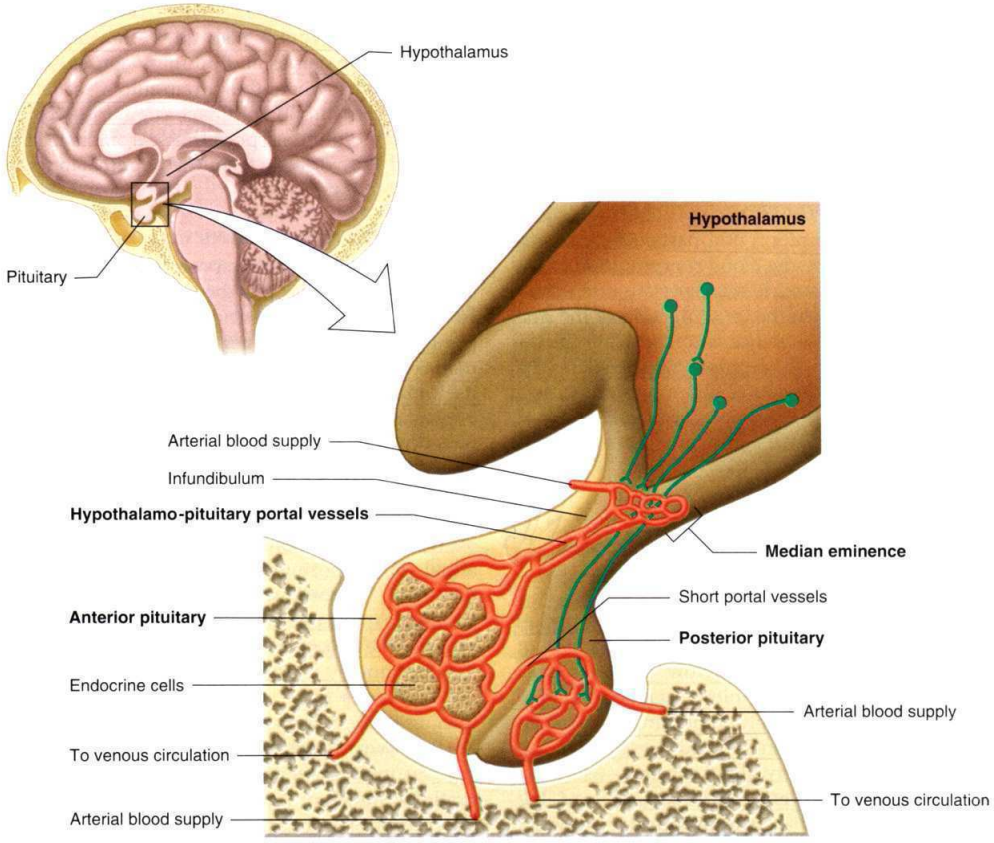

Figure 2.1. Human hypothalamus and pituitary. Reproduced from Vander et al. (2001).

2.2.2 The pituitary

The pituitary gland, or hypophysis, lies in a pocket of the sphenoid bone, known as the sella turcica, at the base of the brain just below the hypothalamus. A stalk known as the infundibulum contains nerve fibres and small blood vessels, and connects the pituitary to the hypothalamus. The

pituitary is comprised of two adjacent lobes, the anterior pituitary and the posterior pituitary, which have distinct embryological origins, functions and control mechanisms (Young and Heath, 2000; Vander et al., 2001).

The posterior pituitary, also known as the neurohypophysis or pars nervosa, is derived from the downgrowth of nervous tissue from the hypothalamus to which it remains joined to by the infundibulum. Axons of the supraoptic and paraventricular hypothalamic nuclei pass down the infundibulum and end in the posterior pituitary. The anterior pituitary arises as an epithelial upgrowth from the roof of the primitive oral cavity, known as Rathke's pouch. This specialised glandular epithelium is wrapped around the anterior aspect of the posterior pituitary and is also known as the adenohypophysis. A vestigial cleft may divide the major part of the anterior pituitary from a thin zone of tissue lying adjacent to the posterior pituitary, known as the pars intermedia. An extension of the adenohypophysis, which surrounds the neural stalk, is known as the pars tuberalis (Young and Heath, 2000).

The anterior pituitary is not connected to the hypothalamus by a neural network, but by a special vascular system. Capillaries of the primary plexus at the base of the hypothalamus, the median eminence, recombine to form the hypothalamo-pituitary portal vessels. These portal vessels pass down the infundibulum and enter the anterior pituitary where they form a second capillary bed, the anterior pituitary capillaries. This blood vessel system is known as the hypothalamopituitary portal system, which ensures that blood from the hypothalamus flows directly to the anterior pituitary (Vander et al., 2001).

The posterior pituitary secretes two hormones, vasopressin, also known as antidiuretic hormone, and oxytocin. Vasopressin is synthesised in the cell bodies of the supraoptic nucleus, while oxytocin is synthesised in the cell bodies of the paraventricular nucleus of the hypothalamus. Both hormones travel down their respective axons through the infundibulum to the posterior pituitary, where they are stored in small vesicles. The release of these hormones by exocytosis is triggered by the depolarisation of the axons (Young and Heath, 2000; Vander et al., 2001). Vasopressin and oxytocin are released into the posterior pituitary capillaries, which drain directly into the main blood stream. Vasopressin increases the permeability of the collecting ducts in the kidney, facilitating the re-uptake of water. Oxytocin acts on the smooth muscles of the breast and uterus to increase contraction (Vander et al., 2001).

Anterior pituitary function is regulated by six hypophysiotrophic hormones which are secreted by various neurons from the hypothalamus that terminate in the median eminence and deliver their hormones to the primary plexus. These hormones are: corticotropin releasing hormone (CRH); thyrotropin releasing hormone (TRH); growth hormone releasing hormone (GHRH); growth hormone-inhibiting hormone (GIH); gonadotropin releasing hormone (GnRH); and prolactin-inhibiting hormone (PIH), also called dopamine. These hypophysiotrophic hormones reach the anterior pituitary by the hypothalamo-pituitary portal system and control the production and secretion of trophic hormones in the anterior pituitary before reaching the main blood stream.

The anterior pituitary hormones are produced by five different secretory cell types: the somatotrophs, mammotrophs, corticotrophs, thyrotrophs and gonadotrophs. Somatotrophs are responsible for growth hormone (GH) secretion and comprise more than 50% of the cells in the anterior pituitary. They are stimulated by GHRH and inhibited by GIH. GH facilitates growth and regulates protein, carbohydrate and lipid metabolism. Mammotrophs secrete prolactin and comprise up to 20 % of the anterior pituitary. Prolactin secretion is inhibited by PIH. Prolactin stimulates growth of mammary glands in breast tissue and milk production in females and may be permissive to certain reproductive functions in the males. Corticotrophs constitute about 20% of the anterior pituitary and secrete ACTH in response to CRH. ACTH is a polypeptide that is cleaved from a larger polypeptide known as pro-opiomelanocortin (POMC). Lipotropins, which are involved in lipid metabolism, and endorphins, which are endogenous opioids, are also derived from POMC and can be secreted in small amounts together with ACTH. ACTH primarily stimulates the release of the glucocorticoids, but also mineralocorticoids and androgens (with the exception of DHEA sulphate), from the adrenal cortex. Thyrotrophs comprise up to 5% of the anterior pituitary and secrete thyrotrophin (TSH) in response to TRH. TSH, in turn, stimulates the release of thyroid hormone from the thyroid gland. Gonadotrophs constitute the remaining 5% of the anterior pituitary and secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) in response to GnRH. Both FSH and LH facilitate gamete production and stimulate the release of androgens and estrogens from the gonads (Young and Heath, 2000; Vander et al., 2001).

2.3 Physiological response to cold stress

Cold stress produces a coordinated response through metabolic, endocrine, autonomic, and behavioral systems. Several brain areas and pathways are involved in response to cold-evoked stress. These responses include decreased activity of the salivary glands, increased activity of the thyroid and adrenal glands, as well as vasoconstriction of cutaneous blood vessels (Pacák and Palkovits, 2001). These responses function together to decrease heat loss and increase the production of metabolic heat (Vander et al., 2001)

2.3.1 The role of the hypothalamus

In mammals, body temperature is controlled by the thermoregulatory centre in the hypothalamus. Bergmann (1845) and Tscheschichin (1866) were the first to report the existence of temperature-sensitive central elements and structures that control body temperature. Liebermeister (1860) suggested homeostatic mechanisms in temperature control by introducing the concept of a "*set-point*" for body temperature. A number of controversial experiments indicated that normal temperature regulation required the integrity of the hypothalamus (Isenschmid and Krehl, 1912; Isenschmid and Schnitzler, 1914; Keller and Hare, 1932; Teague and Ranson, 1936; Clark et al., 1939). It was suggested that other brain areas, such as subthalamic, midbrain, and brainstem regions, were also important structures in thermoregulation (Bligh, 1966).

The preoptic region of the hypothalamus has been identified as the major organizing center for thermoregulation. A variety of thermoregulatory responses can be elicited by activating a group of medial preoptic neurons, which receive synaptic input from the periphery through spinal and medullary thermoreceptive pathways (Pacák and Palkovits, 2001). A detailed mapping of cold-sensitive pathways was attempted by Lipton et al. (1974). Rats were unable to regulate body temperature when the preoptic/anterior hypothalamic region was destroyed or disconnected from brainstem structures. This demonstrated that communication between the preoptic and anterior hypothalamic nuclei was required for normal temperature regulation and that a major portion of the pathways for regulation against cold passes through the medial forebrain bundle in the lateral hypothalamic area.

2.3.2 Activation of the HPA axis and other regulatory pathways

Zoeller et al. (1990) have demonstrated significantly increased expression of CRH mRNA in rat paraventricular hypothalamic nuclei after acute (6 h) and chronic (30 h) exposure to cold stress (5°C). Similarly, Hauger et al. (1988, 1990) demonstrated a 3-fold increase in plasma ACTH levels in rats exposed to chronic cold stress (60 h at 4°C). Interestingly during this study CRH receptor concentrations in the anterior pituitary remained unchanged, but were increased in the neurointermediate lobe and in the median eminence, suggesting that cold stress-induced ACTH release from the anterior pituitary may be at least partly mediated by mechanisms/factors and pathways other than CRH neurons. Angulo et al. (1991) showed that cold

stress (4℃) for three hours daily for four consecutive days increased vasopressin mRNA levels in the paraventricular nucleus. The authors suggested that cold-induced synthesis and subsequent release of vasopressin could have a stimulatory effect on ACTH release at the anterior pituitary level during exposure to cold stress.

Pacák et al (1995, 1998) have, however, demonstrated that in rats, cold stress (4℃ or -3℃) produced much larger proportionate increments in norepinephrine plasma levels than in epinephrine, ACTH, or glucocorticoid plasma levels. These results were consistent with previous reports showing the cold stress-induced depletion of hypothalamic catecholamines and selective activation of the peripheral sympathoneuronal system (Palkovits et al., 1995). The catecholamines act to increase hepatic and muscle glycogenolysis and increase the breakdown of triacylglycerol in adipose tissue in order to provide fuel for the generation of metabolic heat (Vander et al., 2001).

Acute or chronic cold stress has also been shown to increases TRH mRNA expression in the paraventricular nucleus, TRH levels in the hypothalamus, and plasma TSH levels (Ishikawa et al., 1984; Lin et al., 1989; Zoeller et al., 1990; Rondeel et al., 1991; Fukuhara et al., 1996). The effects of cold stress have been shown to be specific for TRH expression in the paraventricular nucleus, as cold stress did not affect the levels of TRH mRNA in other brain regions (Zoeller et al., 1990). In support of these findings, Arancibia et al. (1983, 1986, 1996) have demonstrated the rapid cold stress-induced TRH release from the median eminence using a push-pull perfusion technique. Increased plasma TRH results in the release of thyroid hormones

(thyroxine and triiodothyronine), which acts to increase the basal metabolic rate and thereby the generation of heat (Vander et al., 2001).

From the above discussion it is clear that although the HPA axis is activated during cold stress, it may not be the primary response. Harbuz and Lightman (1989) have shown in rats that, although increased levels of CRH mRNA in the paraventricular nucleus and POMC mRNA in the anterior pituitary were detectable after a short period of exposure (1h at 4°C), the increments were not significantly different from the control animals. Furthermore, Pacák et al. (1995, 1998) found no correlation between the cold stress-induced release of norepinephrine in the paraventricular nucleus and activation of the HPA axis.

Stimulation of the HPA axis results in the production and release of glucocorticoids (cortisol in most mammals, including the goat) from the adrenal cortex. Increased plasma cortisol levels result in increased protein catabolism in the peripheral tissue; increased uptake of amino acids by the liver; increased gluconeogenesis; decreased uptake of glucose by the muscle and adipose tissue; and the stimulation of triacylglycerol catabolism in adipose tissue with the release of glycerol and fatty acids into the blood. (Vander et al., 2001).

As discussed earlier, the catecholamines and thyroid hormones act to generate metabolic heat by using glucose from glycogen stores. The primary cause of Angora goat stock loss during cold spells has been attributed to an energy deficiency resulting from a decrease in blood glucose levels (Wentzel et al., 1979). Therefore, cortisol is important for survival during long term exposure to cold stress as it promotes gluconeogenesis and fatty acid

metabolism. Though the HPA axis is directly stimulated by cold stress, the exhaustion of glycogen supplies and resulting drop in plasma glucose levels, leads to further stimulation of the HPA axis as discussed below.

2.3.3 Hypoglycemia induced activation of the HPA axis

CRH and vasopressin have been shown to play an important role in hypoglycemia-induced activation of the HPA axis as administration of CRH antibodies reduced ACTH responses to insulin-induced hypoglycemia (Plotsky et al., 1985; Suda et al., 1992). Hypoglycemia increases CRH mRNA expression in the paraventricular nucleus (Suda et al., 1988a; Itoi et al., 1996; Brown and Sawchenko, 1997); CRH turnover in the median eminence (Berkenbosch et al., 1989); CRH and POMC mRNA levels in the anterior pituitary (Tozawa et al., 1988; Suda et al.,1992; Robinson et al., 1992); CRH levels in the hypophysial portal and peripheral blood; and the depletion of ACTH content in the anterior pituitary (Sumomito et al., 1987; Suda et al., 1988a; Suda et al., 1988b). In contrast, Plotsky et al. (1985) found unchanged CRH concentrations but elevated vasopressin levels in portal plasma during hypoglycemia. The administration of a vasopressin V1 receptor antagonist attenuated hypoglycemia-induced plasma ACTH increments. In addition the intracerebroventricular administration of vasopressin significantly decreased hypophyseal portal plasma concentrations of CRH, suggesting that hypoglycemia-induced ACTH secretion may be mediated by dynamic changes in portal vasopressin concentrations and that CRH has a permissive role in hypoglycemia-induced plasma ACTH responses.

Rats with hypothalamic deafferentation have been used to investigate insulin-induced activation of the HPA axis. Responses in plasma ACTH and glucocorticoid levels were measured after injection of low (0.04 IU/100 g body weight) or high (0.2 UI/100 g body weight) insulin doses. The high insulin dose resulted in reduced plasma glucose levels (50%) and a significant increase in plasma ACTH and glucocorticoid levels. The lower dose of insulin reduced plasma glucose levels by approximately 30% and elicited a significant adrenocortical response only in the control group and rats with posterior hypothalamic deafferentation. Weidenfeld et al. (1982) concluded that during severe hypoglycemia, the adrenocortical response was mediated by systemic mechanisms that acted directly on the medial basal hypothalamus. In contrast, the activation of neural pathways impinging upon CRH neurons was crucial for the responses of the HPA axis during mild hypoglycemia. Furthermore, hypoglycemia has also been shown to stimulate glucocorticoid responses through pathways independent of the hypothalamus but requiring the pituitary (Kárteszi et al., 1982, Mezey et al., 1984). Mezey et al. (1984) found that insulin-induced hypoglycemia stimulated ACTH secretion from stalk-sectioned rats. It is therefore apparent that both peripheral and central pathways are necessary for activation of the HPA axis, and that the severity of hypoglycemia determines which pathways are activated (Plotsky, 1985).

2.4 The adrenal gland

2.4.1 Anatomy and morphology of the adrenal gland

Most mammals have two bilateral encapsulated adrenal (suprarenal) glands situated on the upper pole of each kidney (Young and Heath, 2000). In mammals, the adrenal gland contains two functionally different types of endocrine tissue with different embryological origin: the outer adrenal cortex and the inner adrenal medulla (Deane, 1962; Rittmaster and Cutler, 1990; Landsberg and Young, 1992; Rittmaster and Cutler, 1992), as shown in figure 2.2(A). The adrenomedullary chromaffin cells originate from neural crest precursor cells that migrate into the adrenal and subsequently differentiate into chromaffin cells (Pohorecky and Wurtman, 1971; Axelrod and Reisine, 1984). Medullary chromaffin cells are organised into interlacing cords and are under the control of sympathetic preganglionic nerve stimulation of the splanchnic nerve (Morrison SF and Cao, 2000). The main secretory products of the chromaffin cells are the catecholamines: epinephrine and norepinephrine (Winkler et al., 1986). Chromaffin cells are characterised by dense-cored catecholamine-containing secretory vesicles (Bornstein et al., 1991; Bornstein et al., 1994; Ehrhart-Bronstein et al., 1998). The medullary cells are separated into two main cell types depending on their secretory product — the epinephrine-secreting type, which have less dense granules; and the norepinephrine type, which have smaller, denser granules. Both cell types may also release smaller amounts of transmitters, neuropeptides and proteins together with the catecholamines (Winkler et al., 1986).

The adrenal cortex is formed from the adrenal primordium during embryogenesis. The adrenal primordium consists of mesodermally derived fetal adrenal cells, which result from the condensation of celomic epithelium at the cranial end of the kidney (Ehrhart-Bronstein et al., 1998). The adrenal cortex secretes three classes of steroid hormones, namely mineralocorticoids, glucocorticoids and androgens. The steroid secreting cells of the adrenal cortex are characterised by large lipid droplets; numerous variably shaped mitochondria with tubulovesicular cristae; and a prolific system of smooth endoplasmic reticulum (Young and Heath, 2000), as shown in figure 2.3. In most mammals the steroid secreting cortical cells are arranged into three distinct zones which differ in morphological features and steroid hormone production (Hardy, 1981; Ehrhart-Bronstein et al., 1998; Young and Heath, 2000). The three zones are, from the outer to the inner layer, the zona glomerulosa, the zona fasciculata and the zona reticularis, as shown in figure 2.2(B). The cortical tissue constitutes 72% of the adrenal mass, of which the zona fasciculata constitutes 50%, the zona glomerulosa 15% and the zona reticularis 7% (Ganong, 1995). The zona glomerulosa often forms an incomplete layer and is the unique source of the mineralocorticoid aldosterone (Ehrhart-Bronstein et al., 1998; Young and Heath, 2000). In this zone, the secretory cells are arranged in irregular ovoid clusters separated by delicate trabeculae containing capillaries (Young and Heath, 2000). The cells of the zona glomerulosa are able to regenerate the zona fasciculata and reticularis when these layers are removed, suggesting an additional role of this zone in the production of new cortical cells (Teebken and Scheumann, 2000). The zona fasciculata produces the glucocorticoids cortisol and corticosterone, and

trace amounts of the androgen DHEA (Ehrhart-Bronstein et al., 1998; Young and Heath, 2000). The secretory cells of this zone are arranged in narrow radially arranged cords, often only one cell layer wide, separated by fine strands of supporting tissue containing capillaries. The zona reticularis consists of an irregular network of branching cords and clusters of cells separated by numerous capillaries (Young and Heath, 2000). These secretory cells produce trace amounts of glucocorticoids and the androgens DHEA, DHEA sulphate and androstenedione (Ehrhart-Bronstein et al., 1998; Young and Heath, 2000). It is the zonation of the steroidogenic enzymes, which will be discussed later in this chapter, that determines the steroidogenic output of each of the three adrenocortical zones.

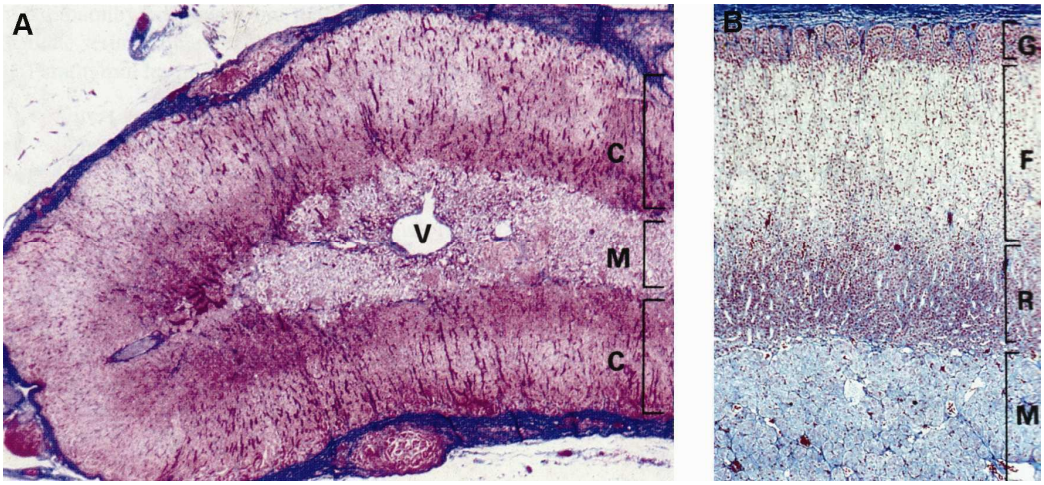

Figure 2.2. A: Cross section through the human adrenal gland. The adrenal cortex (C) is seen surrounded by a dense fibrous tissue capsule which supports the gland. A prominent vein (V) is characteristically located in the centre of the medulla (M). B: Cross section of the adrenal cortex and medulla (M). The three zones of the cortex, the zona glomerulosa (G), the zona fasciculata (F) and the zona reticularis (R) are clearly seen. Reproduced from Young and Heath (2000).

The cortical and medullary cells are in direct contact with each other without separation by connective tissue or interstitium (Bornstein et al., 1991;

Bornstein et al., 1994). Although the adrenal is fundamentally arranged into the cortex and medulla, chromaffin cells are found in all three zones of the adult adrenal cortex, either radiating through the cortex from the medulla or distributed as islets or single cells. Chromaffin cells may also spread and form larger nests in the subcapsular region (Fortak and Kmiec, 1968, Kmiec, 1968; Palacios and Lafraga, 1975; Gallo-Payet et al., 1987; Bornstein et al., 1991). Cortical cells are also found in the medulla as islets, either surrounded by chromaffin tissue or retaining some association with the rest of the cortex (Bornstein et al., 1991; Bornstein et al., 1994). The intimate association and intermingling of the two cell types allows for extensive paracrine interaction, but this is beyond the scope of this discussion.

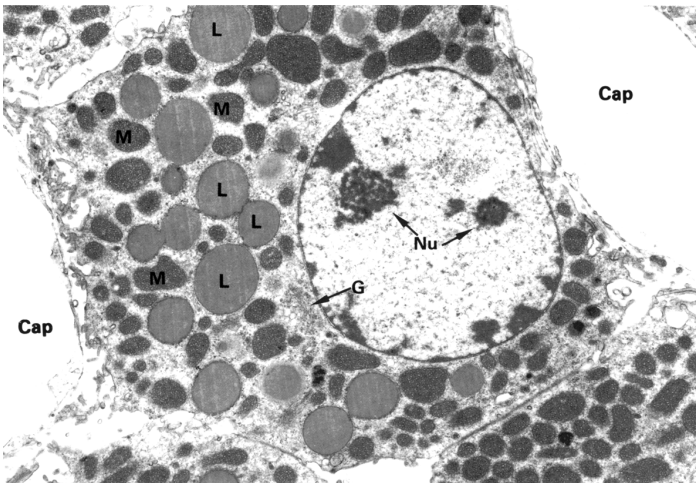

Figure 2.3. Typical steroid-secreting cell intimately associated with capillaries (Cap). A small Golgi apparatus (G) is seen close to the rounded nucleus which is characterized by prominent nucleoli (Nu). The abundant cytoplasm contains many large lipid droplets (L), numerous variably shaped mitochondria (M) and an extensive network of smooth ER (not clearly visible at this magnification). Reproduced from Young and Heath (2000).

2.4.2 Blood supply to the adrenal gland

The adrenal gland receives its blood supply from the superior, middle and inferior suprarenal arteries, which form a plexus just under the capsule of the gland. The cortex is supplied by an astomising network of capillary sinusoids, which are supplied by branches of the subcapsular plexus known as short cortical arteries. These sinusoids descend between the cords of steroid secreting cells in the zona fasciculata into a deep plexus in the zona reticularis before draining into small venules, which converge upon the central vein of the medulla. The central medullary veins contain bundles of smooth muscles between which the cortical venules enter. Contractions of these smooth muscle bundles restrict cortical blood flow as a regulatory mechanism. The medulla is supplied by long cortical arteries, which descend from the subcapsular plexus through the cortex and into a network of capillaries surrounding the medullary chromaffin cells. These capillaries also drain into the central vein of the medulla (Young and Heath, 2000). A schematic representation of the blood supply to the adrenal gland is shown in figure 2.4.

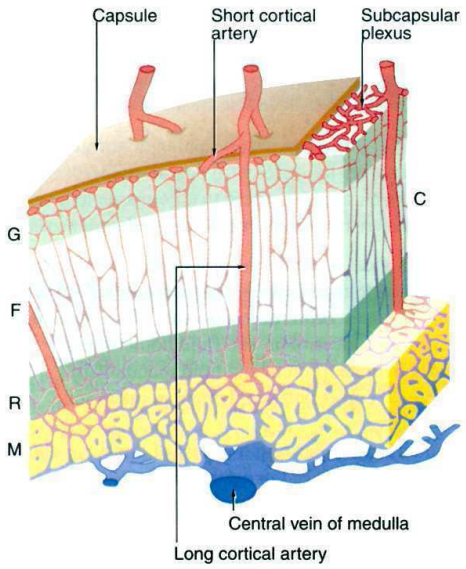

Figure 2.4. Schematic representation of the blood supply to the adrenal gland. C, Cortex; M, Medulla; G, zona glomerulosa; F, zona fasciculata; R, zona reticularis. Reproduced from Young and Heath (2000).

2.5 Hormones of the adrenal cortex

As previously discussed, the adrenal cortex produces three types of steroid hormones — mineralocorticoids, glucocorticoids and adrenal androgens. The mineralocorticoid aldosterone plays an essential role in the regulation of electrolyte concentration in the extracellular fluid. Aldosterone action results in the reabsorption of sodium from urine, sweat, saliva and gastric juices (Cho et al., 1998; Kim et al., 1998; Palmer, 2001). Regulation of aldosterone synthesis is primarily under the control of the renin-angiotensin system and not dependent on the HPA axis (Muller, 1987; Quinn and Williams, 1988; Vander et al., 2000). The adrenal androgens are involved in sexual differentiation and promote protein anabolism and growth (Albright et al., 1942; Albright, 1947; Goldstein and Saenger, 1984; Reiter and Saenger,

1997; Beck and Handa, 2004). The glucocorticoids are primarily the only steroid hormones involved with the stress response of the HPA axis and, as such, will be discussed here in greater detail.

2.5.1 Mechanisms of action

The steroid hormones produced by the adrenal cortex are all lipophylic and can transverse the plasma membrane. Although the primary effects of the steroid hormone stimulation occur via nuclear hormone receptor (NHR) mediated genomic effects, nongenomic effects mediated via receptors on the cell surface have been documented, but are beyond the scope of this discussion (Ganong and Mulrow, 1958; Oberleithner et al., 1987; Oberleithner et al., 1989; Fujii et al., 1990; Funder, 2005).

Once in the cell interior, the mineralocorticoids, glucocorticoids and androgens bind to their corresponding NHR's. All NHR's share common structural features, which include a central DNA binding domain (DBD) and a ligand binding domain (LBD) contained in the C-terminal half of the receptor (Bourguet et al., 2000; Olefsky, 2001). The DBD is highly conserved, composed of two zinc fingers, and is responsible for targeting the receptor to a highly specific DNA sequence comprising a response element (RE). The N-terminal and C-terminal domains are variable with a variable length hinge region between the DBD and the LBD (Evans et al., 1999; Colligwood et al., 1999; Bourguet et al., 2000; Farman and Rafestin-Oblin, 2001). NHR's can exist as homo- or heterodimers with each partner binding to a specific RE sequence that exist as half-sites separated by nucleotide spacers of variable

length. Half-sites may occur as direct or inverted repeats depending on the NHR (Olefsky, 2001).

The mineralocorticoids, glucocorticoids and androgens, bind to specific NHR's, namely the mineralocorticoid receptor (MR), glucocorticoid receptor (GR) and the androgen receptor (AR), respectively (Olefsky, 2001). The mechanisms of action of these three NHR's are similar, however, only the GR will be discussed here.

The GR is located in the cytoplasm of the target cell as part of a larger protein complex, in which it interacts with the heat shock protein HSP90. Upon ligand binding, the GR undergoes a conformational change, dissociates from the protein complex and translocates across the nuclear membrane. In the nucleus the GR binds as a dimer to a glucocorticoid-response element (GRE). This may induce the activation of a target gene by transactivation or the repression of the transcription of a target gene by transrepression. In the case of transrepression, the GRE is known as a negative GRE (nGRE) (Olefsky, 2001; Farman and Rafestin-Oblin, 2001).

The functioning of the NHR's is not, however, always as simple as described above. As a result of alternative splicing of the GR pre-mRNA primary transcripts, two protein isoforms of the GR exist, namely GRα and GRβ. GRβ does not bind glucocorticoids and is an inhibitor of the glucocorticoid induced activity of GRα (de Castro et al., 1996; Oakley et al., 1996; Oakley et al., 1999). Furthermore, a number of regulatory proteins have been identified that form multicomponent assemblies with the NHR's and serve as either coactivators or corepressors. These coregulators can bind to the NHR's via specific amino acid sequence motifs in a ligand-dependent or

ligand-independent manner and provide enzymatic or scaffolding functions. In addition, these proteins influence chromatin remodeling by histone acetylation/deacetylation, methylation and other events that are beyond the scope of this discussion (Rosenfeld and Glass, 2001).

2.5.2 Glucocorticoid action

The two glucocorticoids produced by the adrenal cortex are cortisol and corticosterone. Within different species, one of these glucocorticoids tends to dominate and is secreted at greater concentrations than the other. In goats as well as humans, cortisol is the dominant glucocorticoid (Vander et al., 2001) and all further discussion will deal with cortisol only.

Approximately 90% of the circulating cortisol is bound to plasma albumin and cortisol binding globulin (CBG) (Farman and Rafestin-Oblin, 2001). Although the binding of cortisol to these proteins increases its half-life, it is only the free fraction of cortisol that is active and binds to the GR.

The primary function of cortisol is the regulation of carbohydrate, lipid and protein metabolism (Dallman, 2004). At normal basal concentrations, cortisol has a permissive effect on the action of glucagon and epinephrine in stimulating gluconeogenesis and lipolysis during the postabsorptive state. During starvation and other physiological stress, the adrenal cortex is stimulated by the HPA axis to secrete cortisol. Increased plasma cortisol levels result in increased protein catabolism, increased gluconeogenesis and the stimulation of triacylglycerol catabolism in adipose tissue, as discussed

previously in this chapter. In addition to increasing gluconeogenesis, cortisol also increases the release of glucose from the liver (Vander et al., 2001).

Since the action of cortisol is opposite to that of insulin, individuals with an abnormally high level of cortisol may develop symptoms that are similar to those seen in individuals with insulin deficiency. Conversely, cortisol deficiency can result in hypoglycaemia, serious enough to impair brain function during fasting (Vander et al., 2001).

At basal levels glucocorticoids suppress immune function to a certain extent and are important in preventing the onset of autoimmune diseases (Gold, 2001). Binding of cortisol to GRα, resulting in transactivation, also has an inhibitory effect on certain transcription factors, such as nuclear factor kappa B, involved in immune function. The resulting transrepression decreases the expression of many genes encoding inflammatory mediators (Gagliardo et al., 2001). The immunosuppressive and anti-inflammatory effects of glucocorticoids are significantly increased during periods of high glucocorticoid secretion that is brought about by stress. Cortisol also reduces the number of circulating lymphocytes and decreases both antibody production and the activity of helper T cells and cytotoxic T cells. Glucocorticoid derivatives are therefore administered in high doses to reduce the inflammatory response to injury and infection as well as in the treatment of allergy, arthritis and graft rejection (Vander et al., 2001). It has also been reported that high concentrations of cortisol may cause bone resorption by inhibiting osteoblasts and stimulating osteoclasts; inhibit the secretion of GH by the anterior pituitary (Vander et al., 2001); disrupt preovulatory events (Breen, 2005); lower the number of circulating eosinophils (Kita et al., 2000)

and basophils (Kaliner, 1985); and increase the neutrophil (Strickland et al., 2001), platelet (Sanner et al., 2002) and red blood cell (Reid and Perry, 1991) counts. Since cortisol also facilitates the vasoconstrictive effect of norepinephrine (Vander et al., 2001), adrenal insufficiency therefore prevents vascular compensation for hypovolemia. Other permissive effects of glucocorticoids include the pressor responses and the bronchodilatory effect of the catecholamines (Gagliardo, 2001).

2.6 Enzymes involved in adrenal steroidogenesis

The biosynthesis of the mineralocorticoids, glucocorticoids and adrenal androgens from cholesterol in the adrenal gland involves two distinct groups of enzymes: the cytochromes P450 and the hydroxysteroid dehydrogenases. The general biochemical properties of these enzyme groups will subsequently be discussed.

2.6.1 The cytochromes P450

The cytochromes P450 are a superfamily of haem-containing proteins found in bacteria, fungi, plants and vertebrates, including mammals (Nelson et al., 1996). Over 1200 individual cytochromes P450 have been reported to date (Lewis, 2001). Garfinkel (1958) and Klingenberg (1958) were the first to isolate this unusual cytochrome from mammalian liver microsomes in 1958. The unique spectral properties of these cytochromes were first identified by Omura and Sato in 1962. The reduced cytochrome showed a distinct peak at

450 nm in the presence of carbon monoxide. This peak at 450 nm resulted in the name P450. Detergent treatment of P450 containing microsomes quantitatively converts the cytochrome to a soluble form with a peak at 420 nm, referred to as P420. Omura and Sato (1964) later showed that cytochromes P450 contain a protoporphyrin IX ring structure complexed with iron. The haem iron is always penta- or hexacoordinated, with four of the ligands being contributed by the planar, tetradentate poryphyrin ring. The fifth or proximal ligand is a thiolate sulphur atom contributed by a cysteine residue from in the polypeptide chain. The sixth coordination position of the iron is believed to be occupied by water in the native, substrate free, ferric state. Upon reduction of the iron, the sixth position becomes the site of dioxygen binding (White and Coon, 1980). The unique nature of these cytochromes allows them to catalyse the stereospecific hydroxylation of non-activated substrates (RH) at physiological temperature, a reaction that, uncatalysed, requires extremely high temperatures to proceed. The general reaction is:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The cytochromes P450 function as monooxygenases in this reaction. They utilize reduced NADPH as the electron donor for the reduction of molecular oxygen, with the subsequent incorporation of one oxygen atom into the substrate as a hydroxyl group, while the other oxygen atom is reduced to water. The reaction cycle of the cytochromes P450 has been the subject of many investigations and a general mechanism has been established, as shown in figure 2.5. The resting state of the enzyme is the ferric (Fe^{III}) complex (**1**), with a water molecule as the distal ligand. The entrance of the substrate (RH) to the active pocket displaces the water molecule and

generates the five-coordinate ferric species (**2**). This complex is a good electron acceptor and triggers a single electron transfer from its redox partner, which reduces the iron to the ferrous (Fe^{II}) state (**3**). Ferrous porphyrin is a good dioxygen binder, resulting in the binding of oxygen to form the ternary P450-oxygensubstrate complex (**4**). This species is again a good electron acceptor, triggering a second single electron transfer from its redox partner to give rise to the twice-reduced ferric-dioxo species (**5**). The ferric-dioxo complex is a good Lewis base, and therefore undergoes protonation to yield the ferric peroxide complex (**6**), known as Cpd 0. Since Cpd 0 is still a good Lewis base, it undergoes a second protonation resulting in the splitting of the molecular oxygen. One oxygen atom is lost to water, while the other remains bound to the ferric iron to form the reactive species (**7**) known as Cpd I. This species transfers the distal oxygen atom to the substrate, which is subsequently released and replaced by a water molecule to regenerate the resting state of the enzyme (**1**) (White and Coon, 1980; Shaik and de Visser, 2005). The presence of the ferrous dioxygen and an oxyferryl species was confirmed recently by Schlichting et al., (2000) using trapping techniques and cryocrystallography to investigate the catalytic pathway of cytochrome P450cam (CYP101). Alternative species, such as a $Fe\text{-}OH_2\text{-}O^-$ intermediate (Hata, 2000) and Cpd II that results from the single-electron reduction of Cpd I (Du et al., 1991) have been suggested to participate in the cycle, though no experimental evidence for the existence of these species in the P450 cycle is available. Cpd II is, however, well known in other related haem containing enzymes (Kuramochi et al., 1997).

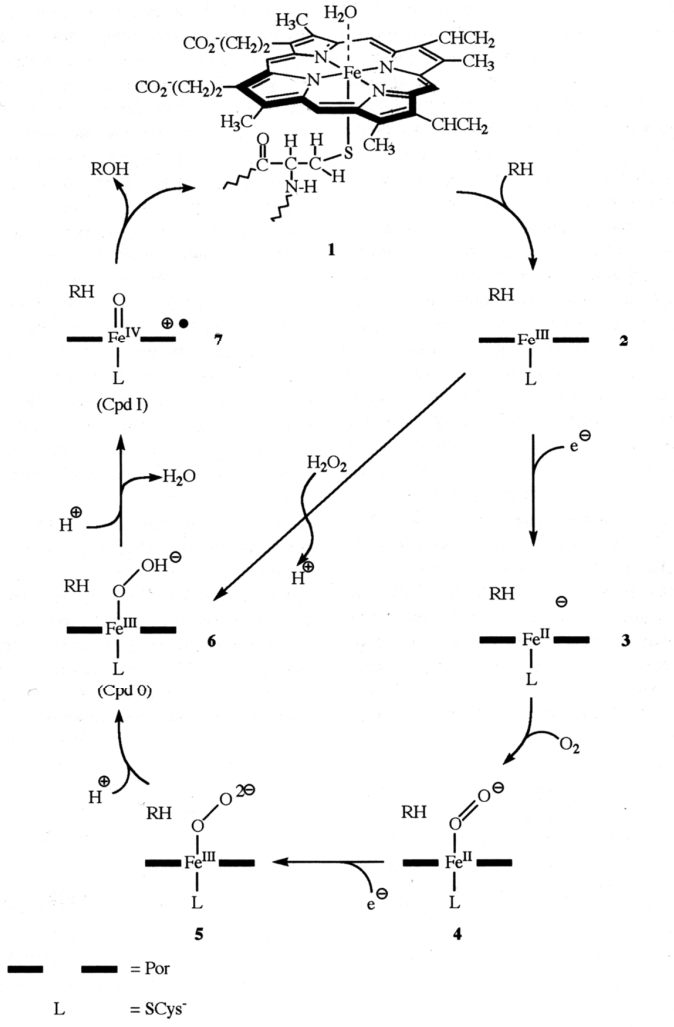

Figure 2.5. Proposed cytochrome P450 reaction cycle. RH represents a substrate and ROH the corresponding hydroxylated product. Reproduced from Shaik and de Visser (2005).

The cytochromes P450 are vital for a number of physiological processes, which include the metabolism of xenobiotics (Porter and Coon, 1991) in the liver and the production of the steroid hormones in the gonads and adrenal cortex. Mammalian cytochromes P450 are all associated with either the endoplasmic reticulum (microsomal) or the mitochondrial (mitochondrial) membranes. The microsomal and mitochondrial cytochromes P450 obtain the

required electrons from NADPH via two different electron transfer systems. The mitochondrial system involves the transfer of the high potential electron to a flavoprotein, adrenodoxin reductase (AdxR), and subsequently to a non-haem ironsulphur protein, adrenodoxin (Adx). Adx acts as an electron shuttle between AdxR and the mitochondrial cytochromes P450. A schematic representation of the mitochondrial electron transfer system is shown in figure 2.6.

The microsomal electron transfer system involves a single protein, cytochrome P450 oxidoreductase (CPR), which contains two flavins. Electrons are transferred from NADPH to flavinadenine dinucleotide (FAD), and subsequently to flavinmononucleotide (FMN) and the microsomal cytochrome P450 (Payne and Hales, 2004). This electron transfer system is discussed in greater detail in the following chapter.

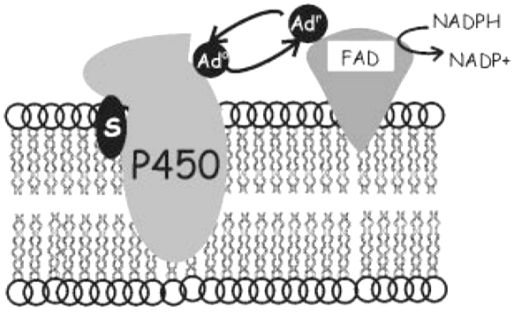

Figure 2.6. Schematic representation of the mitochondrial electron transfer system for cytochrome P450-dependent enzymes. S, substrate; Ad, Adrenodoxin. Reproduced from Payne and Hales (2004).

Three mitochondrial and two microsomal cytochromes P450 are involved in adrenal steroidogenesis, their expression being zone specific, which determines the steroidogenic output of the adrenal gland.

2.6.2 The hydroxysteroid dehydrogenases

Two hydroxysteroid dehydrogenases (HSDs), 3β-hydroxysteroid dehydrogenase (3βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD), are involved in steroidogenesis and are members of the short-chain alcohol dehydrogenase reductase superfamily (Jarabak, 1962; Jarabak and Sack, 1969; The et al., 1989; Lorence et al., 1990; Andersson et al., 1995; Penning, 1997). They are non-metallic enzymes that function as monomers or multimers, but are not known to form complexes with other proteins such as redox partners (Mason, 2002). Only 3βHSD contributes significantly to adrenal steroidogenesis. Two distinct isoforms of 3βHSD have been identified in humans, human 3βHSD I and II (Lorence et al., 1990; Rheaume et al., 1991).

Both of these isoforms function as steroid dehydrogenase/isomerases and catalyse the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids. This conversion requires two sequential reactions. The first reaction is the dehydrogenation of the 3β-equatorial hydroxysteroid, and requires the coenzyme NAD^+, yielding a Δ^5-3-keto intermediate and NADH. In the subsequent reaction, NADH activates the isomerisation of the Δ^5-3-keto-steroid to yield the β4-3-ketosteroid, as shown in figure 2.7 for pregnenolone and DHEA. Both reactions are catalysed without the release of the intermediate or the coenzyme (Thomas et al., 1989; Thomas et al., 1995; Thomas et al., 2003). Unlike the reactions catalysed by the cytochromes P450, the reactions catalysed by the HSDs are reversible, and the reaction

direction is dependent on the concentrations of the substrates, products and cofactors (Mason, 2002).

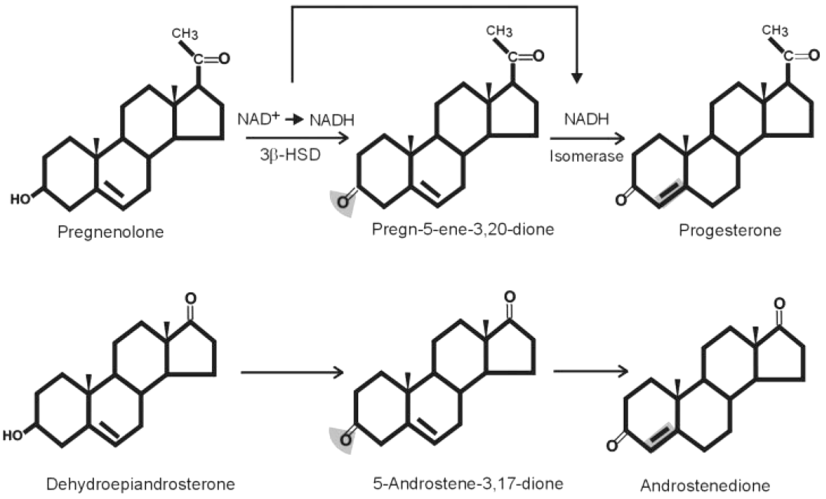

Figure 2.7. Enzymatic reaction catalysed by human 3βHSD. The enzyme catalyses the dehydrogenation of the 3β-hydroxyl group yielding a Δ^5-3-keto intermediate and reduced NADH that activates the isomerisation of the Δ^5-3-keto to yield the Δ^4-3-ketosteroid, as shown here for the conversion of pregnenolone and DHEA to progesterone and androstenedione, respectively. Reproduced from Payne and Hales (2004).

The two isoforms of human 3βHSD are expressed in a tissue specific manner. Only 3βHSD II is expressed in the adrenal, ovary and testis, while 3βHSD I is expressed in the placenta, skin and breast tissue (Rheaume et al., 1991). Similarly multiple isoforms of 3βHSD's have been identified in the mouse (Abbaszade et al., 1997; Payne et al., 1997), and rat (Simard et al., 1991; de Launoit et al., 1992; Simard et al., 1993). In rat adrenals and gonads, 3βHSD was detected mostly in the endoplasmic reticulum, but also in the cristae of the inner mitochondrial membrane (Pelletier et al., 2001). In the human, 3βHSD is expressed in all three zones of the adrenal cortex between 7 months and 8 years of age, followed by a decrease in the zona reticularis with increasing age (Suzuki et al., 2000).

2.6.3 Overview of the adrenal steroidogenic pathway

The steroid hormones produced by the adrenal cortex are all derivatives of cholesterol, containing the cyclopentanoperhydrophenanthrene nucleus shown in figure 2.8. Five cytochromes P450 and a single hydroxysteroid dehydrogenase, 3βHSD, catalyse the conversion of cholesterol to the adrenal steroid hormones. The cytochromes P450 involved are the mitochondrial enzymes: cytochrome P450 side-chain cleavage (CYP11A1); cytochrome P450 11β-hydroxylase (CYP11B1); aldosterone synthase (CYP11B2); and the microsomal enzymes: cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) and cytochrome P450 21-hydroxylase (CYP21).

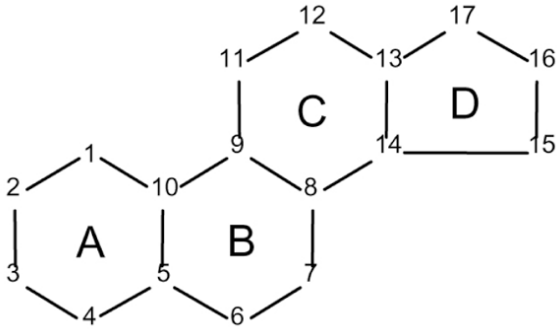

Figure 2.8. Cyclopentanoperhydrophenanthrene nucleus. Reproduced from You (2004).

CYP11A1 catalyses the conversion of cholesterol to pregnenolone, the first step in adrenal steroidogenesis. The pregnenolone produced in the mitochondria moves to the ER where it serves as a substrate for either CYP17 or 3βHSD. CYP17 hydroxylates pregnenolone at C17 to yield 17-hydroxypregnenolone, which can serve as a substrate for the lyase activity of CYP17 to produce DHEA. DHEA and 17-hydroxypregnenolone also act as

substrate for 3βHSD. Pregnenolone, 17-hydroxypregnenolone and DHEA are all dehydrogenated at C3 and converted from the Δ^5 to the Δ^4 isoforms by 3βHSD. The resulting products are progesterone, 17-hydroxyprogesterone and androstenedione, respectively. Progesterone can be hydroxylated at C17 by CYP17 to form 17-hydroxyprogesterone. In addition, the lyase activity of CYP17 can cleave the C17-C20 bond of 17-hydroxyprogesterone to yield androstenedione. Progesterone and 17-20 hydroxyprogesterone also act as substrates for CYP21, which hydroxylates these substrates at C21 to form deoxycorticosterone and deoxycortisol, respectively. These two intermediates move to the mitochondria where CYP11B1 catalyses their 11β-hydroxylation to yield corticosterone and cortisol, respectively. Deoxycorticosterone also serves as substrate for CYP11B2 which catalyses three sequential reactions. These reactions are: the 11β-hydroxylation of deoxycorticosterone; the hydroxylation of C18; and the oxidation of the C18 hydroxyl group to yield the C18 aldehyde group of aldosterone (Payne and Hales, 2004). A map of the adrenal steroidogenic pathways is shown in figure 2.9. It is essentially the distribution of the different steroidogenic enzymes in the three zones of the adrenal cortex that results in each zone's unique steroidogenic output. For example, the zona glomerulosa, which produces only aldosterone, expresses only CYP11A1, 3βHSD, CYP21 and CYP11B2. CYP17 and CYP11B1 are not expressed in this zone, while CYP11B2 is not expressed in either the zona fasciculata or the zona reticularis. The zona fasciculate, which produces mainly cortisol, and small amounts of DHEA and androstenedione, expresses CYP11A1, CYP17, 3βHSD, CYP21 and CYP11B1. Finally the zona reticularis, which produces primarily DHEA and androstenedione, and trace

amounts of cortisol, express CYP11A1, CYP17, 3βHSD, and low levels of CYP21 and CYP11B1 (Payne and Hales, 2004).

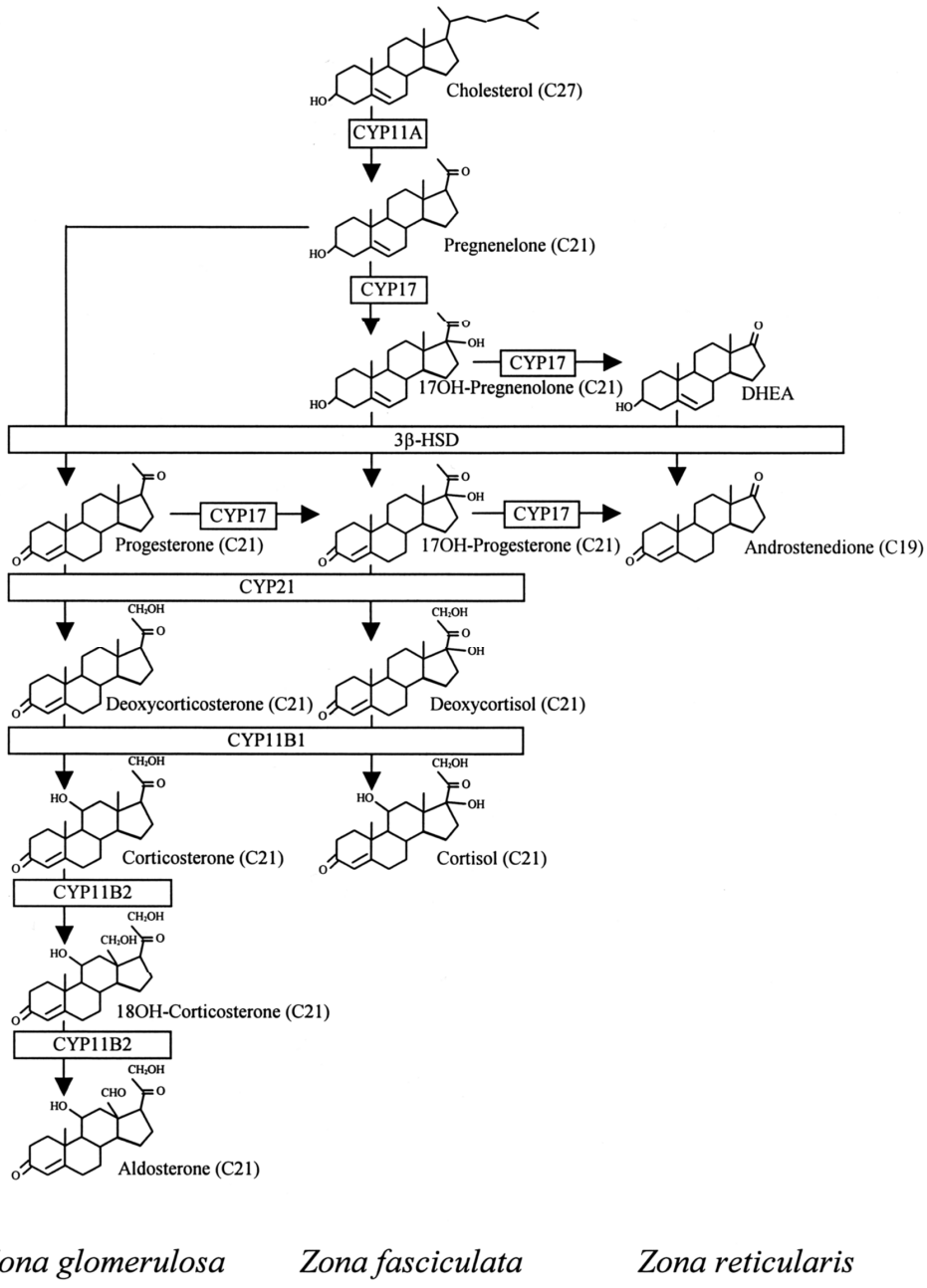

Figure 2.9. Overview of the adrenal steroidogenic pathway. CYP11B2 can also catalyse the conversion of deoxycorticosterone to corticosterone. Reproduced from Slabbert (2003).

2.7 The source of cholesterol for adrenal steroidogenesis

As previously mentioned, cholesterol is the precursor for all steroid hormones. The cholesterol used in steroid biosynthesis is mainly derived from circulating lipoproteins obtained via receptor-mediated uptake, although all steroidogenic cells contain the necessary enzymes to synthesise endogenous cholesterol from acetate.

Adrenocortical cells acquire cholesterol from both low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol esters (CE) circulating in the blood (Martin et al., 1999). LDL uptake is mediated by the LDL receptor. Binding of LDL to the LDL receptor results in the internalisation and degradation of the entire LDL molecule by lysosomal vesicles. CE's are hydrolysed in the lysosome by lysosomal acid lipase with the subsequent release of cholesterol (Liu et al., 2000; Mason, 2002). Vesicles transport most of the released cholesterol to the endoplasmic reticulum (Mason, 2002). Apolipoprotein E (Apo E) is present in high levels on adrenocortical cell membranes, and has been shown to facilitate cholesterol uptake by the LDL receptor (Swarnakar et al., 1998). ACTH promotes LDL mediated CE uptake in the adrenal (Liu et al., 2000). Cholesterol uptake from HDL is mediated by the scavenger receptor class B, type I (SR-BI) receptor, also known as CLA-1 (von Eckardstein et al., 2001). HDL CE uptake is a two step process.

The first step involves the binding of the lipoprotein to the extracellular domain of SRBI, while the second step involves the selective transfer of lipid to the plasma membrane. CE's are delivered to the plasma membrane without the uptake and degradation of the entire HDL particle, as occurs for LDL

uptake (von Eckardstein et al., 2001; Connelly and Williams, 2004). SR-BI expression in the adrenocortical cell membrane results in the formation of microvilli and microvillar channels, the sites for cholesterol delivery. In addition, SR-BI appears to increase membrane fluidity by altering the lipid composition of the cell membrane (Connelly and Williams, 2004). The epitopes in HDL that recognise SR-BI are believed to be various phospholipids and apolipoproteins, specifically apoA-I (von Eckardstein et al., 2001; Connelly and Williams, 2004). CE delivered by SR-BI to the steroidogenic cell is hydrolysed extrasomally by a neutral CE hydrolase, hormone sensitive lipase (HSL) (Kraemer et al., 2004). It would appear that HSL is also involved in the release of cholesterol from CE's stored in lipid droplets within the cytoplasm. The expression of SR-BI is hormonally regulated. Angiotensin II and ACTH promote SR-BI-mediated HDL selective CE uptake in the zona glomerulosa and zona fasciculata, respectively (Connelly and Williams, 2004). However, depletion of adrenal cholesterol stores can promote SR-BI-mediated HDL uptake in the absence of hormone stimulation (Sun et al., 1999). Studies have shown that in humans, and other animals that carry their plasma cholesterol mainly in LDL, cholesterol for adrenal steroidogenesis is derived primarily from LDL uptake via the LDL receptor. Conversely, in animals such as rodents most of the cholesterol is bound to HDL, therefore cholesterol for adrenal steroidogenesis is derived primarily from SR-BI-mediated HDL uptake (Martin et al., 1999; Liu et al., 2000). Esterification of cellular cholesterol with fatty acids is catalysed in the endoplasmic reticulum by acyl-CoA:cholesterol acyltransferase 1 (ACAT1). CE's accumulate in the endoplasmic reticulum and bud off as lipid droplets.

2.8 Regulation of steroidogenesis by the HPA axis

The stimulation of the HPA axis by physiological cold stress and hypoglycemia has been discussed earlier in this chapter. The following section will focus on the effect of the resulting release of ACTH by the anterior pituitary.

2.8.1 Mechanism of ACTH action

Upon binding to the ACTH receptor, ACTH primarily stimulates the secretion of the glucocorticoids, but also the mineralocorticoids and androgens from the adrenocortical cells. The ACTH receptor is expressed mainly in the adrenal cortex and is a member of the superfamily of G-protein coupled receptors with seven transmembrane domains. The ACTH receptor belongs to the melanocortin receptor subfamily, which has characteristic short N-terminal extracellular domains; short intracellular C-terminal domains; and short fourth and fifth transmembrane domains (Beuschlein et al., 2001). This transmembrane hormone receptor is associated with a trimeric signal-transducing G protein on the cytoplasmic side of the cellular membrane. The trimeric G-protein consists of α, β and γ subunits. The α subunit is bound to GDP and complexed with the β and γ subunits when no ligand is bound to the ACTH receptor. Binding of ACTH to the receptor causes a conformational change resulting in the displacement of GDP by GTP in the α subunit. The α subunit dissociates from the β and γ subunits and subsequently associates with adenylate cyclase, which is bound to the inner cell membrane. Adenylate

cyclase is activated by the binding of the α subunit and catalyses the conversion of ATP to cAMP. The α subunit has intrinsic GTPase activity and once the bound GTP is hydrolysed to GDP, the α subunit dissociates from adenylate cyclase and relocates to the β and γ subunits. This activation cycle continues while ACTH is bound to the receptor and is shown in figure 2.10. The displacement of GDP by GTP in the α subunit upon ACTH binding causes a decrease in receptor affinity for ACTH that may result in the dissociation of ligand from the receptor (Lodish et al., 2000). The cAMP produced by adenylate cyclase acts as a second messenger by inducing changes in various metabolic pathways by first binding to and activating cAMP dependent protein kinase (PKA). Activated PKA can phosphorylate a number of enzymes at specific serine and threonine residues, resulting in a change in their catalytic activity. The action of cAMP is terminated by its eventual conversion to AMP by phosphodiesterase (Vander et al., 2001). In the adrenocortical cells, the ACTH induced cAMP and PKA result in a number of effects, which lead to increased steroidogenesis and the secretion of the steroid hormones. Both acute and chronic regulatory effects result from ACTH stimulation. Acute effects occur within a matter of minutes, while chronic effects occur after a few hours, involving increased gene transcription and translation of the appropriate steroidogenic enzymes (Clark and Stocco, 1996; Stocco and Clark, 1996; Clark and Stocco, 1997; Jefcoate, 2002). Only the acute effects of ATCH will be discussed in the following section. An example of the chronic regulation of steroidogenesis, namely the upregulation of CYP17, will be discussed in the following chapter.

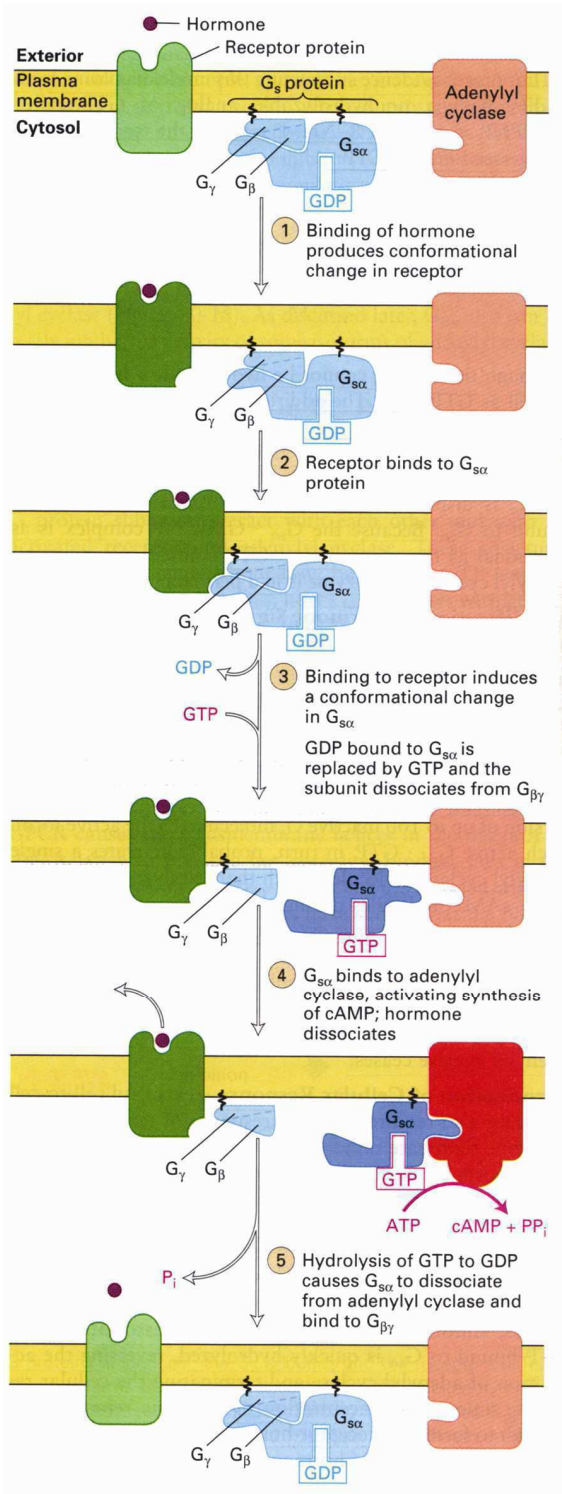

Figure 2.10. Graphic presentation of the ACTH receptor activation cycle. Reproduced from Lodish et al. (2000).

2.8.2 ACTH stimulation of Steroidogenesis

ACTH is the principal hormone secreted from the anterior pituitary that stimulates adrenal steroidogenesis. Acute effects primarily involve the delivery of cholesterol to the inner mitochondrial membrane, the site of the first step in adrenal steroidogenesis, resulting in an increase in the steroidogenic output of all three zones of the adrenal cortex. These effects are, however, limited. Only the production and secretion of the glucocorticoids are significantly increased by ACTH, as ACTH results in an increased expression of CYP11A1, CYP17, CYP11B1 and CYP21 in the zona fasciculata. The expression of CYP11B2 in the zona glomerulosa is not affected by ACTH, as it is controlled primarily by the renin-angiotensin system (Payne and Hales, 2004).

The availability of the cholesterol, however, remains a critical point in the regulation of steroidogenesis. As discussed previously, the adrenal cortex is a highly vascular tissue and contains significantly high levels of lipoprotein receptors, readily providing access to dietary cholesterol (Jefcoate, 2002). Furthermore, ACTH stimulates the delivery of cholesterol to the adrenocortical cells by the upregulation of SR-BI and the LDL receptor (Liu et al., 2000; Connelly and Williams, 2004). The adrenocortical cells maintain CE stores in lipid droplets which are abundant in the cytoplasm of adrenocortical cells (Young and Heath, 2000). In the adrenal cortex, steroidogenesis is acutely regulated by ACTH stimulation, which acts by elevating cAMP levels and activating PKA (Young and Heath, 2000). Increased cholesterol metabolism is observed in adrenocortical cells within 3 minutes of ACTH treatment and peaks 10-15 minutes post treatment. Defects in either PKA or G protein

coupling, preventing cAMP formation, abolish this response. In the adrenal cortex cAMP elevates the expression of the LDL receptor and SR-BI; promotes cholesterol delivery from the plasma membrane and the late endosomes to the mitochondria; and stimulates cholesterol esterase activity, while inhibiting ACAT1. These events favour the release of cholesterol from stored CE's and its accumulation in the outer mitochondrial membrane (Jefcoate, 2002). Another response to ACTH stimulation is the cAMP-dependent dephosphorylation of the cytoskeleton-associated protein paxillin, which is a component of the focal adhesion complex (Rocchi et al., 2000). The dephosphorylation of paxillin is proposed to be involved in a change in cytoskeleton organisation that results in the pronounced rounding of steroidogenic cells within 5 minutes of ACTH stimulation (Stocco and Clark, 1996; Jefcoate, 2002; Rocchi et al., 2000). The reorganisation of the cytoskeleton causes the clustering of the steroidogenic organelles bringing the mitochondria in close contact with CE pools (Soto et al., 1986). ACTH also stimulates endothelin production, acute stimulation of blood flow to the adrenal and enhances the extracellular access of cholesterol and oxygen, the cosubstrate of all cytochromes P450, to CYP11A1, the first enzyme in the steroidogenic pathway (Young and Heath, 2000). The proposed routes through which cholesterol can reach the mitochondria are shown in figure 2.11.

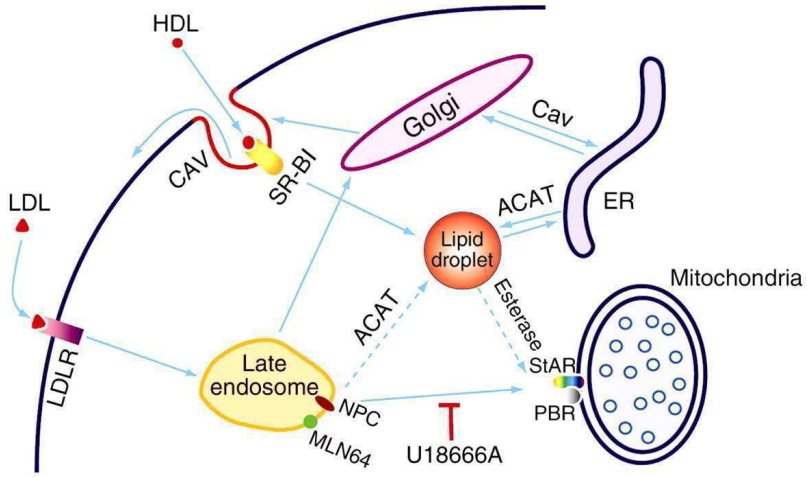

Figure 2.11. Mechanisms of cholesterol transport to the mitochondria. Cholesterol is taken up from both LDL receptors and apoA/HDL receptors (SR-BI) in caveolin-rich domains (CAV). Late endosomes mediate the transfer to the mitochondria via the activities of NPC-1 (inhibited by U18666A) and possibly the StAR-like protein MLN64. Acyl-CoA:cholesterol acyltransferase (ACAT) converts free cholesterol to CE's stored in the lipid droplets. Reproduced from Jefcoate (2002).

2.8.2.1 The role of StAR

Once delivered to the outer mitochondrial membrane, the only remaining barrier for the delivery of cholesterol to the inner mitochondrial membrane, the site at which CYP11A1 metabolises cholesterol to yield pregnenolone, is the aqueous space between the outer and inner mitochondrial membranes (Stocco and Clark, 1996). As the aqueous diffusion of cholesterol is slow and CYP11A1 metabolises relatively soluble hydroxycholesterols in the absence of ACTH stimulation, it was proposed that the acute effects of cAMP and PKA are mediated by StAR, which is required for the transfer of cholesterol from the outer to the inner mitochondrial membrane (Garren et al., 1971; Privalle et

al., 1987; Lambeth et al., 1987; Clark and Stocco, 1996; Stocco and Clark, 1996; Clark and Stocco, 1997; Jefcoate, 2002).

StAR is expressed as a 37-kDa preprotein known as p37 and contains a 68 amino acid mitochondrial targeting presequence, which is cleaved upon mitochondrial uptake to yield the 30-kDa mature protein known as p30. ACTH stimulation results in PKA-dependent phosphorylation of the p37 protein to yield phosphorylated p30 protein (pp30) upon mitochondrial processing (Jefcoate, 2002). Some steroidogenic cells are regulated by hormones other than ACTH and require other pathways to activate StAR. For example, glomerulosa cells, which produce the mineralocorticoid aldosterone, are regulated by the renin-angiotensin system (Clark and Stocco, 1996). Angiotensin II activates the PKC pathway and increases intracellular levels of calcium. StAR responds to the increased calcium levels (Elliot et al., 1993) as it contains consensus sequences for PKA and calmodulin-dependent kinase (Jefcoate, 2002).

The mechanism of action of StAR is not yet fully understood. There has been some debate as to whether the phosphorylated p37 protein (pp37) or pp30 is the active form of StAR, and if StAR acts on the outer or inner mitochondrial membrane. Early models suggested that cholesterol transfer from the outer to the inner mitochondrial membrane occurs during the processing and import of StAR, which results in contact sites being formed between the two membranes, and that pp30 StAR, which is associated with the inner mitochondrial membrane or the mitochondrial matrix, is inactive (Stocco and Clark, 1996). However, deletion of the N-terminal targeting sequence, which directs the StAR to TOM20, the receptor in the outer

mitochondrial membrane where it is cleaved and imported to the inner mitochondrial membrane, does not diminish the steroidogenic enhancing activity of StAR in COS-1 cells (Koehler, 2000; Bose, 2002). In addition, chimeric StAR, bound TOM20, effectively activated cholesterol metabolism in isolated basal steroidogenic MA10 mitochondria (Bose, 2002). Tuckey et al. (2002) have shown that StAR can mediate the transfer of cholesterol between synthetic membranes without other protein components found in mitochondria. This evidence suggests that StAR functions at the outer mitochondrial membrane and remains active in the absence of its N-terminal mitochondrial targeting sequence. Recently a more complex model has been proposed and shows that the activation of StAR, necessary for high cholesterol trafficking, may involve StAR processing as well as other protein factors. Jefcoate (2002) has questioned the relevance of the models used to determine StAR activity, as COS-1 cells are insensitive to changes in the level of StAR expression and process p37 to pp30 at a much slower rate than adrenal cells. During peak cholesterol metabolism in steroidogenic MA10 cells, p37 is scarcely detectable. Furthermore, inhibitors of mitochondrial proteases and protein uptake, which block pp30 formation, prevent the activation of cholesterol metabolism in MA10 cells. However, cholesterol metabolism is unaffected when sufficient pp30 was generated, demonstrating the importance of pp30 in cholesterol transport (Artemenko et al., 2001). It is estimated that each molecule of pp30 mediates the transfer of 400 cholesterol molecules per minute, while Tuckey et al. (2002) showed a stoichiometry of 2.8 molecules of cholesterol per molecule of StAR. This suggests that p37 may well have activity at the outer mitochondrial membrane, but that it is the

processed pp30 form that is crucial for cholesterol uptake during steroidogenesis in the inner mitochondrial membrane. The conversion of newly synthesised p37 to pp30 reaches a steady state between formation and processing within 5 minutes. This is consistent with the time frame for acute regulation of CYP11A1 activity by ACTH. Although cAMP has no measurable effect on these rates, maximal stimulation may ensure near-complete conversion by the phosphorylation of extramitochondrial p37 to pp37 (Jefcoate, 2002). Although StAR transcription is also elevated in response to cAMP, it has been suggested that newly synthesized StAR arises from the translation of pre-existing stable mRNA in the cytoplasm and not from transcription. (Clark and Stocco, 1997; Jefcoate, 2002). StAR is believed to partially denature as it moves through the mitochondrial membranes and then refold after cleavage in the matrix to generate pp30 (Jefcoate, 2002). Assisted by HSP70, which continuously acts on StAR as it enters the mitochondrial matrix, StAR adopts a molten globule structure and associates with the inner mitochondrial membrane (Christensen et al., 2001).

In nonsteroidogenic cells, cholesterol is largely excluded from the inner mitochondrial membrane as it can be detrimental to the activity of mitochondrial proteins involved in cellular respiration (Jefcoate, 2002). The inner mitochondrial membrane of adrenal cells is extensive and vesiculated, with limited direct contact with the outer mitochondrial membrane (Jefcoate, 2002). This implies a further role of StAR in the distribution of cholesterol to locations that are far removed from the outer mitochondrial membrane. Here StAR facilitates the transfer of cholesterol from the cholesterol rich domains in the outer mitochondrial membrane to the inner mitochondrial membrane,

including regions that are far removed from the outer membrane and possible matrix vesicles which contain CYP11A1 (Jefcoate, 2002).

2.8.2.2 The role of the peripheral benzodiazepine receptor in StAR activity

The peripheral benzodiazepine receptor (PBR), which is located in the outer mitochondrial membrane of most mitochondria and is elevated in steroidogenic cells, has been implicated in mitochondrial cholesterol uptake and linked to the activity of StAR (Stocco and Clark, 1996; Jefcoate, 2002). PBR is associated with a member of the porin protein family in the outer mitochondrial membrane. The structure of the porin is altered and a number of mitochondrial functions are affected when PBR is activated by the adrenal acyl-CoA binding protein and other agonists (Jefcoate, 2002). PBR stimulation has also been linked to an increase in cholesterol metabolism in steroidogenic cells, while PBR antagonists inhibit mitochondrial cholesterol transport in the presence of cAMP (Papadopoulos, 1998). Although, cholesterol metabolism is severely impaired in cells where PBR has been deleted, it can be restored by transfection of the cells with PBR constructs (Jefcoate, 2002). In steroidogenic cells, PBR-associated protein 7 (PAP7) has been shown to bind both PBR and PKA, forming a multiprotein complex leading to the phosphorylation of p37, possibly mediating the interaction between PBR and StAR (Li et al., 2001; Jefcoate, 2002). In MA10 cells the suppression of PAP7, using antisense technology, prevents the cAMP-dependent stimulation of steroidogenesis (Jefcoate, 2002). PAP7 is therefore believed to link PKA to PBR and facilitate the phosphorylation of StAR during its mitochondrial uptake. Interestingly, upon agonist binding to PBR, the cholesterol in the outer

mitochondrial membrane aggregates around PBR providing high local concentrations of cholesterol in areas of the outer mitochondrial membrane directly opposite the site of StAR uptake in the inner mitochondrial membrane. The proposed model for the role of PBR and PAP7 in the mitochondrial import of StAR is shown in figure 2.12. Fatty acyl-CoA's formed during the hydrolysis of CE's are able to bind to the acyl-CoA-binding protein, which is responsible for activating PBR, resulting in the interaction of fatty acyl-CoA near StAR (Papadopoulos, 1998). This may promote the translocation of the fatty acyl-CoA's to the inner mitochondrial membrane (Jefcoate, 2002). A mitochondrial thioesterase hydrolyses acyl-CoA to release fatty acids and may be an essential mechanism to maintain membrane fluidity in the presence of high levels of cholesterol (Maloberti et al., 2000).

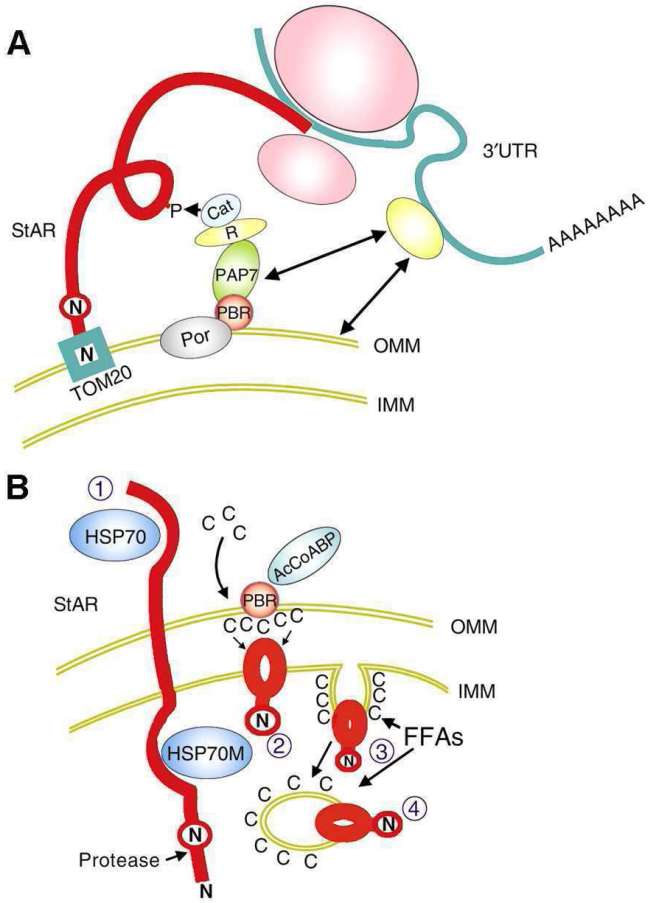

Figure 2.12. Hypothetical model for StAR mediated cholesterol transfer into the mitochondrion. 'N' indicates the N-terminal of p37, while the circled 'N' indicates the N-terminal of p30. (A) The N-terminal of newly synthesized p37 associates with the outer mitochondrial membrane surface protein TOM20. Phosphorylation of p37 by the catalytic unit of PKA (Cat) is facilitated by the interaction of the regulatory subunit (R) with the adaptor protein PAP7. PAP7 binds in turn to PBR which is bound to porin (Por) in the outer mitochondrial membrane. (B) In step 1 following phosphorylation pp37 is denatured aided by the cytosolic HSP70 complex and transported across the outer and inner mitochondrial membranes. This process is assisted by HSP70 protein complexes in the mitochondrial matrix (HSP70M). A mitochondrial protease cleaves pp37 to yield pp30, which is then integrated into the inner mitochondrial membrane. In step 2, cholesterol (C) is transferred to pp30 from domains in the outer membrane formed by PBR, which is activated by acyl-CoA-binding protein (AcCoABP). In step 3, activated inner membrane domains accept the transfer of free fatty acids (FFA) and cholesterol from the cholesterol rich domains in the outer membrane. AcCoABP may also facilitate the transfer of acyl-CoA to the inner mitochondrial membrane, where mitochondrial thioesterase hydrolyses acyl-CoA to FFA. In step 4, pp30 facilitates the relocation of cholesterol to regions in the inner mitochondrial membrane that are far removed from the outer membrane, as well as possible matrix vesicles. Reproduced from Jefcoate (2002).

2.8.3 The HPA negative feedback loop

An important facet of ACTH secretion is the control exerted by the glucocorticoids themselves. The glucocorticoids produced by the zona fasciculata and zona reticularis, in response to ACTH stimulation, have a negative feedback effect on the hypothalamus and pituitary. Rising glucocorticoid levels suppress the release of CRH and ACTH by the hypothalamus and anterior pituitary, respectively. This effect is achieved through negative feedback acting on both the release and synthesis of ACTH and CRH. In the anterior pituitary, the glucocorticoids inhibit the transcription of POMC (Evans et al., 1993). In addition, ACTH itself downregulates CRH secretion from the hypothalamus, forming a short negative feedback loop (Guo et al., 2003). Feedback inhibition is principally mediated by the GR. The negative feedback loops are illustrated in figure 2.13. The circadian rhythm of basal ACTH secretion is characterised by highest levels upon waking, with a decline in these levels throughout the day, reaching their minimum in the evening. The negative feedback of basal activity in the HPA axis is also subject to circadian rhythms, as it requires less glucocorticoid at the trough than at the peak of diurnal rhythm (Bradbury et al., 1994).

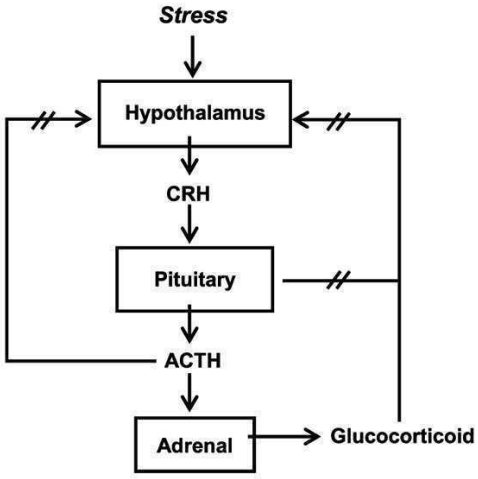

Figure 2.13. Schematic representation of HPA axis regulation. Reproduced from Guo et al. (2003).

2.9 Conclusion

From the above discussion it is clear that the HPA axis plays a central role in mediating the mammalian response to stress (Pacák and Palkovits, 2001). In particular, the HPA axis is critical for stimulating the release of cortisol, which counteracts the hypoglycemic state that occurs in Angora goats during long periods of exposure to cold stress (Munch, 1971). Previous research has, however, demonstrated that South African Angora goats have a reduced ability to produce cortisol relative to other goat species and are thus very susceptible to cold stress (Herselman and van Loggerenberg, 1995; Engelbrecht et al., 2000). Furthermore, Van Rensburg (1971) previously demonstrated that high plasma cortisol levels inhibit hair production, suggesting that selection for high mohair production indirectly resulted in reduced adrenal function and, as a result, reduced cortisol levels. After

investigating adrenal steroidogenesis in the Angora goat, Engelbrecht and Swart (2000) found that the most likely cause of reduced cortisol production was as a result of the enzymatic activity of CYP17, which was significantly different from that of other species investigated. The following chapter will therefore focus on CYP17, addressing the catalytic activity of this key enzyme, its expression, regulation and physiological importance in detail.

CHAPTER 3

CYTOCHROME P450 17α-HYDROXYLASE/17,20-LYASE (CYP17)

3.1 Catalytic activity of CYP17

CYP17 catalyses two distinct mixed-function oxidase reactions, namely the 17α-hydroxylation of the C21 steroids, pregnenolone (Δ^5-steroid) or progesterone (Δ^4-steroid), followed by the cleavage of the C17–20 bond to produce the C19 steroids, DHEA or androstenedione, respectively (Figure 3.1). Each reaction requires one molecule of molecular oxygen and one molecule of NADPH. Initially, it was thought that each reaction was catalysed by two distinct enzymes, until studies revealed that a single enzyme catalysed both reactions (Nakajin and Hall, 1981; Nakajin et al, 1981). This was subsequently confirmed by the cloning and expression of bovine CYP17 in non-steroidogenic COS-1 cells (Zuber et al., 1986).

The hydroxylation and side chain cleavage activities of CYP17 are both catalysed in a common active site. The reaction mechanism for each activity is, however, thought to involve the formation of distinct iron-oxygen complexes. The oxo-intermediate, Fe^{V}=O, is believed to be the active catalytic oxygen-bound cytochrome P450 complex involved in the hydroxylation mechanism (Atkinson and Ingold, 1993), while the iron-peroxo, Fe^{III}-OOH, and iron-oxo, Fe^{V}=O, species have both been suggested as possible candidates involved in the acyl-carbon bond cleavage (Akhtar et al., 1994; Lee-Robichaud et al., 1995).

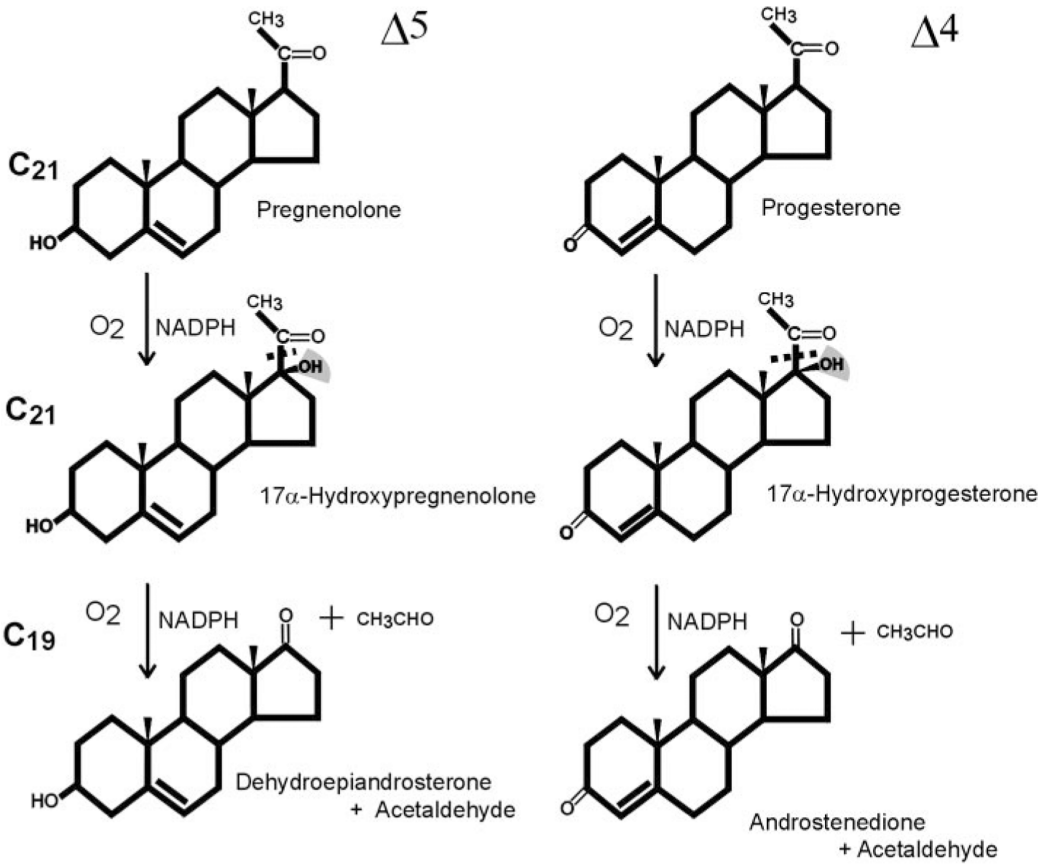

Figure 3.1. Enzymatic reactions catalysed by CYP17. CYP17 catalyses two mixed-function oxidase reactions, a 17α-hydroxylation reaction and a 17,20-lyase reaction. Each reaction requires one molecule of oxygen and one molecule of NADPH and uses the microsomal electron transfer system. Reproduced from Payne and Hales (2004).

It was initially believed that the lyase reaction followed sequentially after the hydroxylase reaction, without the release of the hydroxylated intermediate. Soucy and Luu-The (2000) demonstrated that the transformation of pregnenolone to DHEA by human CYP17 proceeds through two steps in which DHEA is produced from the released intermediate, 17-hydroxypregnenolone.

CYP17 is associated with the endoplasmic reticulum. The electrons required for catalysis are provided by NADPH via a microsomal electron transfer system, as shown in figure 3.2.

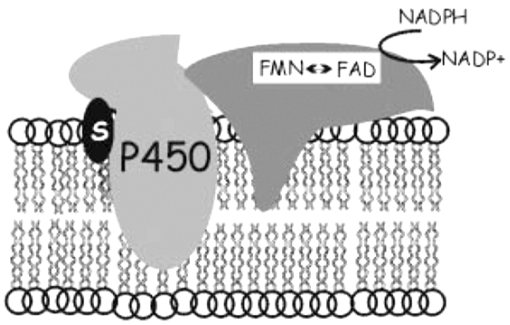

Figure 3.2. Schematic representation of the microsomal electron transfer system for cytochrome P450-dependent enzymes. S, substrate. Reproduced from Payne and Hales (2004).

This system comprises a single protein, CPR, which contains two flavins. The electrons are transferred from NADPH to a FAD, followed sequentially by transfer to FMN and subsequently the cytochrome P450.

The ratio of 17,20-lyase activity to 17α-hydroxylase activity has been shown to be regulated by three distinct post-translational mechanisms. The presence of high molar ratios of CPR to CYP17 favor the 17,20-lyase reaction (Yanagibashi and Hall, 1986; Lin et al., 1993); the presence of cytochrome b_5 enhances the 17,20-lyase activity (Onoda and Hall, 1982; Ishii-Ohba et al., 1984; Katagiri et al., 1982); and serine/threonine phosphorylation of CYP17 increases 17,20-lyase activity without affecting 17α-hydroxylase activity (Zhang et al., 1995; Geller et al., 1999). These mechanisms are discussed in greater detail later in this chapter.

3.2 Species differences in CYP17 catalysis

Although the CYP17 enzyme from various species catalyses both the 17α-hydroxylation and the 17,20-lyase reaction, major species-dependent differences have been observed in 17,20-lyase activity, with different species utilising either 17-hydroxypregnenolone or 17-hydroxyprogesterone as substrate for the lyase activity. Human, baboon, bovine, sheep and goat CYP17 catalyse the hydroxylation of both pregnenolone and progesterone and the conversion of 17-hydroxypregnenolone to DHEA, while the conversion of 17-hydroxyprogesterone to androstenedione is negligible (Chung et al., 1987; Swart et al., 2002; Zuber et al., 1986; Conely and Bird, 1997; Engelbrecht and Swart, 2000). Guinea pig CYP17 cannot convert 17-hydroxypregnenolone to DHEA, preferring the Δ^4-steroid pathway for both the hydroxylase and lyase reactions (Tremblay et al., 1994). Rat, porcine and hamster CYP17 can catalyse both the Δ^5 and Δ^4 hydroxylase and lyase reactions, yielding both DHEA and androstenedione (Fevold et al., 1994; Zang et al., 1992; Cloutier et al., 1995). Recently, Zhou et al. (2007) identified a novel CYP17 expressed in Tilapia (*Oreochromis mossambicus*) and Medaka Fish (*Oryzias latipus*), which possesses 17α-hydroxylase activity, without any detectable 17,20-lyase activity.

Besides catalysing the 17α-hydroxylation of both pregnenolone and progesterone, human CYP17 is able to catalyse the 16α-hydroxylation of progesterone, but not pregnenolone (Swart et al., 1993). In addition, Soucy et al. (2003) have demonstrated a third activity of human CYP17, namely a 16-ene synthase activity, which bypasses the hydroxylation step and directly

cleaves pregnenolone to yield androstadienol, a potential endogenous ligand for orphan receptors such as the constitutive androstane receptor and pregnane-X-receptor.

3.3 CYP17 expression, regulation and physiological importance

3.3.1 CYP17 expression in steroidogenic tissue

In mammals, CYP17 is expressed at all the major sites of steroidogeneis, namely the adrenal cortex, testis, ovary and placenta. There are, however, species related differences in the expression of CYP17 in the adrenal cortex and placenta. In the adrenal glands of human, macaque and guinea pig, CYP17 is expressed in the zona reticularis and the zona fasciculata (Hyatt et al., 1983a; Hyatt et al., 1983b; Endoh et al., 1996), but not in the zona glomerulosa, which consequently produces only mineralocorticoids (Harkins et al., 1982; Crivello and Gill, 1983). CYP17 is only expressed in the zona fasciculata of the Bovine and Ovine adrenal as these species lack a well defined zona reticularis (Conley and Bird, 1997). CYP17 is not expressed in the adrenal glands of mice (Perkins and Payne, 1988) or rats (Brock and Waterman, 1999; Pelletier et al., 2001). The lack of CYP17 expression in the adrenal cortex of these rodents leads to the production of corticosterone as the major glucocorticoid. In contrast, the majority of mammals produce cortisol as the major glucocorticoid.

In the testis of all species, CYP17 is expressed only in the Leydig cell (Saez, 1994; Payne and O'Shaughnessy, 1996; Pelletier et al., 2001), while in

the ovary, CYP17 expression is restricted to thecal cells that are the site of androgen production (Sasano et al., 1989; Tamura et al., 1992; Lauber et al., 1993; Conley et al., 1995). It was generally accepted that CYP17 is not expressed in granulosa and luteal cells (Voutilainen et al., 1986; Sasano et al., 1989; Tamura et al., 1992; Lauber et al., 1993; Conley et al., 1995), however, a recent report suggested that human luteinised granulosa cells in culture do express CYP17 (Moran et al., 2003).

CYP17 is expressed in placenta of mice and rats, starting at midpregnancy and declining just before parturition (Durkee et al., 1992; Arensburg and Payne, 1999). CYP17 is not, however, expressed in the human placenta. Instead the C19 substrate for placental androgens and estrogens is derived from fetal adrenal glands as DHEA-S, which is converted to 16α-hydroxydehydroepiandrosterone sulfate in the fetal liver and subsequently transported to the placenta, where it is acted on by placental steroid sulfatase (Miller, 1998).

Studies of the expression of steroidogenic enzymes in human (Mesiano et al., 1993; Narasaka et al., 2001) and monkey fetal adrenal glands (Mesiano et al., 1993) showed CYP17 expression in the transitional zone and fetal zone throughout gestation, but no expression was detected in the definitive zone. CYP17 mRNA has been detected in mouse fetal adrenal cells between embryonic day 12.5 and 14.5, after which expression disappears (Keeney et al., 1995).

CYP17 expression in mouse fetal testes has been detected from embryonic day 13, the earliest time examined, and throughout pregnancy. No expression has been detected in mouse fetal ovary until the day of birth

(Greco and Payne, 1994). The postnatal expression of CYP17 in mouse testes is low between birth and day 20, rising from between day 20 and day 25, and reaching maximum expression after day 40 (O'Shaughnessy et al., 2002).

Although CYP17 is primarily associated with the classical steroidogenic tissue, its expression has also been reported in the human fetal kidney, thymus and spleen (Casey and MacDonald, 1982). In addition, CYP17 expression has been found in the human heart (Kayes-Wandover and White, 2000) and adipose tissue (Puche et al., 2002). In the mouse, CYP17, which is not expressed in the adrenal, is expressed in the fetal brain beginning at embryonic day 9.5 (Compagnone et al., 1995). DHEA produced in the mouse brain has been shown to stimulate axonal growth from specific populations of neocortical neurons *in vitro*, while DHEA sulphate stimulates dendritic growth from those cells (Blair and Mellon, 2004).

3.3.2 Regulation of CYP17

Several of the steroidogenic cytochromes P450, including CYP17, are transcriptionally activated by ACTH via the PKA signaling pathway (Waterman, 1994). In the adrenal, ACTH directs increased steroid hydroxylase gene transcription via the activation of adenyl cyclase and subsequent increase in intracellular cAMP. This second messenger activates PKA, which in turn leads to the induction of gene transcription by phosphorylating transcription factors, coactivators and/or other proteins in the ACTH signaling pathway (Sewer et al., 2002).

In all species reported to date, CYP17 is the product of a single gene (Nakajin et al., 1981; Chung et al., 1987; Picado-Leonard and Miller, 1987; Sparkes et al., 1991; Youngblood et al., 1991; Fan et al., 1992; Givens et al., 1994). The expression of CYP17 is regulated developmentally, hormonally and in a tissue-specific fashion (Voutilainen et al., 1986; Voutilainen and Miller, 1986; Brentano et al., 1990; Lin et al 2001). The human and rat genes for CYP17 share 76% nucleotide sequence identity in their exons (Givens et al., 1994), but only 43% in the first 1560 bp of the 5´-untranslated region. Furthermore, as mentioned earlier, human CYP17 is expressed in the adrenals, but not in the placenta, whereas rodent CYP17 is expressed in the placenta, but not in the adrenals (Johnson and Sen, 1990; Durkee et al., 1992; Yamamoto et al., 1996). Consistent with these differences, the identity of the *trans*-acting factors that regulate CYP17 expression, differ substantially in the bovine, rat and human genes as seen in figure 3.3 (Lin et al., 2001).

ACTH/cAMP-dependent transcription has been found to occur via two cAMP regulatory sequences (CRS1 and CRS2) in the 5´-flanking region of the bovine CYP17 gene. CRS1 lies approximately -243/-225 upstream of the transcriptional initiation site and has been shown to bind the homeodomain proteins Pbx1, Meis1 and Pknox (Bischof et al., 1998). CRS2 lies approximately -80/-40 upstream of the transcriptional initiation site and binds the orphan nuclear receptors chicken ovalbumin promoter-transcription factor (COUP-TF) and steroidogenic factor-1 (SF-1) in a mutually exclusive manner. COUP-TF acts to suppress transcription, while SF-1 stimulates gene expression (Bakke and Lund, 1995).

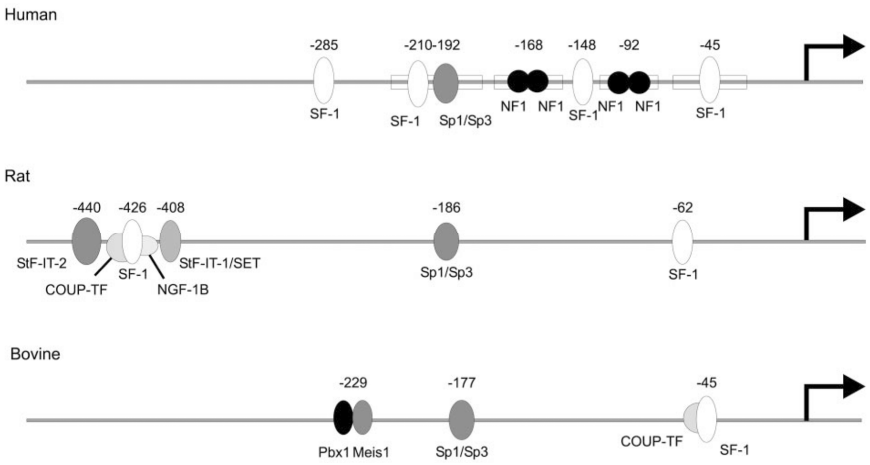

Figure 3.3. Comparison of the reported proximal promoter elements of the CYP17 genes from three species. The numbers indicate the reported positions of each binding site. The promoters of the three species contain an SF-1 site in close proximity to the transcription initiation site as well as conserved SF-1/SF3 sites. The other sites are substantially different for each species as indicated. Reproduced from Lin et al. (2001).

In the rat CYP17 promoter, a proximal element conferring both basal and cAMP-induced activity, is located at position -84/-85 and interacts with SF-1 (Givens et al., 1994; Zhang and Mellon, 1996). A complex machinery of multiple orphan nuclear receptors have been shown to bind a second *cis*-acting element containing three putative recognition sequences for zinc-finger nuclear receptors at bases -447/-399 of the CYP17 promoter. These include SF-1; COUP-TF; nerve growth factor inducible protein B (NGF-IB); and two previously unknown factors, termed steroidogenic factor inducer of transcription-1 and -2 (Zhang and Mellon, 1997). The steroidogenic factor inducer of transcription-1 was recently identified as SET nuclear phosphoprotein (Compagnone et al., 2000).

Studies of human CYP17 gene expression have revealed that cAMP-dependent regulation is dependent on SF-1 binding a -57/-38 CRS2-related

sequence. In the human, however, SF-1 binds as a complex with two other proteins, $p54^{nrb}$/NonO and poly-pyrimidine tract-binding protein-associated splicing factor (PSF). This complex serves as a positive activator via cAMP. In addition, PSF can interact with the corepressor, mSin3A. Basal and cAMP-dependent regulation of human CYP17 are both repressed through mSin3A via histone deacetylase activity (Sewer et al., 2002). Further investigation led to the proposal that intracellular cAMP causes the activation of PKA and the subsequent phosphorylation of a phosphatase, with both serine/threonine and tyrosine activity, possibly a mitogen-activated protein kinase (MAPK) phosphatase (MKP), that is involved in maintaining optimal steroidogenic capacity. Once activated, the phosphatase activates SF-1 by dephosphorylation, thereby alleviating the repression evoked by the binding of the corepressor mSin3A to PSF. The resulting inactivation of the mSin3A/histone deacetylase corepressor system results in increased affinity of the interaction of SF-1, p54nrb/NonO, and PSF with the CYP17 promoter, subsequently increasing transcription as shown in figure 3.4 (Sewer and Waterman, 2002). Alternatively, it has been suggested that the phosphatase (MKP) could be involved in the inactivation of the MAPK that maintains SF-1 in a constitutively phosphorylated state (Hammer et al., 1999; Sewer and Waterman, 2002). A subsequent study confirmed that MKP-1 is essential for the dephosphorylation of SF-1 and suggested that MKP-1 is activated through phosphorylation by PKA. Furthermore, it was found that extracellular signal-regulated kinase (ERK)1/2 plays an essential role in maintaining low constitutive CYP17 expression (Sewer and Waterman, 2003).

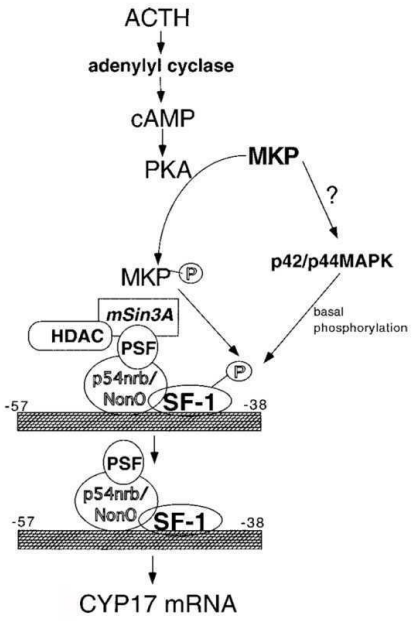

Figure 3.4. Schematic representation of phosphatase activity mediating cAMP-dependent CYP17 transcription. Increased intracellular cAMP activates PKA, which phosphorylates and activates phosphatase(s) with both serine/threonine and tyrosine activities. Activated phosphatase(s) then dephosphorylates $p54^{nrb}$/NonO/SF-1 and promotes increased binding to the -57/-38 region of the hCYP17 promoter. Reproduced from Sewer and Waterman (2002).

It has also been shown that Sp1, Sp3 and nuclear factor-1 (NF-1) are essential for optimal basal transcription of human CYP17. Two NF-1 binding sites have been identified at -107/-85 site and 178/-152. Mutation of both NF-1 sites reduce basal CYP17 transcription by half. In addition, the ubiquitous proteins Sp1 and Sp3 both bind to the -227/-184 region. Mutation of their binding sites reduced transcription by 75%. Mutation of the Sp1/Sp3 site and the two NF-1 sites eliminated almost all detectable transcription (Lin et al., 2001).

Flück and Miller (2004) noted that human placental JEG-3 cells contain Sp1, Sp3 and NF-1, but do not express a transfected CYP17

promotor/luciferase construct (-227/LUC), even when transfected with a vector expressing SF-1. A GATA consensus site was identified in the CYP17 promoter at -64/-58. GATA-4 and GATA-6 were detected in adrenal NCI-H295A cells, but not in JEG-3 cells. Cotransfection of either GATA-4 or GATA-6 without SF-1, which is not present in JEG-3 cells, activated -227/LUC in JEG-3 cells. However, mutation of the GATA binding site in -227/LUC increased GATA-4 or GATA-6 induced activity, whereas mutation of the Sp1/Sp3 site decreased it. This lead Flück and Miller to propose that there is an interaction between Sp1/Sp3 and GATA-4 or GATA-6. Further investigation confirmed the interaction between GATA-4 or GATA-6 and Sp1, but not Sp3. This study furthermore revealed that both the GATA-4 and GATA-6 genes are methylated in JEG-3 cells and therefore not expressed, revealing a possible mechanism for the tissue specific expression of CYP17.

3.3.3 Physiological importance of CYP17

In the adrenal CYP17 is essential for the production of glucocorticoids, which are involved in the regulation of carbohydrate, lipid and protein metabolism (Dallman et al., 2004). CYP17 is also mandatory for the production androgens by the gonads and zona reticularis of the adrenal cortex. In males, androgens induce sexual differentiation before birth; promote sexual maturation during puberty; and maintain male reproductive function in adults (Heinlein and Chang, 2002). In women, androgens serve as precursors for estrogen biosynthesis (Simpson et al., 2000); and also play a significant

role in normal as well as pathologic ovarian development (Ito et al., 1993; Hillier and Tetsuka, 1997; De Leo et al., 1998; Speiser, 2001; Hu et al., 2004).

In humans 17α-hydroxylase/17,20-lyase deficiency, an autosomal recessive disease, causes a rare form of congenital adrenal hyperplasia (CAH) (Patocs et al., 2005). CAH caused by 17α-hydroxylase/17,20-lyase deficiency is characterised by reduced or absent cortisol and adrenal androgen production by the adrenal cortex. The impaired production of cortisol in zona fasciculata leads to hypersecretion of ACTH, which in turn stimulates the overproduction of adrenal 17-deoxycorticosteroids, including the weak glucocorticoid corticosterone and the weak mineralocorticoid 11-deoxycorticosterone. The resulting high plasma levels of 11-deoxycorticosterone typically cause hypertension, hypokalemia, suppressed plasma rennin activity and low plasma aldosterone concentration (Laflamme et al., 1996; Patocs et al., 2005).

In the gonads, 17α-hydroxylase/17,20-lyase deficiency results in the impaired synthesis of DHEA, androstendione, testosterone and estradiol. As a result, genetic males (46,XY) with 17,20-lyase deficiency demonstrate impaired virilisation and develop female external genitalia, pseudohermaphroditism, while genetic females (46,XX) display impaired sexual development characterised by sexual infantilism and primary amenorrhea (Biglieri et al., 1966; Yanase et al., 1991; Kater and Biglieri, 1994; Yanase, 1995; Miller, 1998). Partial deficiencies in CYP17 can cause milder or intermediate phenotypes (Yanase et al., 1991; Miura et al., 1996). In rare instances, only the 17,20-lyase reaction is significantly impaired by mutations, causing isolated 17,20-lyase deficiency, which can result in male

pseudohermaphroditism and lack of progression into puberty in females (Geller et al., 1999; Sherbet et al., 2003).

CYP17 dysfunction has also been associated with various clinical conditions: polycystic ovary syndrome (Qin & Rosenfield 1998, Strauss 2003); CYP17 mutations may be linked to prostate cancer (Lunn et al. 1999, Madigan et al. 2003); endometrial cancer (McKean-Cowdin et al., 2001); and rheumatoid arthritis (Huang et al., 1999).

Blair and Mellon (2004) hypothesised that CYP17 knockout mice would have disordered sex steroid synthesis and disordered brain DHEA production and thus provide phenotypic clues about the functions of DHEA in mouse brain development. However, unexpectedly all mice homozygous for the CYP17 deletion died by embryonic day 7, prior to gastrulation, suggesting that steroid products of CYP17 have essential functions in early embryonic mouse development which are yet to be determined.

3.4. CYP17 Polymorphisms and their functional implications

Since the first description of 17α-hydroxylase/17,20-lyase deficiency by Biglieri et al. (1966), numerous mutations causing complete 17α-hydroxylase/17,20-lyase deficiency, partial forms of this enzymatic deficiency and isolated 17,20-lyase deficiency have been reported (Yanase et al., 1991). These include frame shift mutations (Wei et al., 2006), deletions (Monno et al., 1997; Wei et al., 2006), duplications (Kagimoto et al., 1989) and splice-site mutations (Yamaguchi et al., 1997; Costa-Santos et al., 2004). A number of

the single amino acid changes in CYP17 have provided potentially important insight into key structural domains for enzyme function.

The substitutions H373L (Monno et al., 1993) and P409R (Lam et al., 2001) both abolished CYP17 enzymatic activity as a result of the inability to incorporate the haem prosthetic group correctly, suggesting a critical role for these residues in haem binding. The substitution, R440H, which causes complete enzymatic deficiency is located very close to cysteine 442, which is the fifth ligand of the haem iron and this substitution is therefore thought to interfere with the orientation of this vital haem binding domain (Fardella et al., 1994). The substitution, R440C, at the same position similarly abolished enzymatic activity. A homology model revealed that the arginine at this position forms a hydrogen bond with the propionate group of haem. The mutant is unable to form the hydrogen bond, which renders the mutant enzyme unable to stabilise the correct position of the haem (Patocs et al., 2005).

A S106P substitution abolished both the hydroxylase and lyase activity of CYP17 (Lin et al., 1991). A homology model of CYP17, constructed using the soluble bacterial cytochrome P450, P450cam, as a template (Laughton et al., 1990) revealed that serine 106 lies in close proximity to two putative regions (amino acids 101-102 and 111-116) that form part of the steroid binding pocket (Lin et al., 1991). Lin et al. (1993) demonstrated the serine 106 is crucial for the 17α-hydroxylase and 17,20-lyase activities of human CYP17, but has little effect on 16α-hydroxylase activity. In addition, this group ruled out the possibility that serine 106 participates in the CPR docking site, but rather forms part of the catalytic site. Furthermore, Laflamme et al. (1996)

postulated, on the basis of its proximity to the residues described above, that arginine 96 is also critical in this region as the substitution R96W almost completely abolished the activity of the mutant protein. The importance of this residue was further supported by the observation that this amino acid is highly conserved across species (Laflamme et al., 1996). Brooke et al. (2006) later identified a second substitution, R96Q, at the same amino acid position, which lead to complete inactivity. Homology modeling subsequently substantiated the belief that this residue lies within a substrate-binding region. The nearby substitution F93C results in a mutant CYP17 protein that retains merely 10% of both 17α-hydroxylase and 17,20-lyase activities. The authors suggest that this is a critical domain to the function of CYP17 as the amino acids from 93 to 97 are perfectly conserved in CYP17 of many species (Di Cerbo et al., 2002). Van der Akker et al. (2002) later reported that two substitutions, F114V and D116V, in the putative steroid-binding domain caused complete (F114V), or partial (D116V) 17α-hydroxylase and 17,20-lyase deficiencies.

The substitutions R347H and R358Q, have been shown to selectively ablate more than 95% of 17,20-lyase activity, while retaining approximately 65% of 17α-hydroxylase activity (Geller et al., 1997). Geller et al. (1999) showed that both substitutions selectively interfere with 17,20-lyase activity by altering the interaction of CYP17 with CPR, thus proving that the lyase activity was disrupted by interfering with electron transfer. Furthermore, their data provided evidence that arginine 347 is a crucial component of the site at which cytochrome b_5 interacts with the CYP17·CPR complex. Auchus and Miller (1999) built a homology model of CYP17 based on the soluble bacterial enzyme cytochrome P450BMP. This model confirmed that the two positive

residues R347 and R358 lie on the surface of the protein in the proposed redox-partner binding domain. In addition, the model revealed that the positive residue lysine 89 might contribute to this domain. Subsequent mutation of this residue preferentially disrupted the 17,20-lyase activity, while preserving the majority of the 17α-hydroxylase activity, substantiating its role in the putative redox-binding domain (Auchus and Miller, 1999). Clinical cases of isolated 17,20-lyase resulting from a substitution at this amino acid position have, however, not been reported to date. Sherbet et al. (2003) more recently identified an additional substitution, E305G, which causes 17,20-lyase deficiency. Interestingly, glutamine 305 was shown to lie within the active pocket and primarily alter substrate binding to selectively impair 17-hydroxypregnenolone metabolism, a novel mechanism for 17,20-lyase deficiency.

Although homology modeling has aided the interpretation of data obtained from mutations, it should be noted that all homology models of CYP17 to date, have been based on the known structures of bacterial cytochromes P450 (Laughton et al., 1990; Lin et al., 1994; Burke et al., 1997; Ahmed, 1999; Lewis and Lee-Robichaud, 1999; Schappach and Holtje, 2001; Patocs et al., 2005; Brooke et al., 2006), which share less than 20% identity with the mammalian enzymes (Kemp et al., 2005). The data obtained from these models is therefore speculative. The recent availability of crystal structures of more closely related mammalian cytochromes P450 has contributed towards the increased reliability of homology models for mammalian cytochromes P450 (Kirton et al., 2002; Kemp et al., 2005). The inclusion of mammalian templates in future models of CYP17 will therefore

permit for more accurate predictions to be made about the structure and function of this vital enzyme.

3.5 Differential regulation of hydroxylase and lyase activities of CYP17

3.5.1 NADPH-Cytochrome P450 Reductase (CPR)

All type II cytochromes P450 receive electrons from NADPH via NADPH-Cytochrome P450 Reductase. CPR is an 82-kDa, membrane-associated protein encoded by a single gene of 15 exons spanning 32 kb on chromosome 7q11.2 (Yamano et al., 1989). CPR was first isolated from pig liver, though in a truncated form resulting from the use of proteases during purification (Horecker, 1950). While this form was fully functional and capable of reducing cytochrome *c* (Phillips and Langdon, 1962), the true physiological redox partner was later identified as the microsomal cytochromes P450 (Lu and Coon, 1968; Lu et al., 1969)

CPR contains a molecule of FAD (Horecker, 1950) that accepts a pair of electrons from NADPH. In addition CPR also contains a molecule of FMN, which can accept the electrons from the FAD moiety and donate them one at a time directly to a cytochrome P450 (Miller., 2005). This is a vital feature of CPR, as NADPH is an obligate two-electron donor, but these electrons must be delivered to the cytochromes P450 individually at the appropriate time in the reaction cycle (Paine et al., 2005). Furthermore, the first electron is always transferred more rapidly than the second (Oparian and Coon, 1982). CPR is,

therefore a self-contained electron transfer system that does not need another protein such as the ferredoxin used by type I P450 enzymes.

The crystal structure of a soluble, amino-terminally deleted form of rat CPR revealed that the catalytic region of CPR comprises three distinct domains: an FMN-binding domain; an NADPH/FAD-binding domain; and a linker domain or hinge region as shown in figure 3.5 (Wang et al., 1997). The hinge region plays an important structural role in positioning the FMN- and FAD-binding domains correctly for direct electron transfer. Binding of NADPH and the resulting receipt of electrons by the FAD moiety elicits a conformational change in the hinge, resulting in the alignment of the isoalloxazine rings of the FAD and FMN moieties so that electrons can pass from FAD to FMN. On doing so, the hinge undergoes a second conformational change, which permits the FMN domain to become associated with the redox partner binding site of the cytochrome P450 (Miller, 2005).

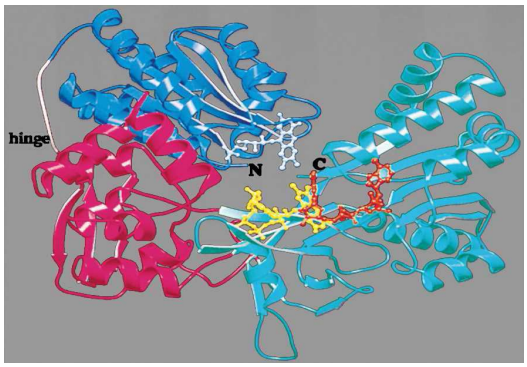

Figure 3.5. Ribbon diagram showing the structure of CPR. The FMN-binding domain is shown in blue, the connecting domain in red, and the FAD and NADP(H)-domains are represented in turquoise. The cofactors are shown as ball and sticks, with the FMN (white), FAD (yellow), and NADPH (orange). Reproduced from Wang et al. (1997).

The surface charge of the FMN domain of CPR is negative as a result of a number of acidic residues (Wang et al., 1997; Shen and Kasper, 1995; Estabrook et al., 1996). These negative residues are believed to form ionic interactions with positive surface residues of cytochromes P450 (Yamazaki et al., 2002). The redox partner binding site of the cytochromes P450 is on the opposite side of the plane of the P450 haem group from the substrate-binding site. The electrons from the FMN moiety of CPR must therefore travel about 18 Å to reach the haem iron (Sevrioukova et al., 1999). The N-terminal hydrophobic membrane-anchoring domain of CPR is essential for the correct spatial orientation of the redox centres for effective electron transfer. The removal of the hydrophobic anchoring peptide of mammalian CPR yields a soluble form, which is incapable of coupling with cytochrome P450 (Paine et al., 2005).

The availability of electrons from CPR is limiting in most microsomal cytochrome P450 reactions. The microsomal cytochrome P450 component is found in a great molar excess to CPR of up to 20:1 in both liver and steroidogenic tissue (Estabrook et al., 1971) and has a profound influence on steroidogenesis. Yanagibashi and Hall (1986) investigated the comparatively low levels of 17,20-lyase activity shown in adrenal microsomes, as compared to testicular microsomes. They found that the addition of purified CPR to either adrenal or testicular microsomes increased the lyase activity relative to that of the hydroxylase reaction. The same effect was seen when CPR was added to the purified enzyme. As the concentration of CPR was increased, the 17,20-lyase activity increased relative to the hydroxylase activity until the rates of the activities became almost equal. Furthermore, the ratio of CPR to

CYP17 was shown to be 3- to 4-fold higher in testicular microsomes than adrenal microsomes. Both enzymatic activities of CYP17 were inhibited when antibodies to CPR were added to testicular microsomes, with the inhibition of lyase being greater than that of hydroxylase.

This key finding was later confirmed for human CYP17 by Lin et al. (1993) who examined the influence of the ratio of CPR to CYP17 on enzymatic activity by cotransfecting nonsteroidogenic COS-1 cells with varying amounts of vectors expressing each protein. While the endogenous CPR of COS-1 cells was sufficient to confer maximal 17α-hydroxylase activity, 17,20-lyase activity was increased 3-fold by the additional CPR produced by the expression vector. The authors concluded that the availability of reducing equivalents is a crucial factor in regulating 17,20-lyase activity.

3.5.2 Cytochrome b_5

Cytochrome b_5 is a ubiquitous electron transfer hemoprotein that catalyses a wide range of reactions in mammalian tissue. Initially cytochrome b_5 was identified in microsomes and thus named cytochrome *m* (Strittmatter and Ball, 1954). Later two different forms of the protein were shown to exist, an amphipathic membrane-bound form and a water-soluble cytosolic form. The membrane-bound form is further divided into two species, one of which is located in the endoplasmic reticulum, while the other is located in the mitochondria. (Strittmatter 1963). In humans the three versions of cytochrome b_5 are encoded by two genes. The first gene is located on chromosome 18q23 and has six exons that undergo alternative splicing. Exons 1, 2, 3 and 4

encode the 98 amino acid soluble form that is primarily found in erythropoietic tissues, while exons 1, 2, 3, 5 and 6 encode the widely expressed 134 amino acid microsomal form (Giordano and Steggles, 1991; Giordano et al., 1993). The second gene is located on chromosome 16q22.1 and consist of five exons that encode the mitochondrial form (OMb_5), which consists of a 146 amino acid. (Kuroda et al., 1998).

3.5.2.1 Structure of cytochrome b_5

The membrane bound forms of cytochrome b_5 consist of two domains. The large globular domain comprises the cytosolic haem-containing, amino-terminal, hydrophylic region, while the smaller tail domain comprises the hydrophobic, membrane-binding, carboxyl portion of 14-18 amino acid residues. The hydrophobic tail is connected to the haem-containing catalytic domain by a proline containing hinge region of ~7 amino acid residues and is followed by 7 polar amino acids at the carboxyl terminal (Mathews, 1985). Proteases can cleave before or after the hinge region to release the soluble, haem-containing catalytic domain. This domain has a cleft between four α-helices into which the haem is inserted, with the haem edge exposed to the environment. The haem iron is coordinated with the side chains of two conserved histidine residues at positions 44 and 68. The interaction between the fifth and sixth coordinates of the haem iron with the histidine residues prevents its direct interaction with molecular oxygen. The different mammalian species of cytochrome b_5 show over 80% sequence identity. Twenty-three of the 134 amino acids are either glutamate or aspartate and are conserved in

all mammals. Three acidic residues, glutamate 49, glutamate 53 and aspartate 65, and one of the haem propionate residues protrude into a common plane in the solvent and have been implicated in charge-paring interactions between cytochrome b_5 and other electron transfer proteins (Scenkman and Jansson, 2003).

Studies have shown that proteolytically solubilised cytochrome b_5 is unable to affect the activities of microsomal cytochromes P450 (Imai and Sato, 1977; Sugiyama et al., 1980; Chiang, 1981). The remainder of this discussion will therefore focus on the 134 amino acid microsomal form as it is the only form known to play a significant role in the regulation of CYP17 activity, which is a microsomal enzyme. Furthermore, microsomal cytochrome b_5 is the most abundant form of the enzyme expressed in the adrenal (Pandey and Miller, 2005).

3.5.2.2 Stimulation of the 17,20-lyase reaction

Microsomal cytochrome b_5 is a multifunctional protein participating in a variety of electron-transfer reactions, including the desaturation of fatty acids (Oshino et al., 1971) and cholesterol biosynthesis (Fukushima et al., 1981). In addition, this form of cytochrome b_5 has also been shown to augment the activities of numerous cytochromes P450 (Schenkman and Jansson, 2003).

Early studies revealed a strong positive correlation between DHEA production and the co-localisation of CYP17 and cytochrome b_5 in the endoplasmic reticulum of the gonads and the zona reticularis of the adrenal cortex, implicating cytochrome b_5 as a possible modulator of the 17,20-lyase

activity of CYP17 (Lu et al., 1974, 1975). Subsequently, using purified enzymes, Katagiri et al. (1982) demonstrated that cytochrome b_5 enhanced the lyase reaction of CYP17 and that this stimulation was dependent on the concentration of cytochrome b_5 present. Onoda and Hall (1982) later confirmed this finding by demonstrating that purified cytochrome b_5 stimulated the lyase reaction of CYP17 purified from neonatal pig testicular microsomes. Furthermore, the hydroxylase and lyase activities of purified CYP17 were suppressed in the presence of cytochrome b_5 antibodies (Ishii-Ohba et al., 1984; Kominami et al., 1992). Further evidence for the involvement of cytochrome b_5 was obtained from studies of patients with Cushing's syndrome. High expression of cytochrome b_5 in adrenocortical adenomas from patients with Cushing's syndrome has been associated with a significant increase in the secretion of adrenal androgens (Sakai et al., 1994; Yanase et al., 1998).

Katagiri et al. (1995) later substantiated the involvement of cytochrome b_5 in the selective stimulation of the lyase activity of CYP17 by using a number of reconstituted assays. The presence of cytochrome b_5 enhanced the conversion of 17-hydroxypregnenolone to DHEA 13-fold, while the conversion of 17-hydroxyprogesterone to androstendione was increased 10-fold. Although no significant change was observed for the hydroxylation of progesterone in the presence of cytochrome b_5, the hydroxylation of pregnenolone was, however, 2-fold greater when cytochrome b_5 was present.

3.5.2.3 Mechanism of action

It was initially suggested that cytochrome b_5 may act as an alternative donor for the second electron in the cytochrome P450 catalytic cycle (Correia and Mannering, 1973; Hildebrandt and Estabrook, 1971). The faster rate of the second electron for the completion of the catalytic cycle was thought to reduce the likelihood of the spontaneous decay of the oxyhaemoprotein complex during the P450 oxidoreduction cycle (Hildebrandt and Estabrook, 1971). Subsequent studies have revealed that although cytochrome b_5 acts as an electron transfer component in a number of systems (such as lipid metabolism) among the cytochromes P450 it has a number of different effects depending on the enzyme involved. These effects include: the transfer of a rate-limiting electron; complex formation with cytochrome P450 to permit two-electron transfer during a single interaction with reductase; and direct effector actions without the transfer of electrons (Schenkman and Jansson, 2003).

The enhancement of the lyase activity of CYP17 by cytochrome b_5 was investigated by Auchus et al. (1998), using a yeast expression system. The abundance of putative electron transfer proteins were varied in yeast microsomes containing human CYP17. Maximum stimulation of lyase activity was observed when the cytochrome b_5:CYP17 ratio ranged from 10:1 to 30:1, while maintaining CPR at a fixed concentration. The stimulated lyase activity, however, decreased rapidly in the presence of excess cytochrome b_5. This suggested that at high ratios, cytochrome b_5 scavenged electrons from CPR, reducing the potential of the first electron derived from CPR to reduce CYP17. This was confirmed by using an alternative CPR electron acceptor,

cytochrome *c*, which at an equimolar amount to cytochrome b_5 gave a similar pattern of lyase inhibition. The redox role of cytochrome b_5 was investigated using a recombinant cytochrome b_5 that was devoid of haem (apo-cytochrome b_5). Interestingly, the apo-cytochrome b_5 yielded a similar stimulatory profile to that of the holo-cytochrome b_5. In addition, the lyase activity of CYP17 was not inhibited when the apo-cytochrome b_5:CYP17 ratio exceeded the optimum expressed by the holo-cytochrome b_5. Similar results were obtained by Lee-Robichaud et al. (1998), using Mn^{2+}-substituted cytochrome b_5. Using modified forms of cytochrome b_5 the stimulatory effect of cytochrome b_5 on the lyase activity of CYP17 was demonstrated to be mediated by cytochrome b_5 exerting an allosteric effect on CYP17 and not by electron transfer from cytochrome b_5. Auchus et al. (1998) hypothesised that cytochrome b_5 alters the CYP17·CPR complex allosterically, possibly optimising its geometry for the more sensitive 17,20-lyase reaction as shown in figure 3.6.

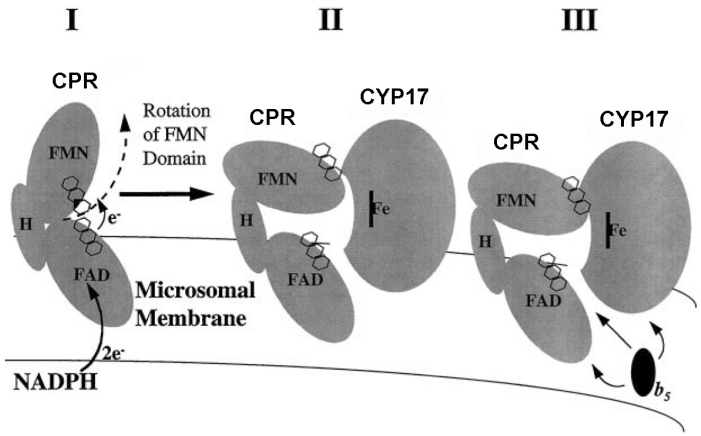

Figure 3.6. Proposed function of cytochrome b_5. I, NADPH donates two electrons to the FAD domain of CPR, which pass to the FMN moiety. II, the FMN domain of CPR, which is connected to the FAD domain by a connecting domain and a hinge region (*H*) must rotate about 90° (counterclockwise in the figure) to dock with the redox-partner binding site of CYP17. The interaction of CYP17 and CPR is adequate to support 17α-hydroxyation, but this complex rarely adopts the geometry required to catalyse the 17,20-lyase reaction. III, the presence cytochrome b_5 favors the interaction of CPR and CYP17 in an orientation that satisfies the more stringent conformational restrictions required by the 17,20-lyase reaction, facilitating productive electron transfer from CPR to CYP17 and subsequent catalysis. Reproduced from Auchus et al. (1998).

Lee-Robichaud et al. (1995) had proposed that the interaction of cytochrome b_5 with the CYP17·17-hydroxypregnenolone complex causes a conformational change which results in the optimal positioning of the peroxide iron with respect to C-20 of the substrate, with the ensuing nucleophilic attack at the carbonyl group producing a tetrahedral intermediate that follows a side-chain cleavage path.

Allosteric effects of cytochrome b_5 have subsequently been demonstrated in a number of catalysis by other cytochromes P450, including CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP3A4 and CYP3A5 (Yamazaki et al., 2002).

3.5.2.4 Role of the membrane-anchoring domain

NMR studies have shown that the membrane-anchoring domain of cytochrome b_5 may be responsible for the stimulation of 17,20-lyase activity, as this domain is structurally indistinguishable in the apo- and holo-forms of the enzyme. In contrast, the globular haem-binding domain of the apo-form is significantly more structurally disordered in comparison to the holo-form (Muskett et al., 1996; Falzone et al., 1996). Furthermore, the membrane-anchoring domain retains its overall topology during molecular dynamics simulations of apo-cytochrome b_5, while the haem-binding domain loses secondary structure and exhibits conformational mobility (Storch and Daggett, 1996). Lee-Robichaud et al. (1997) investigated the importance of the membrane-anchoring domain of cytochrome b_5 in the modulation of CYP17 lyase activity using a number of genetically engineered forms of cytochrome b_5. These included the native pig cytochrome b_5 (native pig), its genetically engineered rat counterpart (core-tail), the soluble core form of the latter (core), the core with the secretory signal sequence of alkaline phosphatase appended to its N-terminal (signal-core) and the latter containing the C-terminal tail of the native rat protein (signal-core-tail). The engineered cytochrome b_5 isoforms, apart from the core form, were constructed in such a way that each form, when anchored in the membrane, would impose a distinct orientation of the haem-containing domain for its interaction with CYP17. The results revealed that the native pig and core-tail enzymes were equally efficient at enhancing the 17,20-lyase activity of human CYP17, while the signal-core-tail enzyme was 55% as efficient. The core and signal-core

constructs did not have any effect on the CYP17 lyase reaction (Figure 3.7). The authors concluded that a productive interaction between cytochrome b_5 and CYP17 is governed not only by the presence of a membrane-insertable hydrophobic segment but also by a defined spatial orientation of the exposed haem-containing domain.

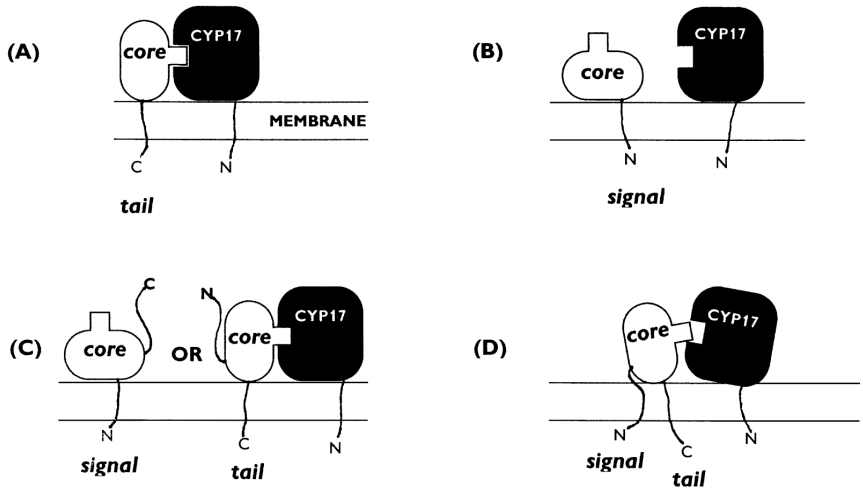

Figure 3.7. Proposed model showing the interaction with the various cytochrome b_5 derivatives with CYP17 (A) The core domain of the wild-type cytochrome b_5 is shown to be anchored to the membrane via its C-terminal hydrophobic tail for optimal interaction with CYP17. (B) The core domain of the signal-core derivative is anchored to the membrane via its N-terminal signal and is incorrectly orientated for productive interaction with CYP17. (C) Only half of the signal-core-tail molecules are anchored to the membrane via their C-terminal tails and have their core domain spatially orientated for optimal interaction with CYP17. (D) The core domain is anchored by both its N-terminal signal and C-terminal tail which restricts movement of the core with respect to the plane of the membrane and leads to a weaker interaction with CYP17. Reproduced from Lee-Robichaud et al. (1997).

3.5.2.5 Nature of interaction between cytochrome b_5 and CYP17

The interaction of the cytochromes P450 to their redox partners such as CPR and cytochrome b_5, which contain clusters of surface negative charges,

is generally believed to proceed via ionic interactions with the positive surface residues of cytochromes P450 (Yamazaki et al., 2002). Male patients with isolated lyase deficiency have been identified with homozygous mutations for R347H and R358Q (Geller et al., 1997). These mutants were expressed in nonsteroidogenic COS-1 cells in the presence of cytochrome b_5 and yielded approximately 65% of hydroxylase and 5% lyase activity (for pregnenolone metabolism) relative to cells transfected with the wild type enzyme. A subsequent study by Lee-Robichaud et al. (1998) revealed that mutation of lysine 83, arginine 347 and arginine 358 produced proteins that were deficient in their responsiveness to cytochrome b_5 with the effect being the most pronounced for the two arginine residues. The same group later showed that an additional mutation, R449A, also leads to selective lyase deficiency (Lee-Robichaud et al., 1999). Interestingly, replacing these residues at positions 347, 358 and 449 with lysine residues could restore the stimulatory effect of cytochrome b_5 on the CYP17 lyase reaction, indicating the importance of positively charged residues at these positions.

Bridges et al. (1998) used site directed mutagenesis to determine the redox partner binding sites of CYP2B4 and showed that out of the 24 mutations used in the study, only mutations of those residues located on the proximal surface of CYP2B4 resulted in decreased binding to cytochrome b_5 as well as CPR. The authors therefore concluded cytochrome b_5 and CPR bind to the proximal surface of CYP2B4 and that the binding areas for both redox partners partially overlap. The cationic arginine residues at positions 347 and 358 of CYP17 are, therefore, thought to be positioned similarly on the

proximal surface of the molecule where they electrostatically dock with CPR and/or cytochrome b_5 (Auchus and Miller, 1999).

3.5.2.6 Aggregation of cytochrome b_5

The aggregation state of ovine cytochrome b_5, isolated from ovine liver, was investigated in solution, in adrenal microsomes and *in situ* by Lombard et al. (2002). SDS-Polyacrylamide gel electrophoresis indicated the presence of a prominent tetrameric form of the protein, even after stringent detergent and mercaptoethanol pretreatment with only a minor band corresponding to the monomer. The tryptic removal of the membrane binding region abolished aggregation. Anti-cytochrome b_5 IgG raised against the truncated protein recognised only the truncated form and not the full length protein or the tetrameric complex. Subsequent immunoblot analyses of sheep adrenal microsomes with the same antibody were also negative. Furthermore, immunoblot analyses and immunocytochemistry of adrenal tissue with antibodies raised against full length cytochrome b_5 indicated that the tetrameric form of the protein was the dominant specie *in vivo*. More recently Lombard et al. (unpublished data) showed that while the tetrameric form is the dominant specie in the adrenal, the monomeric form is the dominant specie in the gonads where more C19 steroids are synthesised. The aggregation of cytochrome b_5 may therefore play a role in regulating its interaction with CYP17 and therefore warrants further investigation.

3.5.3 Serine/threonine phosphorylation of CYP17

Zhang et al. (1995) have shown that human CYP17 serine and threonine residues are phosphorylated by a cAMP-dependent protein kinase. Furthermore, phosphorylation of CYP17 was found to increases the 17,20-lyase activity, while dephosphorylation virtually eliminated this activity. Pandey et al. (2003) later demonstrated that treating human adrenal NCI-H295A cells with the phosphatase inhibitors: okadaic acid, fostriecin and cantharidin increased 17,20-lyase activity, suggesting the involvement of protein phosphatase 2A (PP2A) or 4 (PP4). Subsequent investigations revealed that PP2A, but not PP4, inhibited 17,20-lyase activity in microsomes prepared from cultured cells, but neither affected 17α-hydroxylation. The inhibition of 17,20-lyase activity by PP2A was found to be concentration-dependent and could be inhibited by okadaic acid. The 17,20-lyase activity could, however, be restored by endogenous protein kinases. In addition, suppression of PP2A by small interfering RNA increased 17,20-lyase activity. It was proposed that the modified negatively charged phosphorylated residues served to enhance electrostatic associations of CYP17 and its redox partners so as to engage a stronger interaction of CPR with CYP17. Biason-Lauber et al. (2000a) investigated the phosphorylation state of wild-type and mutant CYP17 proteins, deficient in 17,20-lyase activity. No phosphorylation was detected for the F417C mutant, which was shown to be associated with the lack of electron transfer. This provided evidence for the link between the electron transfer system and the phosphorylation state of the CYP17 enzyme in the control of 17,20-lyase activity.

Pandey and Miller (2005) subsequently investigated if the enhancement of the 17,20-lyase activity of CYP17 by serine phosphorylation and cytochrome b_5 were dependent on one another or acted by independent mechanisms. Okadaic acid treatment of NCI-H295A cells expressing decreased amounts of cytochrome b_5, resulting from the expression of a small interfering RNA against cytochrome b_5, increased the 17,20-lyase activity of the cells. The increase in 17,20-lyase activity in control cells was similar to that in cells with reduced cytochrome b_5 expression. The overexpression of cytochrome b_5 in NCI-H295A cells resulted in a moderate increases in 17,20-lyase activity. Okadaic acid treatment of NCI-H295A cells, overexpressing cytochrome b_5, further increased the 17,20-lyase activity, however, the increase was similar to that of the control cells treated with okadaic acid. Using microsomes isolated from NCI-H295A cells, the effect of cytochrome b_5 and of *in vitro* protein phosphorylation on 17α-hydroxylase and 17,20-lyase activities were determined. Adding purified cytochrome b_5 to the prepared microsomes increased the 17,20-lyase activity 2-3-fold, but had no effect on 17α-hydroxylase activity. Similarly, *in vitro* phosphorylation of microsomal CYP17 increased 17,20-lyase activity 3.5-fold, but had no effect on 17α-hydroxylase activity. The addition of cytochrome b_5 to microsomes that had been phosphorylated *in vitro* increased 17,20-lyase activity 5-fold, with no effect on 17α-hydroxylase activity. It was therefore concluded that protein phosphorylation and cytochrome b_5 can enhance 17,20-lyase activity independently of each other and that their effects are neither additive nor cooperative.

Further investigations using human CYP17 purified from a bacterial expression system, demonstrated 17α-hydroxylase activity, but very little 17,20-lyase activity in the absence of cytochrome b_5 or serine phosphorylation (Pandey and Miller, 2005). The addition of purified cytochrome b_5 at a ratio of 3:1 (cytochrome b_5 to CYP17) yielded a 3-4-fold increase in 17,20-lyase activity. Upon phosphorylation of CYP17, the 17,20-lyase activity increased 4-5-fold in the absence of cytochrome b_5. The addition of cytochrome b_5 to phosphorylated CYP17, at the same ratio described above, further increased the lyase activity. These data confirmed that the effects of cytochrome b_5 and phosphorylation are neither additive nor cooperative.

In addition, neither phosphorylation nor the addition of cytochrome b_5 affected the kinetic parameters of the 17α-hydroxylase reaction. The catalytic efficiency (V_{max}/K_m) of the 17,20-lyase reaction was increased 4-fold upon the addition of cytochrome b_5, while CYP17 phosphorylation increased the catalytic efficiency 6-fold. However, addition of cytochrome b_5 to phosphorylated CYP17 increased the 17,20-lyase activity only 7-fold, demonstrating that this effect was not significantly different from the action of phosphorylation alone. The authors concluded that the effects of cytochrome b_5 and P450c17 phosphorylation are not additive and that most of the effect on the lyase activity is due to an increase in reaction velocity, as changes in K_m were less than 2-fold (Pandey and Miller, 2005).

The specific signaling pathway responsible for the phosphorylation of CYP17 is still unknown, as are the relevant kinases. Biason-Lauber et al. (2000b) showed that low doses of leptin caused a significant increase in 17,20-lyase activity in adrenal NCI-H295R cells expressing the leptin receptor

and that this influence is specific and dependent on the integrity of the signal transduction pathway. Tee et al. (2008) searched for possible kinases involved in the signaling pathway by testing a series of kinase inhibitors. Two inhibitors that target the Rho-associated, coiled-coil containing protein kinase (ROCK)/Rho pathway were found to suppress both the 17,20-lyase activity and phosphorylation of CYP17 in NCI-H295A cells and in COS-1 cells transfected with a CYP17 expression vector. Although ROCK1 was able to phosphorylate CYP17 *in vitro*, phosphorylation did not, however, affect 17,20-lyase activity. The authors concluded that members of the ROCK/Rho pathway act upstream from the kinase that phosphorylates CYP17 in a fashion that augments 17,20-lyase activity, possibly acting to catalyse a priming phosphorylation.

3.6 Conclusion

From the above discussion it is clear that the dual activity of CYP17 plays a vital role in determining the steroidogenic output of the adrenal cortex. Changes in either activity can therefore have significant physiological effects. As mentioned in the previous chapter, studies have demonstrated that the South African Angora goats' susceptibility to cold stress is as a result of its inability to produce sufficient levels of cortisol (Van Rensburg, 1971; Herselman and van Loggerenberg, 1995; Engelbrecht et al., 2000). The following chapter will therefore give an overview of the research that has been conducted on Angora goats which led to the identification of hypocortisolism

in these goats and the subsequent implication of CYP17 as the possible cause of this condition.

CHAPTER 4

THE SOUTH AFRICAN ANGORA GOAT: A BRIEF HISTORY AND A SUMMARY OF RESEARCH INTO ITS VULNERABILITY TO PHYSIOLOGICAL STRESS

4.1 The origin of the Angora goat

It is commonly accepted that the goat, together with the sheep and the dog, was one of the first animals to be domesticated by man. The domestication of goats is believed to have played an important role in the development of man, as they are able to produce meat, milk, fibre and skins. The white fleeced goat — now recognised as the Angora — owes its name to the capital city of Turkey, Ankara, which was once known as Angora. Ankara is situated on the Anatalian Plains of Asia Minor, which lies between the Black and Mediterranean seas.

The Angoras fleece became known as mohair from the Arabic word "*mukhayyar*", which means "cloth of bright hair from a goat". Although the mohair industry owes its beginning to this region of Turkey, the exact origin of the Angora goat is unclear. Some believe that all domesticated goats, *Capra hircus*, are descended from the wild goat *Capra aegagrus*. However, there is some evidence to suggest that the Angora goat is, in fact, descended from the Falconer's goat, *Capra falconeri*, which was first identified in central Asia (Hayes, 1882).

It is unclear who was responsible for the introduction of the Angora into Asia Minor. Through the ages, Asia Minor has been home to a number of

important civilisations, including the Hittites (2000-1200 BC), Greeks, Persians and Romans, amongst others. During the 14th and 15th centuries the Ottoman Turks, originally from central Asia, conquered Asia Minor, which remained part of the Ottoman Empire until the establishment of the republic of Turkey in 1923. It is believed that these nomadic Turks may have been responsible for the introduction of the Angora into Asia Minor (Hayes, 1882). This time frame is well supported by the observation that the first reference to Angora goats was made by Pierre Belon, a French Naturalist, who made a brief, but characteristic description of the Angora goat in his book "*L'Histoire de la nature des oyseaux*" published in 1555.

Although the exact origins of the Angora remain unclear, there is no doubt that it was not until these goats were introduced into the Anatalian Plains surrounding Ankara that these goats started to flourish with the resulting birth of the mohair industry. After his travels through Asia Minor, the French botanist, Joseph Pitton de Tournefort (1656-1708) wrote the following about the Angora goat in his book "*Relation d'un voyage du Levant*": "*They breed the finest goats in the world in the champaign of Angora. They are of a dazzling white and their hair, which is fine as silk, naturally cured in locks of eight or nine inches long, is worked up into the finest stuffs, especially camlet. Bet they do not suffer these fleeces to be exported from this place, because the people of the country gain their livelihood thereby… However it be, these fine goats are to be seen only within four or five day's journey of Angora and Beibazar. Their young degenerate if they are carried further…All this country is dry and bare except the orchards. The goats eat nothing except the young shoots of herbs, and perhaps it is this which, as Brusbequia*

observes, contributes to the consummation of the beauty of their fleece, which is lost when they change their climate and pasture."

The region around Ankara is situated on the high Anatolian Plateau at an altitude of 850 meters above sea level. The climate is hot and dry in summer and cold and snowing in winter. The rainy season is in spring, especially in May.

The first Angoras believed to be exported from Turkey, were by Charles V, who imported a pair of Angoras into Europe in 1554. Subsequently Angoras were exported to a number of countries, including Sweden (1740), Spain (1765), Venice (1778) and France (1785), in an attempt to establish a mohair industry in these areas. None of these importations were particularly successful, leading to the common belief that Angoras could not be successfully maintained outside of the cool and dry plateaus of Turkey (Hayes, 1882). In 1838, Queen Victoria of England put pressure on the Sultan of Turkey to lift the ban that had previously been placed on the export of raw mohair in order to protect the local industry. England's industrial revolution resulted in a demand for raw mohair that could not be met by Turkey.

4.2. Importations to other countries

4.2.1 Importations into the United States of America

It was not until the middle of the 19th century that mohair industries were successfully started outside of Turkey. In 1849, the Sultan of Turkey rewarded Dr. James B. Davis of Columbia, South Carolina with nine head of Angoras

for his work as a cotton and agricultural consultant in Turkey. The Angoras were mistaken for Cashmere goats by Dr Davis, until he sold his herd in 1853 to Colonel Richard Peters of Atlanta, Georgia, who correctly identified the animals as Angoras. Further importations of Angoras to the United States were made in the subsequent years.

A significant contribution towards the establishment of the mohair industry in the United States was made by William Landrum. He bought Angoras from Colonel Peters, amongst others, while living in California from 1850 to 1883. In 1883 he successfully introduced Angoras into Texas. By 1900 it was estimated that of the 900 000 Angoras in the United States, about 250 000 were found in Texas. Approximately 95% of mohair production in the United States during the period of 1940 to 1980 occurred in the Edwards Plateau in Texas. Angoras have subsequently been spread throughout the United States, though the majority of mohair is still produced in Texas (Shelton, 1993). Today, together with Turkey and South Africa, the United States is one of the three largest producers of mohair in the world.

4.2.2 Importations into the Cape Colony

The first importation of Angoras into the Cape colony is reported to have occurred in 1838 by Colonel John Henderson, a former British officer. This shipment included 12 rams and 1 ewe. However, all the rams on this shipment had been sterilised as a result of the Turkish exporter's intent to safeguard their industry. Fortunately, the ewe was pregnant and gave birth to a ram kid during the sea voyage to South Africa via India. The ram kid was

used to establish the Angora in South Africa. Additional consignments of goats were imported during the period of 1857 to 1868, but in 1880 the Sultan of Turkey prohibited further export of Angoras worldwide (Kettlewell, 1983; Terblanche, 1987). The first shipload of mohair from the Cape colony was exported for R20 in 1857 (Potgieter, 1992). By 1868 there were approximately 500 000 Angoras in the Cape colony (Grobler, 1975). In 1881 a total of 8 498 bales of mohair, weighing approximately 1.7 million kg, were exported to England (Hayes, 1882). By 1912 the goat population had grown to approximately 4 million goats and more than 10.5 million kg of mohair was exported (Grobler, 1975).

Figure 4.1. South African Angora goat with its kid.

The success of the mohair industry in South Africa was attributed to two factors: firstly, the similarity of the climate in the Karoo region of the Eastern Cape, where the goats were farmed, to the Anatalian Plains of Turkey; and secondly, crossbreeding of purebred Angoras with the native goats of the

region. These points are highlighted in the following letter written by Sir Samuel Wilson in 1882, who quotes directly from a "*gentleman who is acquainted with the Cape*": "*The country at the Cape, where Mr. J.B. Evans, the manager of the Cape Stock Farming Company, breeds his Angora goats, is called 'Zwart Ruggens,' and is situated in the division of Graaff Reinett. In this and the neighboring divisions of Cradock and Somerset East, comprising that portion of the Cape Colony lying between the 24° and 27° of East Longitude, and 30° and 33° of South Latitude, the best mohair is produced… The success of Mr. Evans and other breeders in the districts named above, is perhaps mainly due, first, to the pure breed imported from Angora; and second, to the wonderful similarity existing between the nature and climate of this district and Angora itself. There are those who even prefer the climate of the former. This district is not subject to any very sudden changes. It has its regular rain season. It is a very dry climate; in fact, were it not so, the goats would not thrive, for it has been found next to impossible to breed them on the sea-bound districts of the Colony, as the damp kills them. The nature of the country is of a mixed description, both mountainous and level. In the mountainous parts the goats thrive best. In the plains they exist on what is known as 'Karoo' country. This country is covered largely with the small mimosa tree, growing about six feet high…The advantage of breeding Angoras in the Colony is that they scarcely interfere with the pasture of the sheep flocks, and will often live on herbage which the later discards."*

In his letter Sir Samuel Wilson goes on to write that: "*The success is so great that millions of acres, but ten years ago of no value, now give sustenance to flocks of goats, the quality of whose mohair is now quoted in*

the English and our own markets as equal to the best from the Angora district of Asia Minor. And this result has been attained by the crossing of the common goats of the country with the imported Angoras, carefully selected in Asia by Mr. Evans himself; the sires used always being, however, the imported thoroughbreds, or their pure progeny.“

The use of the native goat in breeding practices is confirmed in a letter written by Amos Crabtree in 1882: “*There is no doubt this great increase in the number of goats has been obtained by crossing the pure-bred Angora males with the common native goat of the country. This is the practice still, and I learn that most of the farmers breed a number of pure Angoras entirely for stud purposes. They have now been so successful, that now they say they could do without any fresh importation from Turkey. The common goat is very numerous in the country; at every kraal, I am told, a good number to be found, and they are mostly white; so that, besides the suitable climate, the farmers appear to have had great facilities for raising their present large flocks*.”

The native goats referred to above is most likely a reference to the Boer goat, which owes its name to the Dutch word “boer” meaning farm. This name is believed to be employed to distinguish the farm goat from the Angora goat (Teh and Gipson, 1993). The Boer goat originated in the Eastern Cape region, the same region in which the Angoras were thriving. As early as 1800, farmers used the indigenous goats, which they acquired from the Namaqua Hottentots and of the southward migrating Bantu tribes, to select for desirable characteristics. By the beginning of the 20th century a distinct breed had emerged (Casey and Van Niekerk, 1988). The Boer goat, albeit in the early stage of its development, fits the description of the native goat referred to

above as it was widespread in the area and is predominantly white, having just a light red head.

These breeding practices, coupled with careful selection for fine mohair, lead to the continual growth of the mohair industry in South Africa. By the late 1970's, South Africa became the undisputed leader in mohair production in the world, a status that it has kept until today. By 1982, South Africa was producing more than 40% of the total world production. This increased to over 50% by 1996. Currently, South Africa produces approximately 3.5 million kg of mohair annually, of which more than 95% is exported primarily to Europe and Asia. This constitutes approximately 55% of the global mohair production. The mohair production values of the three largest mohair producing countries, namely South Africa, Turkey and the United States, are shown in table 4.1.

Table 4.1. Mohair production data of the three largest mohair producing countries since 1972. Data provided by Mohair South Africa.

Year	Mohair Production (million kg)					Percentage total global production			
	SA	Turkey	USA	Other	Total	%SA	%Turkey	%USA	%Other
1972	**3.70**	4.10	4.60	1.80	14.20	**26.06**	28.87	32.39	12.68
1973	**3.40**	4.10	4.50	1.60	13.60	**25.00**	30.15	33.09	11.76
1974	**3.70**	4.10	3.80	1.60	13.20	**28.03**	31.06	28.79	12.12
1975	**3.80**	3.90	3.90	1.60	13.20	**28.79**	29.55	29.55	12.12
1976	**4.10**	4.00	3.60	1.60	13.30	**30.83**	30.08	27.07	12.03
1977	**4.60**	4.10	3.60	1.40	13.70	**33.58**	29.93	26.28	10.22
1978	**4.90**	4.50	3.70	1.50	14.60	**33.56**	30.82	25.34	10.27
1979	**5.40**	4.50	4.20	1.50	15.60	**34.62**	28.85	26.92	9.62
1980	**6.10**	4.50	4.00	1.60	16.20	**37.65**	27.78	24.69	9.88
1981	**6.90**	4.50	4.50	1.60	17.50	**39.43**	25.71	25.71	9.14
1982	**7.60**	4.50	4.50	1.60	18.20	**41.76**	24.73	24.73	8.79
1983	**7.20**	3.80	4.80	1.80	17.60	**40.91**	21.59	27.27	10.23
1984	**8.10**	3.50	5.00	1.70	18.30	**44.26**	19.13	27.32	9.29
1985	**9.20**	3.50	6.00	1.80	20.50	**44.88**	17.07	29.27	8.78
1986	**11.00**	3.00	7.20	1.80	23.00	**47.83**	13.04	31.30	7.83
1987	**11.50**	3.00	7.30	2.80	24.60	**46.75**	12.20	29.67	11.38
1988	**12.20**	2.90	7.80	3.10	26.00	**46.92**	11.15	30.00	11.92
1989	**11.70**	2.00	7.80	3.40	24.90	**46.99**	8.03	31.33	13.65
1990	**10.70**	1.80	7.30	2.00	21.80	**49.08**	8.26	33.49	9.17
1991	**7.60**	1.20	7.40	2.20	18.40	**41.30**	6.52	40.22	11.96
1992	**6.70**	1.20	7.10	1.80	16.80	**39.88**	7.14	42.26	10.71
1993	**6.00**	0.80	6.50	1.70	15.00	**40.00**	5.33	43.33	11.33
1994	**5.70**	0.80	5.40	1.40	13.30	**42.86**	6.02	40.60	10.53
1995	**5.40**	0.60	4.80	1.60	12.40	**43.55**	4.84	38.71	12.90
1996	**5.60**	0.40	3.50	1.50	11.00	**50.91**	3.64	31.82	13.64
1997	**5.20**	0.40	2.50	1.30	9.40	**55.32**	4.26	26.60	13.83
1998	**5.00**	0.40	1.50	1.30	8.20	**60.98**	4.88	18.29	15.85
1999	**4.50**	0.40	1.20	1.10	7.20	**62.50**	5.56	16.67	15.28
2000	**4.30**	0.40	1.00	1.20	6.90	**62.32**	5.80	14.49	17.39
2001	**4.20**	0.30	0.80	1.50	6.80	**61.76**	4.41	11.76	22.06
2002	**4.20**	0.30	0.75	1.35	6.60	**63.64**	4.55	11.36	20.45
2003	**3.95**	0.25	0.90	1.45	6.55	**60.31**	3.82	13.74	22.14
2004	**3.70**	0.20	0.85	1.30	6.05	**61.16**	3.31	14.05	21.49
2005	**3.60**	0.30	0.80	1.50	6.20	**58.06**	4.84	12.90	24.19
2006	**3.35**	0.30	0.80	1.60	6.05	**55.37**	4.96	13.22	26.45

From this data it is clear that mohair production in South Africa has decreased from 12.2 million kg in 1988 to approximately 3.5 million kg at present. Similarly the number of goats in South Africa has decreased from 3 million in 1989 to 0.8 million today. There are a number of reasons attributed to this decline. These include an increase in game farming in the regions that Angoras are traditionally farmed; unstable mohair prices; increased stock theft; the production of finer high quality mohair as opposed to high quantities and stock loss due to cold stress (Grobler, 2007). The remainder of this chapter will focus on the vulnerability of the Angora goat to stress.

4.3. Vulnerability of the Angora goat to stress

The high quality of fleece produced by the Angora goat is due to careful selection. Unfortunately, according to Vogt and Specht (1889), extensive in-breeding was used to accomplish this. During the first half of the 20th century, the global demand for mohair, as dictated by fashion, was low. Consequently, in South Africa only the animals with the best production characteristics were retained (van Heerden, 1963). In a study of the genetic background of the South African Angora goat, Delport (1982) noted that the South African mohair industry was established with the import of less than 1000 animals. As previously mentioned, there is evidence that early imports of Angora goats were crossed with native goats, pure bread Angoras were, however, maintained for stud purposes (Hayes, 1882). By 1912, the number of Angora goats in South Africa had increased to 4.4 million. Due to economic reasons, this number decreased substantially to 580 000 by 1949, with only the goats

that produced the best quality and quantity of hair being maintained. Little to no emphasis was placed on fitness during the selection process (Delport, 1982).

During the second half of the 20th century there was a sharp increase in the demand and price of mohair. It therefore became profitable for farmers to increase the size of their flocks in order to capitalise on the increased demand. Even though more animals are reported to have been imported during the period of 1912 – 1961, a lot of inbreeding occurred in order to increase the size of the flocks. It is documented that the offspring of a ram that was imported during 1927, namely “White” was used in most of the studs in the country (Delport 1982).

During this period of increased mohair demand, the farmers were, however, handicapped by the emergence of the increased prevalence of abortions, which reached incidences of more than 50%. In addition, normal flock replacement was hampered by a low rate of conception and a high rate of mortality among young goats (Wentzel, 1986a).

Similar abortion rates of up to 40% have been reported in Texas, USA, but only in flocks that consist predominantly of the smaller South African goat, which have been selected for quality and quantity of hair. The flocks consisting of the larger, more robust type that produce smaller quantities of fairly course hair, rarely abort (van Rensburg, 1971). This supports the opinion that the breeding of Angora goats for quality and quantity of hair has resulted in less hardy goats with poor reproductive qualities. This problem is not, however, limited to the South African population, as low conception rates and high incidence of abortion have been reported elsewhere (Escobar et al.,

1998; Huston, 1994). In a comparative study of the reproductive parameters of young Angora and Cashmere goats in Australia, Ritar et al., (1994) found that almost all Cashmeres were ovulating by 8-10 months of age at a live weight of at least 18 kg. In contrast, the Angoras grew slower, fewer ovulated, ovulation commenced later, ovulation rates were lower and the breeding season was shorter.

Lupton (1996) made the following observation about the high fibre producing Angoras in the USA: "*Angora goats seem to give top priority (or second, after maintenance) to fiber production, even aborting their offspring under stressful conditions such as when nutrition is inadequate or when predators are in the vicinity. Compared with other types of goat under the same range conditions, the percentage of kids raised by Angoras is low*".

In the traditional mohair producing regions of South Africa, goats are dependent on natural pasture as the primary source of nutrition. Natural grazing does not, however, always meet the nutritional requirements of the animals, especially during critical periods of the reproductive cycle. Herselman (1990) reported that energy supplementation before conception and during the third term of pregnancy had ensured a birth rate of 100% during a period of drought in 1984. Conversely, the birth rate during this period was only 20% in instances where no additional feed was given.

In addition to having poor reproductive qualities, Angora goats are notoriously sensitive to cold stress, in particular, wet and windy cold conditions. In addition to causing abortions, such conditions result in the death of new born and young animals, especially after they have been shorn. During the winter months the weather can change unexpectedly, leaving goats

exposed in the large pastures. Farmers are often unable to provide artificial shelter large enough for all their animals or are unable to gather large flocks under shelter timeously. The resulting exposure to wet and windy cold conditions leads to large numbers of stock losses. During August 1933, approximately 50 000 Angora goats were lost in the Jansenville and nearby districts due to cold conditions. During February 1962, approximately 5 000 goats died in the districts of Aberdeen and Graaff-Reinet, while in May 1977, an estimated 10 000 goats died in the Karoo. During a three day period of cold and wet weather in 1983, it is reported that 70 000 goats were lost to cold stress in the Eastern Cape (Terblanche, 1987). More recently, Mohair South Africa has estimated that 9000 goats died during the winter of 2007.

Several studies have been undertaken over the years to investigate the high incidence of abortion and the inability of Angora goats to cope with stress. An overview of these will be given during the rest of this chapter.

4.3.1 Abortions

One of the biggest problems that has hampered the progress of the mohair industry in South Africa is the low reproductive rates resulting from low conception rates, abortions and a relatively high kid mortality rate. Van Heerden (1963) reported that some farms lost up to 80% of the potential kid crop to abortions, while on average, kidding rates of less than 60% were generally accepted as normal. Initial investigations into this phenomenon eliminated the possibility of infectious disease as well as mineral and vitamin deficiencies as a cause of abortions in the population (van Heerden, 1963).

Furthermore, during an investigation into the possible causes of the high abortion rate, Marais (1968) showed it to be unlikely that feeding influenced the successful attachment of the embryo. The possibility of thyroid malfunction was also excluded by Wentzel et al (1976). Van Heerden (1963) described marked regression of the corpus luteum and exhaustion of the acidophilic cells of the anterior hyophysis, leading to the conclusion that the observed abortions were due to a hereditary defect of the anterior hypophysis regarding the maintenance of the corpus luteum through luteotrophic hormone secretion. Van Heerden (1964) subsequently implemented a breeding program designed to eliminate the defect. Although the abortion rate was successfully reduced, this practice only limited the incidence of abortions to first time aborters and failed to eliminate the defect. After nine years of strict culling of all aborters, the abortion rate was approximately 9.4%. Based on these results, Van der Westhuysen and Roelofse (1971) concluded that only minimal genetic progress was possible as the endocrinological abnormality could not be determined prior to abortion.

Several comprehensive studies of the endocrinology of pregnancy and abortion in the South African Angora goat were conducted and led to the identification of two distinct types of abortion (Van Heerden, 1963, 1964; Van der Westhuysen and Wentzel, 1971; Van Rensburg, 1963, 1964, 1965, 1970; Wentzel, 1973; Wentzel and Botha, 1976; Wentzel et al., 1976).

Studies showed that the first type of abortion is triggered by nutritional stress or other physiological stress conditions, such as cold stress, which cause a drop in maternal blood glucose concentration. (Wentzel, 1973; Wentzel et al., 1976). This condition causes a similar hypoglycemic condition

to occur in the foetal circulation, thereby activating the foetal HPA-axis, which results in an elevated output of adrenal steroids, including oestrogen precursors. This in turn results in an excessive placental secretion of oestrogens into the maternal circulation, which in turn mediates the regression of the corpus luteum through the release of uterine prostaglandins. The consequent reduction of the progesterone level permits the onset of labour and the delivery of the foetus (Wentzel, 1973). During a study into the effect of adverse nutrition on the incidence of abortion during the last trimester of pregnancy, Wentzel et al. (1974) noted that all the abortions caused by submaintenance energy nutrition were characterised by the expulsion of apparently normal fetuses with normal appearance. In this study, a group of confirmed aborters were compared to a group of normal does. Both groups were fed a ration that contained approximately twice the energy and protein requirement for maintenance. During the last trimester a random sample of each group was limited to three quarters of the requirement for maintenance. The low energy ration significantly ($p < 0.01$) affected the incidence of abortion among the confirmed aborter ewes (Table 4.2). No abortions were encountered among the normal ewes, while the incidence of abortion was 52.4% among the aborter ewes, with the majority of abortions occurring in the third week following the change in diet.

Table 4.2. Reproductive data of normal and confirmed aborter ewes kept on a high or low plane of energy nutrition during the last trimester of pregnancy. Reproduced from Wentzel et al. (1974).

	High energy	Low energy
Number of normal does	25	15
Number pregnant	21	12
Number aborted	0	0
Perinatal losses	0	0
Number of confirmed aborters	20	25
Number pregnant	13	21
Number aborted	1	11
Perinatal losses	1	2

Subsequently, Wentzel et al. (1975) confirmed that the expulsion of the foetus during this type of abortion is closely related to a low level secretion of progesterone, and it was proposed that regression of the corpus luteum is invariably necessary for the expulsion of the foetus, but not for causing foetal death *in utero*. Furthermore, Wentzel and Viljoen (1975) demonstrated a similarity between experimentally induced abortions, via oestrogen, and those resulting from sub-nutrition. In both cases foetal heart rate remained normal virtually up to the expulsion of the foetus, the abortuses were expelled in a fresh state and in some cases the heart was still beating after expulsion.

The second type of abortion was found to be completely different to the first. It was postulated that a condition of chronic hyperadrenocorticism interferes with the normal regulation of the water and electrolyte balance of the body, causing placental dysfunction and the progressive accumulation of excessive intra-uterine fluids (Wentzel et al., 1975). This creates an unfavourable foetal environment, which results in foetal oedema, a retarded foetal heart and eventually congestive heart failure (Wentzel, 1973, 1986a). From this it was evident that the mechanism involved in abortion of this type is

primarily responsible for intra-uterine foetal death. It was therefore believed that expulsion of the dead foetus is merely consequence of the latter event and was mediated through uterine prostaglandin release, induced by autolysis of the dead foetus, which in turn led to luteal regression. The resultant cessation of progesterone secretion subsequently permitted the delivery of the foetus (Wentzel, 1986a). Hyperadrenocorticism is therefore the primary cause of gestational termination and the expulsion of the dead foetus, which followed after a varying length of time, appeared to be a secondary consequence of the defect.

The majority of abortions that occurred under natural conditions were of the stress-induced type (Wentzel,1986a) rendering the second type of abortion identified of very little practical significance. Furthermore, this observation provided an explanation for the more severe spates of abortion usually occurring during droughts and following adverse weather conditions. Further observations in farming practices led to the identification of additional conditions which increase the susceptibility of the animals to abortions. These include: inclement weather conditions; exposure of ewes to cold climatic conditions after sheering, together with the increased demand for energy not being met; moving animals to pastures consisting of different vegetation, resulting in temporary energy deficiency being introduced by a period of ruminal adaptation to the new diet; and sheltering animals for long periods of time without the provision of feed. Furthermore, it was noted that young or old and relatively small ewes are more prone to abort as a result of stressful conditions than well grown adult ewes (Wentzel, 1986b). The practical

implementation of energy supplementation, as discussed later in this chapter, led to a significant decline in the incidence of abortion (Wentzel, 1986b).

4.3.2 Cold stress

A large number of South African Angora goats are lost during the months of July and August as a result of the cold weather conditions (Terblanche, 1987). This is also a time of year when the goats are sheared and the ewes are pregnant, with September usually being the month in which ewes give birth. In a study where mature pregnant and young shorn Angora ewes were subjected to wet and windy cold conditions, Wentzel et al. (1979) showed that the primary cause of stock loss during cold spells is due to an energy deficiency resulting from a decrease in blood glucose levels. This physiological condition, together with the low ambient temperature, subsequently causes a drop in body temperature and subnormal heart function. During this study the mature pregnant ewes were able to endure the cold stress for a significantly longer period than the young Angora ewes. Furthermore, all animals which collapsed during this study were subsequently given an intravenous glucose injection and were able to get up within four hours. By twelve hours, these animals could not be distinguished from the control group.

A possible solution to cold related deaths was to increase blood glucose through nutrition. Results revealed that an increase in circulating blood glucose was directly related to the amount of starch ingested by the goat. The application of this concept was, however, limited by the increased risk of

acidosis in free-grazing ruminants not adapted to high concentrate diets (Wentzel, 1986a). In order to overcome the problem of acidosis, an alkali-ionophore-treated whole grain known as “chocolate grain” was developed (Wentzel, 1982, 1983). This permitted the ad lib intake of treated grain by goats not previously adapted to such diets without the danger of acidosis or other digestive disorders. Angora goats that had free access to the treated grain showed an average increase of 50-60% in circulating glucose levels within 48 hours after offering the grain. The implementation of this practice has proved to be a valuable preventative measure which rapidly increases the energy status of the goat and thereby increases its tolerance to cold. In addition, the treated grain has also been used successfully to revive cold stressed animals.

Treated grain has not only been used successfully in the prevention of losses during cold spells, but also in reducing the incidence of abortions by the strategic supplementation during critical periods such as: droughts or cold spells; when pregnant ewes are being sheltered in enclosures; after shearing; and during changes in the diet due to a change in pasture (Wentzel, 1986a). Though supplementary feeding can reduce occurrence of abortions and cold-stress related deaths, these problems were not eliminated. Research was therefore continued to further the understanding of the underlying cause of these problems.

Fourie (1984) subsequently investigated the influence of wet and windy cold conditions, as well as the role of feeding, on sheared Angora goats. In this study, Angora goats where compared to Boer goats, which are generally accepted as being more hardy. Initially the effect of hair length and different

ambient temperatures (1.5℃ - 20℃) on heat production was investigated under dry, wet and wet, windy conditions. The most dramatic increase in heat production was found for shorn Angora goats under low ambient temperatures in wet, windy conditions. The effects of the wet and windy cold conditions, were also more severe in the Angora goats than the Boer goats.

The ability of the shorn Angora goats to cope with severe weather conditions for prolonged periods was subsequently investigated. Exposure to wet and windy cold (3℃) conditions resulted in an increased heat production of 233 $kJ^{-1}day^{-1}$ (20℃) to 719 $kJ^{-1}day^{-1}$. Similarly, blood glucose concentrations increased from 104.5 mg/100 ml to 127.9 mg/100 ml during the same period. After three hours of exposure to wet and windy cold conditions, the goats were unable to survive further without assistance. The body temperature dropped to 34.8℃, heat production and blood glucose concentration decreased to 364 $kJ^{-1}day^{-1}$ and 66.5 mg/100ml, respectively.

The influence of feeding levels on the ability of the animals to withstand the wet and windy cold conditions was also investigated. Fourie showed that Angora goats on a low feeding levels could only withstand these conditions for 1.5 hours, while goats on high feeding levels could survive for up to three hours. In contrast, on a low feeding level, Boer goats could withstand the conditions for three hours. Boer goats on the high feeding level were still comfortable after six hours. These results clearly demonstrated that supplementary feeding, as proposed by Wentzel (1982, 1983), increased the Angora goat's tolerance of cold, but could not eliminate the problem of cold-stress related deaths, especially during long periods of exposure.

Fourie attributes the difference between the Angora and Boer goat to the difference in heat loss and the ability to produce heat and sustain heat production. Heat loss in the shorn Angora was found to be more rapid than in the short haired Boer goat. In addition, the Angora is unable to produce sufficient heat to compensate for the loss. The author concludes that the Angora goat does not have the metabolic capacity to produce enough heat, a problem that is further compounded by the weak insulation of the short hair after shearing.

Cronje (1992) later showed that Angora ewes have a lower blood glucose concentration; a slower response of the glucose synthesis rate to dietary energy increments; and a slower acetate clearance rate than Boer goat ewes, providing further evidence of the inability of the Angora goat to mobilise glucose precursors. Subsequent studies demonstrated lower glucose concentrations in Angora goats than in both Alpine and Nubian goats (Hart et al., 1993).

Van Rensburg (1971) previously suggested that selection for mohair growth rate had, in reality, been for low level adrenal function, eliminating the inhibitory effects of cortisol on hair production. Herselman and Pieterse (1992) later investigated the hypothesis that the high fibre production in the Angora goat was possibly due to decreased adrenal activity. They suggested that lower cortisol production in these animal resulted in altered metabolism which, while permitting greater fibre growth, conversely made the animals less hardy. This was investigated by treating a test group of Angora goats with an intravenous injection of dihydroxycortisolacetate every second day for six months, after which the animals were slaughtered and compared to a control

group. The greasy hair mass of the test group (0.976 kg) was significantly (p<0.01) lower than that of the control group (1.292 kg). The internal fat mass and back fat depth was 927.4 g and 3.45 mm for the test group and 566.3 and 0.75 mm for the control group respectively. These results indicate that cortisol favours fat synthesis over protein synthesis. Furthermore, cortisol treatment was able to change the metabolism of the Angora goat to resemble that of more hardy breads with a concomitant decrease in fibre production. These results implicated the decreased cortisol production in Angora goats as a possible cause of cold related deaths, as lower cortisol levels would affect metabolism and limit the glucose available in the blood for heat production. Furthermore, this study showed that reduced cortisol levels in Angora goats favoured protein biosynthesis over fat biosynthesis, resulting in a decrease in the amount of insulating fat compared to other more hardy goats. This finding substantiated Fourie's (1984) observation that Angora goats loose heat quicker than Boer goats.

Cronje (1995) investigated plasma glucose levels in Angora goats before and after insulin stimulation and fasting. Two lines of goats were compared, namely 'high producers', which produce a relatively large quantity of mohair of a larger diameter and 'low producers', which produce less mohair and of a smaller diameter. Basal plasma glucose levels were measured prior to intravenous insulin injection and prior to a 48 hour fast. Glucose concentrations were consistently 6-9% lower in the high producers, in addition high producers took 22% longer to restore basal glucose after insulin injection and glucose concentrations declined 28% more rapidly during fasting. Fasting for as little as 24 hours after shearing had previously been shown to

precipitate cold-stress related deaths (Shelton and Terrill, 1989). Cronje concluded that the high producing phenotype is unable to mobilise glucose precursors during times of increased demand due to an altered metabolism.

In a study by Herselman and Loggerenberg (1995) cortisol production was compared in a number of small ruminant breeds, varying in potential for fibre production. The mean plasma cortisol concentration of Angora goats was found to be significantly lower than that of Afrino, Dorper and Namaqua Africana sheep. Intravenous insulin injection caused a drop in blood glucose concentration in all breeds, with a resulting increase in the plasma cortisol concentration. The peak plasma cortisol concentration was, however, three to five times smaller in the Angora when compared to the other breeds. Similarly, plasma cortisol, in response to intravenous CRF injection, was three to four times smaller in the Angora than in the other breeds. This study concluded that a form of hypocortisolism significantly contributes to the disorders in carbohydrate metabolism observed in the Angora.

4.3.3 Poor growth in young animals

An additional concern for mohair producers has been impaired growth in young animals, resulting in the direct loss of mohair production and more importantly the impaired reproduction experienced in such animals once they become adults (Wentzel, 1986b). Live body mass has been shown to be one of the primary factors affecting reproduction, especially in the maiden ewe at the first mating. A kidding rate of 86% was recorded in well-fed young ewes when compared to a kidding rate of only 19% in unsupplemented young ewes

on natural pasture (Shelton, 1961; Van der Westhuysen and Roelofse, 1971). Research was therefore focused on the effects of energy supplementation on growing kids grazing on the natural pasture. The results clearly indicated an energy insufficiency in goats grazing on natural pastures and a remarkable response in growth rate due to energy supplementation (Wentzel, 1986b).

4.4 The energy requirement of the Angora goat

From the above discussions it is clear that the natural pasture where Angoras are farmed cannot support the energy requirements of these animals. Selection for mohair has placed a high metabolic priority on fibre production. In a study, where the turnover of feed to fibre was investigated, Gallagher and Shelton (1972) found that the turnover in Angora kids were 3.2 times more effective than Rambouillet ewes. Conversely, the turnover of feed to body weight was 34% more effective in the Rambouiller ewes than the Angora kids. Similar results were found for adult animals.

Herselman (1990) subsequently quantified the energy requirements of the Angora goat and investigated whether these requirements were influenced by hair production. This study showed that the production of high quality mohair was the cause of the total energy metabolism being less effective when compared to other goats, and that hair production might be at the expense of other physiological functions.

The inhibitory effect of high plasma cortisol levels on hair production, identified by Van Rensburg (1971) led the author to suggest that selection for mohair indirectly resulted in reduced adrenal function and as a result reduced

cortisol levels. Herselman (1990) stated that this phenomenon would result in lowered blood glucose levels; lowered liver glycogen; delayed mobilisation of fat and protein; and a lowered effectiveness of the role of the catecholamines during stress. Herselman further postulated that lower levels of glycogen storage in the liver, resulting from decreased adrenal activity, may contribute to the high abortion rate found in the Angora population. In instances when an increased demand for nutrition by the growing foetus occurs and the blood glucose levels cannot be maintained by high starch intake, the glycogen reserves in the liver are quickly exhausted resulting in an abrupt drop in glucose, as demonstrated in other studies (Wentzel et al., 1979). Low plasma cortisol levels also lower the mobilisation of fat for metabolism. Therefore, factors, which include low quality feeding, that will lead to the exhaustion of the glycogen reserves in the liver during pregnancy, would increase the abortion rate (Wentzel et al., 1974; Wentzel et al., 1976).

In addition, Herselman (1990) reasoned that low glycogen reserves may also explain the Angoras vulnerability to cold stress. As discussed earlier in this chapter, a study by Wentzel et al. (1979) showed a sharp increase in cortisol levels in Angora goats subjected to cold. Blood glucose levels also increased initially, but after 12 hours decreased to approximately half of the normal levels, together with a lowered heart rate. The animals subsequently recovered quickly after an intravenous administration of glucose. Herselman (1990) attributes the drop in glucose levels after 12 hours to the depletion of glycogen reserves.

Similarly Herselman (1990) ascribed the retarded growth of Angora kids to the lowered adrenal function for the following reasons — the low fat

reserves found in animals kept on natural pastures, could be explained by low glucocorticoid levels; low plasma cortisol levels would lead to lowered blood glucose concentrations, accounting for the drastic effect on the growth rate of the Angora goat observed during energy addition through supplementary feeding (Wentzel et al., 1976); and retarded growth would be further influenced by the suppressive effect of low corticosteroid levels on voluntary food intake (Bassett, 1963).

These observations, together with previous studies described in this chapter, clearly demonstrate that the primary cause of abortions, cold deaths and retarded growth in young animals, can therefore be attributed to a lowered activity of the Angora adrenal gland.

4.5 Investigating hypocortisolism in the South African Angora goat

Possible causes of the observed hypocortisolism in the South African Angora goat have been investigated by our laboratory. Engelbrecht et al. (2000) compared the adrenal response of Angora goats, Boer goats and Merino sheep to insulin-induced stress, as well as ACTH and CRH stimulation. Insulin-induced stress mimics cold stress conditions by inducing a hypoglycaemic condition, which serves as a signal to the hypothalamus. Cortisol released by the adrenal, as a result of the stimulation of the HPA-axis, should rectify the hypoglycaemic condition as discussed in chapter 2. Insulin induced a hypoglycaemic condition in all three of the abovementioned species (Figure 4.2(A)).

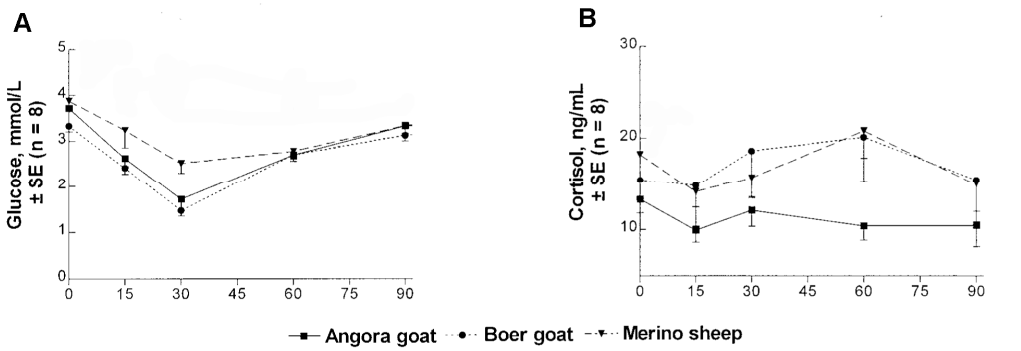

Figure 4.2 Effect of an intravenous injection of human insulin (0.1 IU/kg body mass) on (A) plasma glucose and (B) cortisol concentrations in the Angora goat, Boer goat and Merino sheep. The cortisol levels for each species were compared by a repeated measures ANOVA. The plasma cortisol levels, in response to intravenous insulin injection, were significantly ($p < 0.001$) less in the Angora goat than in the other species. Reproduced from Engelbrecht et al (2000).

Plasma cortisol levels increased significantly in the Boer goat and sheep, after insulin injection, with a peak level being reached 60 min post injection. The Angora goat, however, did not display any significant increase in plasma cortisol levels (Figure 4.2 (B)), confirming the hypocortisolism reported by previous studies (Van Rensburg, 1971; Herselman and Pieterse, 1992; Herselman and Loggerenberg, 1995).

The ability of ACTH and CRH to elicit a response in plasma cortisol levels was also investigated. Sheep CRH produced a response in the Merino sheep, but not in the two goat species, possibly due to the inability of sheep CRH to bind to the goat CRH receptor efficiently enough to elicit a subsequent response. ACTH stimulation caused a rise in plasma cortisol concentration in the three species, however, the response was highest in the Merino sheep and lowest in the Angora goat. This data indicated that the HPA-axis was functional in all three species, with the Angora goat again demonstrating a weak cortisol response upon HPA-axis stimulation when compared to the

other two species. These results led to the investigation of adrenal steroidogenesis in the Angora goat in order to identify the possible cause of the observed hypocortisolism.

Engelbrecht and Swart (2000) used subcellular adrenal fractions (microsomes and mitochondria) to investigate and compare adrenal steroidogenesis in the same three species previously investigated, namely the Angora goat, Boer goat and Merino sheep.

Pregnenolone was used as a substrate in the prepared microsomes and the production of glucocorticosteroid precursors (deoxycorticosterone and deoxycortisol) and androgens (DHEA and androstenedione) was compared (Table 4.3). During the 10 minute incubation period, significantly less glucocorticosteroid precursors were produced in Angora goat microsomes than in Boer goat and Merino sheep microsomes. The Angora adrenal microsomes converted 36% of the pregnenolone to glucocorticoid precursors, while the conversion in the other two species was 79 and 82%, respectively. In contrast, the Angora goat adrenal microsomes produced significantly more 17-hydroxypregnenolone and dehydroepiandrosterone than did the other two species. Unfortunately, 17-hydroxypregnenolone and dehydroepiandrosterone could not be quantified individually during this study.

Table 4.3. Pregnenolone (10µM) metabolism in Angora goat, Boer goat and Merino sheep microsomes. Metabolite distribution was analysed with one-way ANOVA, followed by Bonferroni's multiple comparison test. Reproduced from Engelbrecht and Swart (2000).

Species	% Glucocorticoid precursors formed (deoxycorticosterone and deoxycortisol)	% DHEA and 17-hydroxypregnenolone formed	% androstenedione formed	% pregnenolone remaining
Angora goat	35.6 ± 8.9[a]	34.7 ± 4.2[c]	5.5 ± 2.3	1.1 ± 1.1
Boer goat	78.5 ± 13.9[b]	8.8 ± 1.7[d]	-	6.3 ± 4.0
Merino sheep	82.0 ± 6.5[b]	0.0 ± 0.0[d]	-	-

[a,b]Within columns, values without common superscripts differ ($p < 0.05$)
[c,d]Within columns, values without common superscripts differ ($p < 0.001$)

Progesterone was completely metabolised within 15 minutes when used as a substrate in all the microsomal preparations. Significantly more deoxycorticosterone and less deoxycortisol was produced in Angora preparations, while androstenedione and 17-hydroxyprogesterone production was less than 5% in the preparations from all three species.

The differences in steroid output in the microsomal preparations from the three species suggested that there was a difference in activity of one or more of the enzymes in the adrenal steroidogenic pathway. The activities of these enzymes in the different species were subsequently studied and compared. Pregnenolone metabolism was manipulated by the selective addition of cofactors. The activity of 3βHSD was investigated by measuring the conversion of pregnenolone to progesterone using NAD^+ as cofactor in the absence of NADPH. The addition of NAD^+ resulted in the formation of progesterone in all three species. No difference in activity was observed between species (Figure 4.3(A)). CYP17 activity was studied by measuring the conversion of pregnenolone to dehydroepiandosterone using NADPH as cofactor in the absence of NAD^+. CYP17 in the Angora goat adrenal

microsomes converted pregnenolone to dehydroepiandosterone significantly faster than in the other two species (Figure 4.3(B)).

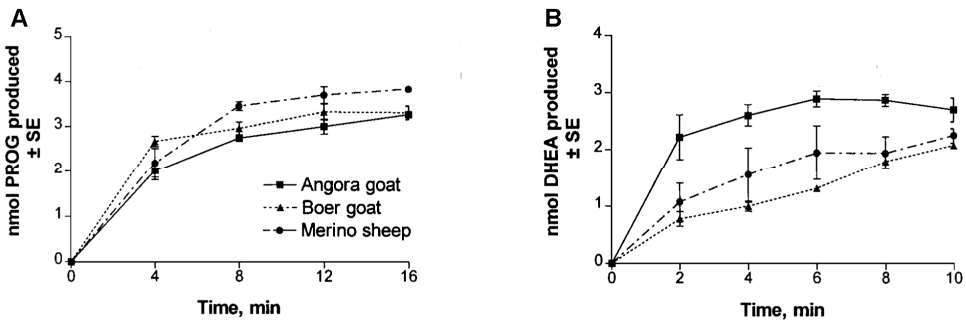

Figure 4.3. Comparison of the activity of (A) 3βHSD and (B) CYP17 in Angora goat, Boer goat and Merino sheep adrenal microsomes. The activity of 3βHSD was measured by following the metabolism of pregnenolone (PREG, 10µM) to progesterone (PROG) in the presence of NAD^+ as cofactor, while the activity of CYP17 was measured by following the metabolism of pregnenolone (10µM) to dehydroepiandosterone in the presence of NADPH as cofactor. The results were analysed using repeated measures ANOVA followed by Dunnets' post test. No significant difference in 3βHSD activity was observed between species. The activity of Angora goat CYP17 was significantly ($p < 0.01$) greater than that of the other species. Reproduced from Engelbrecht and Swart (2000).

CYP21 activity was investigated by incubating the adrenal microsomes with 17-hydroxyprogesterone. All three species converted more than 90% of the 17-hydroxyprogesterone to deoxycorticosterone, with negligible conversion to androstenedione. There was no significant difference in the activity of CYP21 between the species. Furthermore, the activities of the mitochondrial enzymes CYP11A1 and CYP11B1 were investigated, but revealed no significant differences that might contribute towards the impaired glucocorticoid production in Angora goats.

From these results it was clear that the only significant difference in enzymatic activity between species lay with CYP17. Angora CYP17 had a

lower affinity for progesterone than the other two species, but also a significantly higher affinity for pregnenolone.

Engelbrecht and Swart (2000) concluded that the preference exhibited by Angora CYP17 for the Δ^5-steroid pathway during adrenal steroidogenesis would likely result in an increased production of adrenal androgens *in vivo*, with a resulting decrease in the production of glucocorticoids when compared to the other species.

This study clearly implicated abnormal activity of CYP17 as the possible cause of hypocortisolism in the South African Angora goat. These findings led to a new hypothesis that inbreeding practices could have preserved a mutation in the CYP17 gene that caused the altered adrenal steroidogenic activity (Slabbert, 2003). Slabbert (2003) suggested that since some Angora breeders had reported that their breeding stocks comprise of goats that are not as susceptible to environmental stress as others, there may be more than one allele for CYP17 in the Angora goat gene pool. Total RNA was subsequently isolated from Angora goat adrenocortical tissue and used to prepare Angora CYP17 cDNA. Sequencing analysis of the cDNA yielded a sequence that shared 99.74% homology with Boer goat CYP17 (GenBank accession no. **AF251387**). Only four nucleotide bases differed in the 1530 bp coding region of the two sequences. Double peaks were observed at four nucleotide positions in the Angora CYP17 cDNA sequence when sequenced with forward and reverse primers, indicating the presence of two isoforms. Interestingly, the four double peaks were observed at the same four positions where the Angora CYP17 cDNA differed from that of the Boer goat, viz. positions 17, 122, 637 and 1065. Analysis of the cDNA reading frame, in

terms of the genetic code, revealed that the first three nucleotide substitutions resulted in amino acid substitutions in the translated protein, namely Ala/Gly, Pro/Leu and Val/Ile, respectively.

Furthermore, the nucleotide substitution at position 637 caused an alteration in the recognition site for the endonuclease ACS I from 'AAATTC' to 'AAGTTC', which is not a substrate for endonuclease cleavage by ACS I. Slabbert used this observation to develop a restriction based genotyping method for the two CYP17 isoforms. Genomic DNA was isolated from blood and a 1.2 kb region, which included the ACS I in exon 3, was amplified by PCR. The PCR product was purified, concentrated and subsequently digested with ACS I and analysed by gel electrophoresis. Eighty three goats were genotyped in this study, and of these 24 were homozygous for CYP17 without the ACS I site, and 59 were heterozygous. No goats homozygous for CYP17 with the ACS I site were detected. A typical banding pattern for the ACS I restriction digest assay is shown in Figure 4.4 for both a homozygous and heterozygous sample.

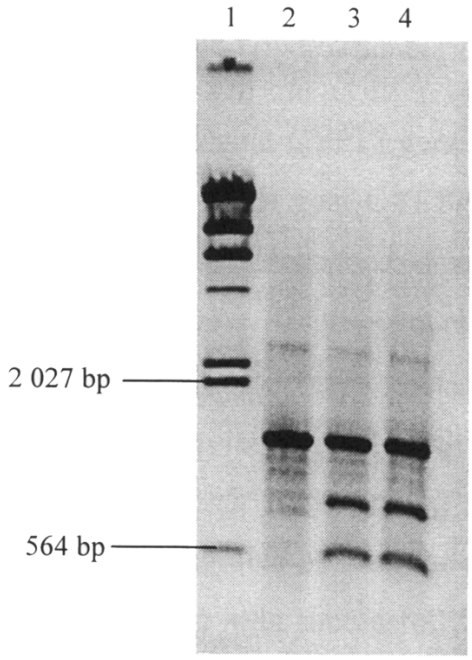

Figure 4.4. Typical result obtained using the restriction based genotyping assay developed by Slabbert (2003). Lane 1, DNA marker DNAλ/Hind III; Lane 2, typical banding pattern of a homozygous sample; Lanes 3 and 4, typical banding pattern of a heterozygous sample. Reproduced from Slabbert (2003).

Slabbert (2000) attempted to identify possible correlations between the different CYP17 genotypes and a physical characteristic of the Angora goat associated with stress intolerance. According to a breeder from the Little Karoo, he could identify two types of Angora goat — goats with so called oily skins and goats with so called dry skins. According this breeder, goats with the oily skins are more hardy compared to the goats with dry skins. The breeder provided 20 goats for this study, and of these only six could conclusively be categorised in terms of oily or dry skins. Three of these goats had oily skins and were genotyped as heterozygotes, while the remaining

three goats genotyped as homozygotes with no ACS I site had dry skins. Unfortunately, even though a positive correlation was observed, the sample size in this experiment was too small to draw any definitive conclusions. Interestingly though, six Boer goats, which are generally regarded as being more hardy, were also genotyped and were all heterozygous. This finding suggested, firstly, that the heterozygote genotype may be more hardy than the homozygote with no ACS I site. Secondly, this implied that two CYP17 isoforms also existed in the Boer goat. Slabbert concluded that if the heterozygote did not cause hypoadocorticism, or at least not a severe form thereof, it could produce a selective advantage that would explain its high genotypic frequency.

Subsequently, Swart (Unpublished data) investigated the ability of the genotypes, identified by Slabbert (2003), to produce cortisol in response to insulin induced hypoglycemia. For this study 15 heterozygous and 13 homozygous goats, without the ACS I site, were identified. Intravenous insulin injection resulted in a hypoglycemic state in both groups within 30 min of insulin injection. Plasma cortisol concentrations increased significantly in both groups within 15 min with no significant difference found between the groups. Swart did, however, note that the cortisol concentrations of the heterozygous animals showed a significantly greater variation than those of the homozygous animals (Figure 4.5).

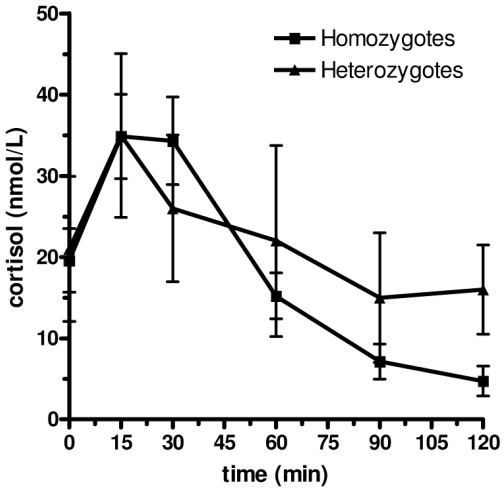

Figure 4.5. Comparison of plasma cortisol concentrations of two CYP17 genotypes (heterozygous and homozygous with no ACS I site) in response to intravenous insulin injection (0.1 IU/kg). The results were analysed using repeated measures ANOVA followed by Dunnets' post test. No significant difference was found between the groups. Results provided by Swart (Unpublished results).

4.6 Conclusion

From the above studies it is therefore evident that Angora CYP17 has an altered activity when compared to that of Boer goats and Merino sheep. Furthermore, Slabbert (2003) clearly demonstrated that there are in fact at least two CYP17 isoforms within the South African Angora goat population. Although there was no conclusive evidence, both Slabbert (2003) and Swart (unpublished data) demonstrated that one of the CYP17 isoforms may be the primary cause of the observed hypocortisolism in the South African Angora goat. An in-depth investigation of the enzymatic activities of the individual CYP17 isoforms and their role in the inability of the Angora goat to produce high levels of cortisol, will therefore be described in the following chapters.

CHAPTER 5
THE DEVELOPMENT OF AN UPLC-COUPLED ATMOSPHERIC PRESSURE CHEMICAL IONIZATION MASS SPECTROMETRY ASSAY FOR SEVEN ADRENAL STEROIDS

5.1 Introduction

The aim of this study was to address the role played by the two identified CYP17 isoforms in cold stress related deaths in the South African Angora goat population. In order to accomplish this both CYP17 isoforms were cloned and characterised, as will be discussed in the following chapter. The enzymatic activities of the two cloned isoforms were subsequently compared by expression of each CYP17 isoform in non-steroidogenic COS-1 cells.

In the adrenal cortex, CYP17 and 3βHSD compete for the same substrates. Together the catalytic properties and ratio of these enzymes determine steroidogenic output of the adrenal. Therefore, in order to determine the physiological effect of the two CYP17 isoforms, each isoform had to be coexpressed with 3βHSD. Such an experiment would yield a complex mixture of steroid metabolites to be analysed. A new rapid and accurate LC-MS method was therefore developed in order to separate and quantify these steroids. The method was validated and the results were submitted to Analytical Biochemistry and presented here in published form (Storbeck et al., 2008a).

Available online at www.sciencedirect.com
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Analytical Biochemistry 372 (2008) 11–20
ANALYTICAL BIOCHEMISTRY
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The development of an ultra performance liquid chromatography-coupled atmospheric pressure chemical ionization mass spectrometry assay for seven adrenal steroids

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Received 12 April 2007
Available online 1 October 2007

Abstract

An ultra performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (UPLC–APCI–MS) method was developed for the separation and quantification of adrenal steroid metabolites from heterologous expression media. Steroids were extracted by liquid–liquid extraction, separated on a Waters UPLC BEH C18 column, ionized by APCI, and detected using a triple quadrupole mass spectrometer in APCI positive mode with single ion monitoring. The incorporation of UPLC enabled the detection of seven structurally closely related steroids at between 5 and 40 ng/ml using run times of 11 min. The adrenal steroidogenic enzyme cytochrome P450 17-hydroxylase/17,20-lyase (CYP17) was expressed in the yeast *Pichia pastoris* and in nonsteroidogenic COS-1 cells, and used as a model system to evaluate the detection and quantification of adrenal steroid metabolites by UPLC–APCI–MS.

Keywords: Cytochrome P450 17-hydroxylase/17,20-lyase (CYP17); Atmospheric pressure chemical ionization (APCI); Ultra performance liquid chromatography (UPLC)

The mammalian adrenal gland is an important source of steroid hormones. The three end products of adrenal steroidogenesis are the mineralocorticoids, glucocorticoids, and adrenal androgens. The adrenal steroidogenic cytochromes P450 are a unique group of enzymes responsible for the biosynthesis of these hormones that are vital for the control of water and mineral balance, stress management, and reproduction. Within the adrenal steroidogenic pathway, cytochrome P450 17-hydroxylase/17,20-lyase (CYP17)[1] catalyzes two distinctly different reactions, the 17α-hydroxylation and the C17-C20 lyase reaction [1], placing this enzyme at a key branch point in the biosynthesis of aldosterone, cortisol, and the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione (A4). The 17α-hydroxylation of pregnenolone (PREG) and progesterone (PROG) yields 17-hydroxypregnenolone (17-OHPREG) and 17-hydroxyprogesterone (17-OHPROG), respectively. The 17,20-lyase reaction catalyzes the cleavage of the C17,20

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[1] *Abbreviations used:* CYP17, cytochrome P450 17-hydroxylase/17,20-lyase; DHEA, dehydroepiandrosterone; A4, androstenedione; PREG, pregnenolone; PROG, progesterone; 17-OHPREG, 17-hydroxypregnenolone; 17-OHPROG, 17-hydroxyprogesterone; 3βHSD, 3β-hydroxysteroid dehydrogenase; 16-OHPROG, 16α-hydroxyprogesterone; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; UPLC, ultra performance liquid chromatography; APCI, atmospheric pressure chemical ionization; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; MS/MS, tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification.

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doi:10.1016/j.ab.2007.09.036

bond of 17-OHPREG and 17-OHPROG to yield DHEA and A4, respectively. 3β-Hydroxysteroid dehydrogenase (3βHSD) converts the 3β-hydroxy-Δ^5-steroid precursors PREG, 17-OHPREG, and DHEA to the corresponding Δ^4 3-ketosteroids PROG, 17-OHPROG, and A4 (Fig. 1). In addition, it is possible for human CYP17, as well as CYP17 of other primate species, to convert PROG to 16α-hydroxyprogesterone (16-OHPROG) [2,3]. These steroids are structurally closely related, and in some instances differ only by the position of a double bond or an OH group, complicating their separation and quantitation in biological extracts.

The detection and quantification of steroid metabolites from various heterologous expression systems expressing steroidogenic cytochromes P450 are commonly preformed by thin layer chromatography (TLC) [3,4–7] and high-performance liquid chromatography (HPLC) [2,8,9]. Because a number of the steroids, in particular the Δ^5 steroids, have poor UV absorption [10], both of these methods rely on the use of expensive tritiated steroids. Although it is possible to separate either the Δ^5 or Δ^4 steroid metabolites of CYP17 adequately by TLC, it is impossible to separate a complex mixture containing both the Δ^5 and Δ^4 steroid metabolites. Even though a better chromatographic separation can be achieved by HPLC, the resolution remains unsatisfactory because metabolites coelute and run times of up to 30 min are required [8].

The detection of adrenal steroids in biological fluids is critical for diagnostic purposes because clinical conditions, such as congenital adrenal hyperplasia and polycystic ovary syndrome [11], are characterized by abnormalities in adrenal steroid production. Gas chromatography–mass spectrometry (GC–MS) and immunoassays are employed regularly for the detection of steroids in serum [12–16]. Although GC–MS methods exhibit good specificity, efficiency is impeded by low throughput and the requirement of large sample volumes [17,18]. Immunoassay analyses, on the other hand, are hampered by cross-reactivity of immunoglobulins and matrix effects [19]. Furthermore, immunoassays often are method specific and cannot be used interchangeably [20–25].

Recently, Nithipatikom and coworkers [26] developed a liquid chromatography–mass spectrometry (LC–MS) assay for the simultaneous detection of several adrenal steroids, demonstrating that LC–MS is a viable alternative to conventional methods. However, this assay made use of conventional HPLC resulting in run times of 50 min.

We have developed a method of LC–MS for the simultaneous detection of seven adrenal steroids obtained in the conversion of PREG and PROG catalyzed by CYP17 expressed in *Pichia pastoris,* a yeast expression system, and in COS-1 cells, a mammalian expression system. Steroids were extracted by liquid–liquid extraction, separated by ultra performance liquid chromatography (UPLC), ionized by atmospheric pressure chemical ionization (APCI), and detected by a tandem quadrupole mass spectrometer.

Materials and methods

Reagents

PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, A4, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical (St. Louis, MO, USA). COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Penicillin–streptomycin, trypsin–EDTA, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal calf serum was purchased from Highveld Biological (Lyndhurst, South Africa). *P. pastoris* strain GS115 was purchased from Invitrogen (Carlsbad, CA, USA), and all yeast media components were obtained from Difco Laboratories (Detroit, MI, USA). Nucleobond AX plasmid preparation kits were purchased from Macherey–Nagel (Duren, Germany). A bicinchoninic acid (BCA) protein

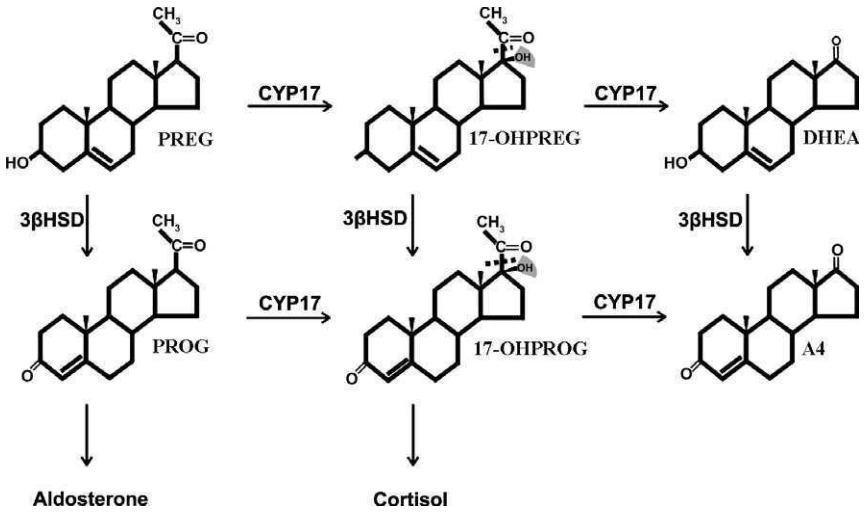

Fig. 1. Enzymatic reactions catalyzed by CYP17 and 3βHSD.

assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest analytical grade and were purchased from scientific supply houses.

Standards

Stock solutions of PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, and A4 (2 mg/ml) were prepared in ethanol. A series of standards (2, 20, 200, and 2000 ng/ml) were prepared in methanol from the stock solutions.

UPLC–APCI–MS conditions

A mixture of the Δ^5 steroids (PREG, 17-OHPREG and DHEA) and Δ^4 steroids (PROG, 16-OHPROG, 17-OHPROG and A4) was separated by UPLC (ACQUITY UPLC, Waters, Milford, MA, USA) using a Waters UPLC BEH C18 column (2.1×50 mm, 1.7 μm) at 50 °C. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile). A linear gradient from 85% A to 80% B in 3.5 min, followed by a linear gradient from 80% B to 100% B in 0.1 min and an isocratic elution with solvent B for 1 min, was applied. A linear gradient returned the column to 85% A in 1 min. The total run time per sample was 5 min at a flow rate of 0.4 ml/min. The injection volume of standards and samples was 5 μl.

Samples containing both the Δ^5 steroids and Δ^4 steroids (PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, and A4) were separated by UPLC (ACQUITY UPLC) using a Waters UPLC BEH C18 column (2.1×100 mm, 1.7 μm) at 50 °C. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (3:1 acetonitrile/methanol with 1% isopropanol). The column was eluted isocratically with 56% A and 44% B for 6 min, followed by a linear gradient from 44% B to 80% B in 0.01 min and a subsequent linear gradient from 80% B to 100% B in 2.49 min. A linear gradient returned the column to 56% A and 44% B in 0.5 min. The total run time per sample was 11 min at a flow rate of 0.3 ml/min. The injection volume of standards and samples was 5 μl.

An API Quattro Micro tandem mass spectrometer (Waters) was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters) was used for the APCI interface in positive mode. The corona pin was set to 7 μA, the cone voltage to 30 V, and the APCI probe temperature to 450 °C. All other settings were optimized to obtain the strongest signal possible. Calibration curves were constructed by using weighted ($1/x2$) linear least squares regression. Data were collected with the MassLynx 4.0 software program.

Extraction procedure

Steroids were extracted from the samples (0.5 ml) by liquid–liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 2 min and centrifuged at 500*g* for 5 min. The water phase was aspirated off, and the dichloromethane phase was transferred to a clean test tube. The samples were dried under a stream of nitrogen. The dried steroids were reconstituted in 100 μl of methanol prior to analysis.

Validation of UPLC–APCI–MS assay

A standard curve (10, 20, 40, 100, 200, 400, and 1000 ng/ml) was generated ($n = 6$). The reproducibility of the assay was determined by replicate analysis ($n = 6$) of cell culture media (0.5 ml) and yeast culture media (0.5 ml) that had been spiked with each steroid, extracted, and subsequently quantified. Absolute recoveries were calculated at a concentration of 200 ng/ml for all of the steroids, from spiked cell culture media and yeast culture media, by comparing the peak areas of the extracted samples with standard solutions.

Enzymatic assays in P. pastoris expressing CYP17

Single colonies of *P. pastoris* strain GS115 (Invitrogen), previously transformed with wild-type human CYP17 [27], were grown in 25 ml BMGY growth media (1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer [pH 6.0], 1.34% yeast nitrogen base without amino acids, 4×10^{-5}% biotin, and 1% [v/v] glycerol) for 48 h ($OD_{600} = 10.5 \times 10^7$ cells·ml^{-1}) in a shaking incubator at 30 °C.

Cell suspension aliquots (8 ml) were harvested by centrifugation at 2000*g* for 5 min at room temperature, washed with PBS, and resuspended in 2 ml of fresh BMMY induction media (1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer [pH 6.0], 1.34% yeast nitrogen base without amino acids, 4×10^{-5}% biotin, and 0.5% [v/v] methanol). Cells were preincubated for 15 min at 30 °C in an Erlenmeyer flask (100 ml). PREG and PROG metabolism was assayed after the addition of an equal volume of BMMY media supplemented with either PREG (10 μM) or PROG (10 μM). Aliquots of 50 μl were removed at specific time intervals to assay substrate conversion. Control reactions were preformed using the parent vector strain (GS115/pPIC3.5K), which had not been transformed, following the same protocol.

Enzyme assays in COS-1 cells expressing CYP17 and 3βHSD

COS-1 cells were grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 1% penicillin–streptomycin, 4 mM L-glutamine, and 25 mM glucose. Cells were plated in 12-well dishes at 1×10^5 cells/ml 24 h prior to transfection. Cells were transiently transfected with 0.5 μg DNA using GeneJuice transfection reagent (Novagen, Darmstadt, Germany) and were incubated for 72 h before adding steroid substrate. PREG metabolism was assayed in COS-1 cells cotransfected with Angora goat CYP17 (0.25 μg) and 3βHSD (0.25 μg) plas-

mid constructs. PROG metabolism was assayed in COS-1 cells transfected with either Angora goat, baboon, or human CYP17 constructs (0.5 μg). Control transfection reactions were preformed using the mammalian expression vector pCI-neo (Promega, Madison, WI, USA) containing no insert. Substrate conversion was assayed by removing aliquots (0.5 ml) at specific time intervals. On the completion of each experiment, the cells were washed with and collected in 0.1 M phosphate buffer (pH 7.4). The cells were homogenized with a small glass homogenizer, and the protein content of the homogenate was determined by the Pierce BCA method according to the manufacturer's instructions.

Results and discussion

Separation and characteristics of steroids by UPLC–APCI–MS

This study aimed to develop a new method to separate and quantify complex mixtures of seven adrenal steroids that form the substrates and metabolites of CYP17. Current methods use conventional HPLC and rely on retention times alone for the identification of the steroids. This complicates the detection and quantification of steroids from complex biological samples and results in run times of up to 30 min [8]. However, coupling HPLC with MS ion extraction analysis allows the detection and quantification of steroids from complex samples as well as the detection and quantification of steroids that were not well resolved by conventional HPLC previously [28]. In addition, incorporating the new technology of UPLC as an analytical tool has the potential to reduce run times significantly.

A UPLC method was initially developed for the separation of the Δ^5 steroids (PREG, 17-OHPREG, and DHEA) and Δ^4 steroids (PROG, 16-OHPROG, 17-OHPROG, and A4). A cone voltage of 30 V and a capillary voltage of 3.5 kV in MS mode were used for the detection of the Δ^5 steroids. PREG and DHEA exhibited a high abundance of protonated molecules due to the loss of water ($[M - H_2O + H]^+$), whereas 17-OHPREG exhibited high abundance of protonated molecules due to the loss of both a single water molecule ($[M - H_2O + H]^+$) and two water molecules ($[M - 2H_2O + H]^+$) (Fig. 2). The ionization of PREG was further increased by reducing the cone voltage to 15 V. Tandem mass spectrometry (MS/MS) mode resulted in reduced sensitivity due to quantitatively inconsistent daughter ions. Therefore, MS mode was the preferred mode of detection and quantification. Good separation (Fig. 3) was achieved for PREG within 5 min, whereas 17-OHPREG and DHEA eluted within 0.10 min of each other (Table 1). Comparing the spectra of these two steroids revealed that characteristic ion m/z 297 of 17-OHPREG is absent in the spectra for DHEA and similarly that the characteristic ion m/z 271 of DHEA is absent in the spectra for 17-OHPREG (Fig. 2). Therefore, these extracted ions were chosen for quantification.

Using the same system, the Δ^4 steroids exhibited mass spectra with a high abundance of protonated molecules ($[M + H]^+$) (Fig. 2). Good separation was achieved for PROG and 16-OHPROG (Fig. 3), whereas 17-OHPROG and A4 had similar retention times (Table 1). The extracted ions m/z 331, unique to 17-OHPROG, and m/z 287, unique to A4, were chosen for quantification.

The separation of a mixture of the Δ^5 and Δ^4 steroids, using this method, resulted in the coelution of 17-OHPREG, DHEA, 17-OHPROG, and A4 (data not shown). Therefore, the separation of the Δ^5 and Δ^4 steroids necessitated an alternative UPLC method using a longer column and a different solvent system, resulting in an increased run time of 11 min. This method achieved good resolution for all of the steroids, including 17-OHPREG, DHEA, and 17-OHPROG (Fig. 4). Although the retention time of A4 overlapped negligibly with the retention times of 17-OHPREG and DHEA, the extracted ion m/z 287 of A4 was not detected in the 17-OHPREG and DHEA spectra. Similarly, neither of the extracted ions for 17-OHPREG (m/z 297) or DHEA (m/z 271) was detected in the A4 spectra (Fig. 2). PREG and PROG shared the same retention time using this method. Although these steroids could be separated by increasing the gradient elution time, this would unnecessarily increase the run time because each of these steroids is characterized by a unique extracted ion (PREG, m/z 299; PROG, m/z 315).

Recovery efficiencies

The recoveries of steroids were determined by the extraction of standards from both COS-1 cell culture media and yeast cell culture media, followed by UPLC–APCI–MS analyses. The recoveries are shown in Table 2. These data were calculated from the peak areas of the extracted steroids as compared with standards not extracted by media. Steroid recovery from mammalian cell culture media always was higher than that from yeast culture media, with the exception of the recovery of PREG. There are no interfering chromatographic peaks from the mammalian cell culture media (data not shown). Therefore, this effect most likely is due to a matrix effect resulting from the more complex mammalian cell culture media.

The use of an internal standard is not necessary when determining the relative amounts of steroids extracted from heterologous expression media and, therefore, was not used in this study. However, including an internal standard may reduce the variability of the recovery and is recommended, especially when working with low steroid concentrations.

Standard curves

Standard curves were generated for each steroid for concentrations ranging from 10 to 1000 ng/ml. The calibration curves were linear over these concentration ranges, with regression correlation coefficients (r^2) always greater than 0.99.

The limit of detection (LOD) for each steroid was determined by the lowest concentration at which a signal/noise

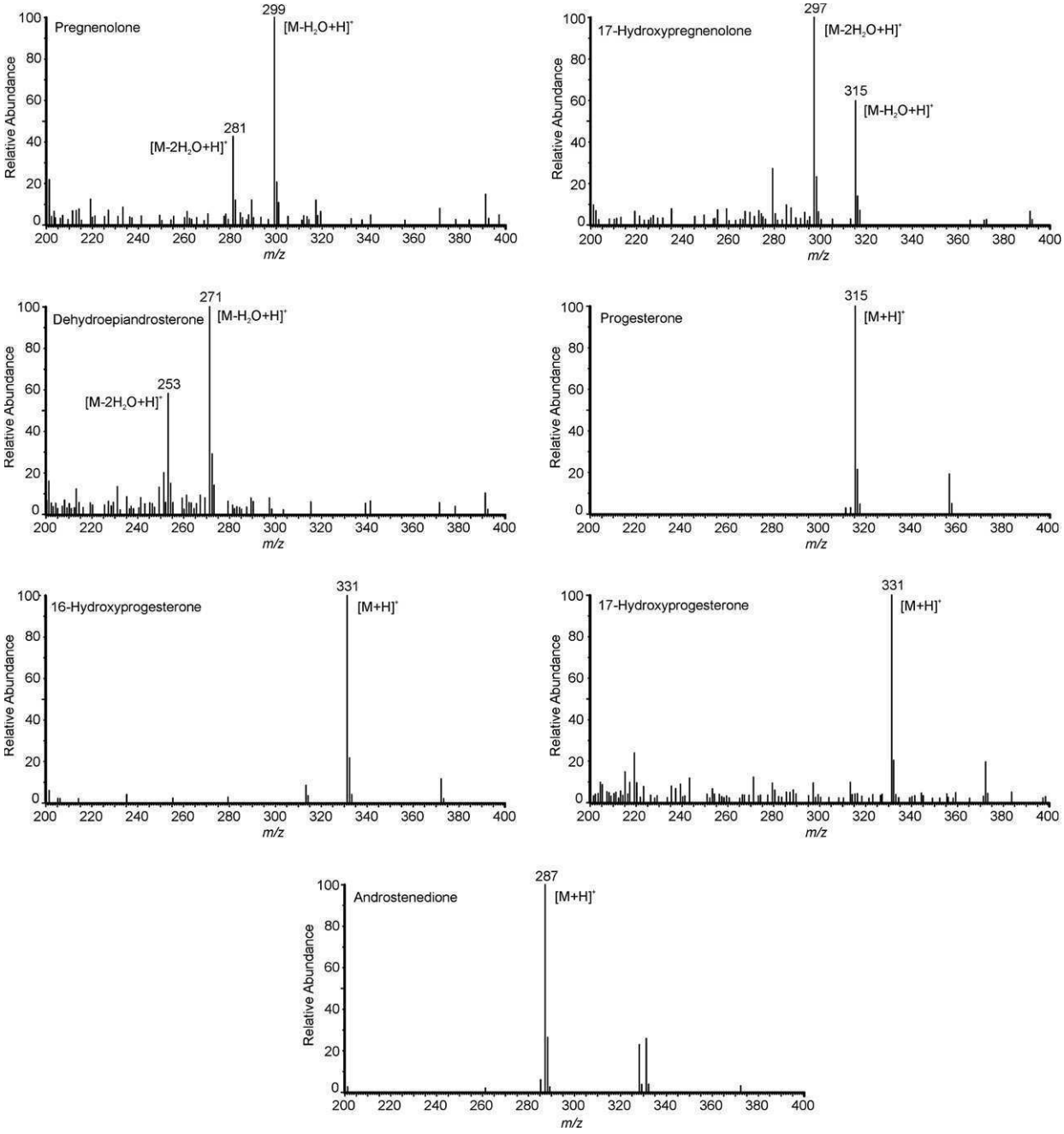

Fig. 2. APCI–MS mass spectra of steroid standards.

ratio greater than 3 was detected. The limit of quantification (LOQ) was determined by the lowest concentration that was detected with a signal/noise ratio greater than 10 and at which the accuracy was within ±20%. The data for LOD and LOQ are shown in Table 2. These values are relevant for the detection of steroids assayed in the heterologous expression systems used in this study.

PREG and PROG metabolism of CYP17 expressed in P. pastoris

Wild-type human CYP17 (GenBank accession number NM000102) was expressed in *P. pastoris,* and the conversion of PROG (10 μM) and PREG (10 μM) was assayed. After 60 min, PROG was metabolized to approximately 63% 17-OHPROG and 37% 16-OHPROG, with no detectable A4, whereas PREG metabolism yielded approximately 76% 17-OHPREG and 14% DHEA (Fig. 5). No PREG or PROG metabolites were detected in *P. pastoris* cells transformed, with the pPIC3.5K vector containing no insert. The 16α-hydroxylation of PROG by CYP17 is characteristic of human CYP17, with 16-OHPROG being identified in humans and other primate species [2,3]. Expression of CYP17 in COS1 cells yielded a 16-OHPROG: 17-OHPROG ratio of 1:4 [2]. In the

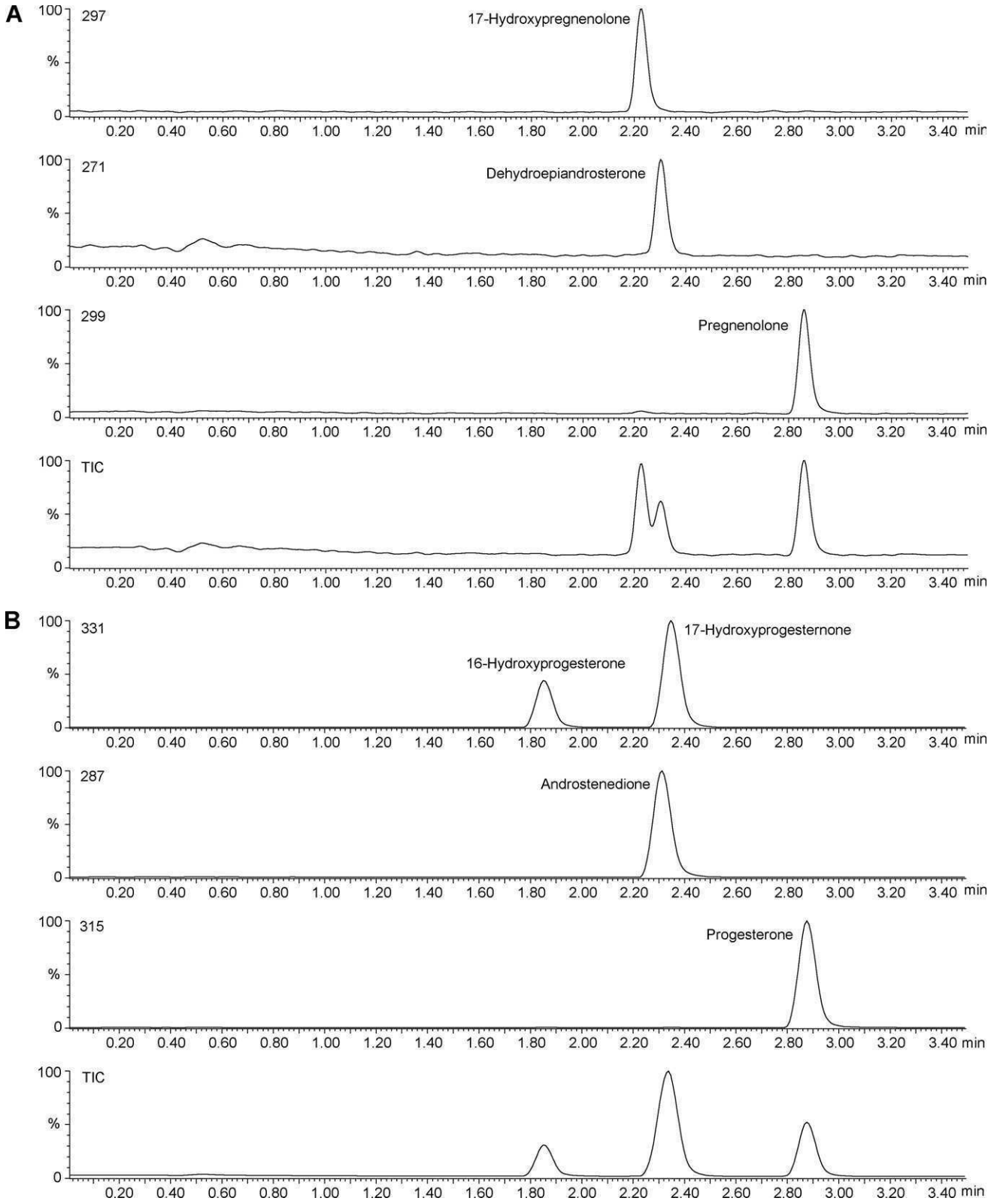

Fig. 3. Selected ion chromatogram of Δ^5 steroid (A) and Δ^4 steroid (B) standards separated on a Waters UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) at 50 °C as described in Materials and methods. Total ion current (TIC) of m/z 200 to 400 for mixtures of standards is also shown. Specification of individual ions (m/z) is as in Table 1.

current study, the ratio obtained in the expression of human CYP17 in *P. pastoris* was approximately 1:2 (16-OHPROG:17-OHPROG).

Table 1
Peak identification, extracted ions, and retention times of adrenal steroids

Steroid	Extracted ion (*m*/*z*)	Retention time (min)	
		50-mm column	100-mm column
PREG	299	2.9	7.6
17-OHPREG	297	2.2	4.8
DHEA	271	2.3	5.2
PROG	315	2.9	7.5
16-OHPROG	331	1.9	2.8
17-OHPROG	331	2.3	5.7
A4	287	2.3	4.9

PREG and PROG metabolism of CYP17 expressed in COS-1 cells

Angora goat CYP17 (GenBank accession number EF524063) and 3βHSD (GenBank accession number EF524065) were transiently cotransfected in nonsteroidogenic mammalian COS-1 cells, and the conversion of PREG (1 μM) was assayed. In the metabolism of PREG, the major metabolites detected were 17-OHPROG (∼52%) and A4 (∼32%) (Fig. 6). The PROG levels remained low during the entire time course, reaching a maximum of approximately 7% at 3 h, indicating that on formation PROG was metabolized to 17-OHPROG by CYP17. Similarly, 17-OHPREG reached a maximum of

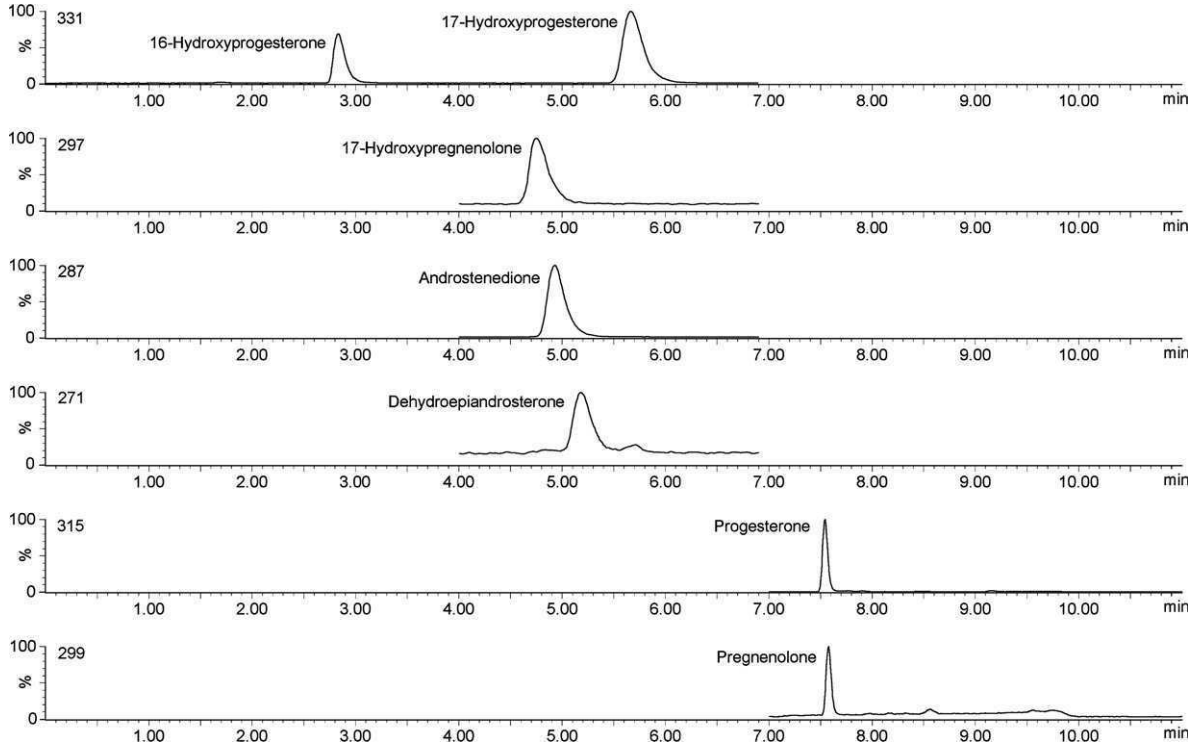

Fig. 4. Selected ion chromatogram of PREG, 17-OHPREG, DHEA, PROG, 16-OHPROG, 17-OHPROG, and A4 standards separated on a Waters UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) at 50 °C as described in Materials and methods. Specification of individual ions (*m*/*z*) is as in Table 1.

Table 2
Summary of method performance characteristics

Steroid	LOD (ng/ml)	LOQ (ng/ml)	Reproducibility[a] (200 ng/ml) (% RSD)		Recovery[b] (200 ng/ml) (%)	
			COS-1 cells	Yeast	COS-1 cells	Yeast
PREG	20	30	2.6	9.7	77 ± 2	111 ± 10
17-OHPREG	20	40	4.8	14.9	110 ± 5	91 ± 13
DHEA	40	110	5.0	15.6	118 ± 6	72 ± 11
PROG	5	10	17.3	13.6	111 ± 6	102 ± 17
16-OHPROG	10	10	12.9	15.8	116 ± 3	68 ± 1
17-OHPROG	5	10	14.1	19.4	120 ± 1	90 ± 12
A4	10	10	11.2	15.5	114 ± 5	83 ± 4

[a] $n = 6$.
[b] $n = 3$.

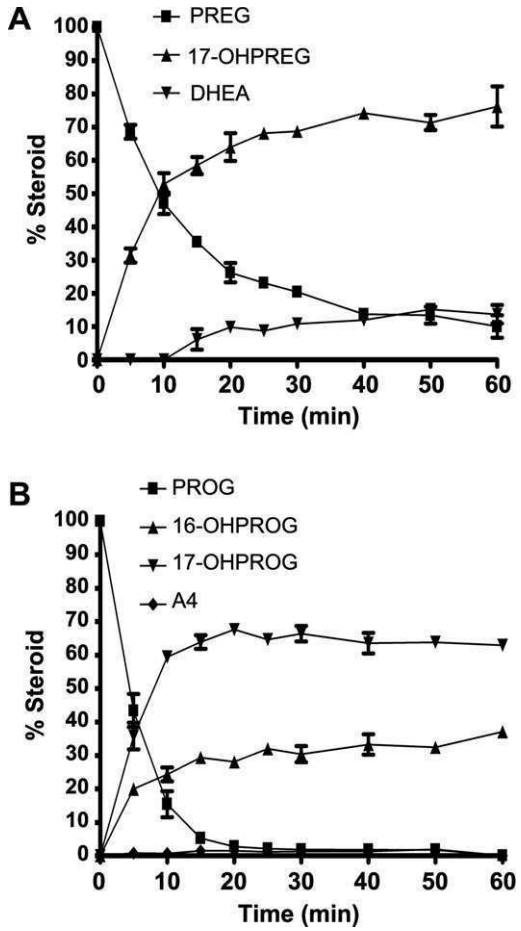

Fig. 5. Time course of PREG (10 μM) (A) and PROG (10 μM) (B) metabolism by human CYP17-expressed *P. pastoris*.

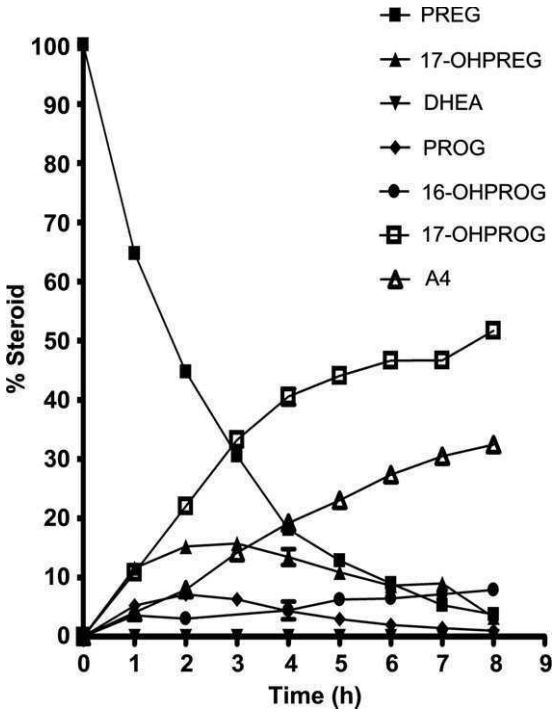

Fig. 6. Time course of PREG (1 μM) metabolism by Angora goat CYP17 and 3βHSD coexpressed in COS-1 cells.

approximately 15% at 3 h, demonstrating that on formation 17-OHPREG was metabolized to DHEA by CYP17 and to 17-OHPROG by 3βHSD. Because Angora goat CYP17 is unable to metabolize the conversion of 17-OHPROG to A4 (data not shown), 17-OHPROG is the end product of the Δ^4 pathway. No DHEA was detected due to the immediate conversion of DHEA to A4 by 3βHSD.

COS-1 cells expressing Angora goat CYP17 and 3βHSD also produced low, yet detectable, levels of 16-OHPROG (Fig. 6). To date, only CYP17 from primate species has been shown to convert PROG to 16-OHPROG [2,3]. Human CYP17 (GenBank accession number NM000102), baboon CYP17 (GenBank accession number AF297650), and Angora goat CYP17 were subsequently expressed in COS-1 cells and assayed for activity with PROG (1 μM) as substrate. The 17-OHPROG/16-OHPROG ratio was determined after 8 h (Fig. 7). As expected, human CYP17 produced the highest levels of 16-OHPROG, with a 17-OHPROG/16-OHPROG ratio of 2. Baboon CYP17 produced significantly less 16-OHPROG (17-OHPROG/16-OHPROG ratio of 7), whereas Angora goat CYP17 produced significantly less 16-OHPROG than did both primate species, with a 17-OHPROG/16-OHPROG ratio of 22. Our data indicate that CYP17 of other species may well produce small amounts of 16-OHPROG but that the detection methods used previously were unable to detect these low levels. The physiological implications of this finding will be investigated in the future.

Conclusion

We have developed a UPLC–APCI–MS method for the rapid and accurate quantification of seven adrenal steroids that are structurally very similar and thus, difficult to separate effectively. The incorporation of UPLC allows for higher throughput and smaller sample volumes than do current methods, whereas the use of MS offers better selectivity as well as better LODs and LOQs. This method was used successfully for the quantification of both PREG and PROG metabolites formed by CYP17 expressed in the yeast *P. pastoris* and in nonsteroidogenic COS-1 cells. In addition, 16-OHPROG production by CYP17 was indicated for the first time in a nonprimate species.

Acknowledgments

The authors thank Patricia Storbeck for help with the preparation of this manuscript. This work was financially supported by the South African Mohair Council, National Research Foundation (NRF), University of Stellenbosch, and Wilhelm Frank Bursary Fund.

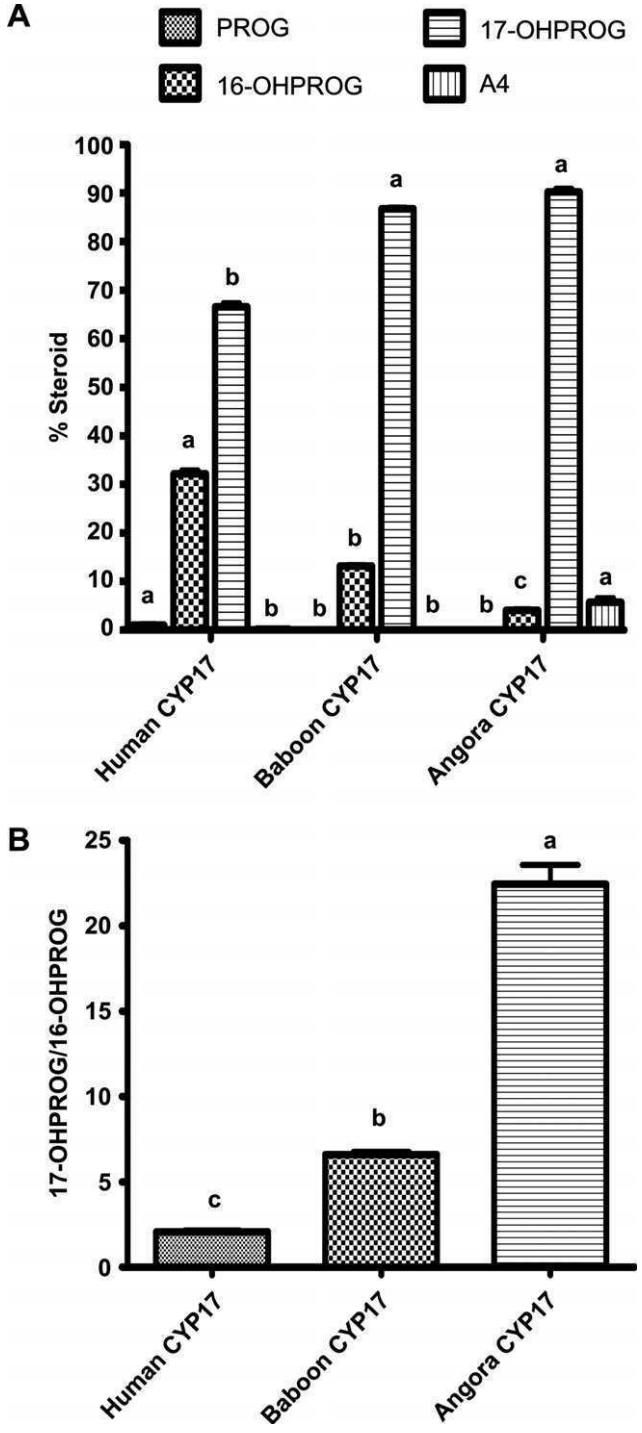

Fig. 7. (A) Steroid profile of PROG (1 μM) metabolism after 8 h by human, baboon, and Angora goat CYP17 expressed in COS-1 cells. Individual steroids were compared for each construct by a one-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. The letters above the columns indicate the statistical difference among the three species for each individual steroid; columns with the same letter have no significant difference, whereas columns with different letters are significantly different ($P < 0.05$). (B) Ratios of 16-OHPROG and 17-OHPROG formation during PROG (1 μM) metabolism by human, baboon, and Angora goat CYP17 expressed in COS-1 cells. Ratios were compared for each species by a one-way ANOVA followed by a Bonferroni's multiple comparison test ($P < 0.001$). Results are representative of at least three independent experiments.

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5.2 Conclusion

This chapter presented the development and validation of a novel UPLC-LC-MS method for the quantification of seven adrenal steroids. These steroids have traditionally been difficult to resolve chromatographically and detect spectrophotometrically. The combination of UPLC with mass spectrometry has greatly simplified the simultaneous analysis of these metabolites. This method has therfore provided the analytical tool necessary for investigating the role of CYP17 in the hypocortisolism observed in the South African Angora goat. The cloning, sequencing and *in vitro* expresion of two Angora CYP17 isoforms are presented in the following chapter.

CHAPTER 6
THE IDENTIFICATION OF TWO CYP17 ALLELES IN THE SOUTH AFRICAN ANGORA GOAT

6.1 Introduction

Evidence indicating the existence of two CYP17 isoforms in the South African Angora goat suggested that one of these isoforms may contribute towards the observed hypocortisolism (Slabbert, 2003). This chapter describes an investigation into the validity of this hypothesis. Both CYP17 isoforms, as well as 3βHSD and cytochrome b_5, were subsequently cloned and expressed in nonsteroidogenic COS-1 cells. The K_m and *V*-values for the metabolism of pregnenolone and progesterone were determined. In addition, the influence of cytochrome b_5 on the activity of the two CYP17 isoforms was investigated. Each isoform was subsequently coexpressed with 3βHSD to investigate the possible effects that the catalytic activity of the two isoforms may have on the outcome of adrenal steroidogenesis. The results were published in Drug Metabolism Reviews and are presented in this chapter (Storbeck et al., 2007). For completeness the sequencing data and restriction digest based genotyping results obtained by Slabbert (unpublished data) were included in this paper. The majority of the work was, however, novel to this study.

Drug Metabolism Reviews, 39: 467–480, 2007

ISSN: 0360-2532 print / 1097-9883 online
DOI: 10.1080/03602530701468649

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THE IDENTIFICATION OF TWO CYP17 ALLELES IN THE SOUTH AFRICAN ANGORA GOAT

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South African Angora goats* (Capra hircus) *are susceptible to cold stress, due to the inability of the adrenal cortex to produce sufficient levels of cortisol. Two CYP17 isoforms were identified, cloned and characterized in this study. Sequence analysis revealed three amino acid differences between the two CYP17 isoforms, which resulted in a significant difference in 17,20 lyase activity of the expressed enzymes in both the presence and absence of cytochrome b_5. Furthermore, cotransfections with 3βHSD revealed that one CYP17 isoform strongly favours the Δ^5 steroid pathway. Our data implicates CYP17 as the primary cause of the observed hypoadrenocorticoidism in the South African Angora goat.

Key words: Cytochrome P450; 17α-hydroxylase/17–20 lyase; CYP17; Angora goat; Hypoadrenocorticoidism; Stress.

INTRODUCTION

South African Angora goats (*Capra hircus*) are the most efficient fibre producing, but least hardy, small stock breed in Southern Africa. South Africa produces approximately 4 million kg mohair annually, of which more than 95% is exported, supplying close to 60% of the international demand for mohair. However, severe loss of young, newly shorn Angora goats, which occur during cold spells, hamper the industry.

Previous research revealed that an abrupt drop in glucose concentration was the crucial factor responsible for the inability of the Angora goat to produce the metabolic heat required to survive cold spells (Wentzel et al., 1979; Wentzel, 1987). In mammals physiological stress stimulates the release of glucocorticoids from the adrenal cortex, via the hypothalamic-pituitary-adrenal (HPA) axis, which favours glucose production at the expense of glycolysis (Munch, 1971). Van Rensburg (1971) reported that selection for mohair production resulted in reduced adrenal function and hypoadrenocorticoidism. Furthermore, Engelbrecht et al. (2000) showed that the stimulation of the HPA axis with insulin and ACTH *in vivo* resulted in less cortisol being produced in Angora goats when compared to Boer goats (*Capra hircus*) and Merino sheep (*Ovis aries*). These results clearly indicated that adrenocortical insufficiency was a major contributing factor to the vulnerability of the South African Angora goat to stress.

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In addition to the production of the glucocorticoids, the adrenal cortex is also responsible for biosynthesis of the mineralocorticoids and adrenal androgens. Pregnenolone (PREG), the common precursor for each of these hormones, serves as the substrate for both cytochrome P450 17α-hydroxylase/17–20 lyase (CYP17) and 3β-hydroxysteroid dehydrogenase (3βHSD). The 17α-hydroxylation of the Δ^5- and Δ^4-steroids, PREG and progesterone (PROG), by CYP17 yields 17-hydroxypregnenolone (17-OHPREG) and 17-hydroxyprogesterone (17-OHPROG), respectively. The 17,20 lyase reaction of CYP17 catalyzes the cleavage of the C17,20 of 17-OHPREG and 17-OHPROG to yield dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively (Nakajin and Hall, 1981; Nakajin et al., 1981; Zuber et al., 1986) In addition, it is possible for human CYP17, and that of other primate species, to convert PROG to 16α-hydroxyprogesterone (16-OHROG) (Swart el al., 1993; Arlt et al., 2002). 3βHSD converts the 3β-hydroxy-Δ^5-steroid precursors PREG, 17-OHPREG and DHEA to the corresponding Δ^4 3-ketosteroids, PROG, 17-OHPROG and A4 (Thomas et al., 1989). PROG and 17-OHPROG are substrates for cytochrome P450 21-hydroxylase (CYP21), which commits PROG to aldosterone biosynthesis and 17-OHPROG to cortisol biosynthesis. Due to the competition between CYP17 and 3βHSD for the same substrates, the ratio and substrate specificities of these two enzymes play a critical role in determining the steroidogenic output of the adrenal cortex.

Using subcellular fractions, prepared from adrenocortical tissue, Engelbrecht and Swart (2000) demonstrated that the Angora goat produced significantly more DHEA and less glucocorticoid precursors when compared to Boer goats and Merino sheep. Furthermore, this study selectively compared the enzymatic activity of CYP17, 3βHSD and CYP21 between the three species and found no difference in the activities of 3βHSD and CYP21. The 17,20 lyase activity of CYP17, resulting in DHEA production, was significantly greater in the Angora goat than in the other two species, resulting in the conclusion that the catalytic properties of Angora CYP17 may contribute to the observed hypoadrenocorticoidism.

CYP17 is the product of a single gene, expressed in both the adrenals and gonads (Nakajin et al., 1981). The hydroxylase activity of CYP17 is similar amongst different species, while marked differences occur in the 17,20 lyase activity (Brock and Waterman, 1999). The dual activity of this unique enzyme originates from a single active site, although the precise mechanism of catalysis is still unknown. The activity of CYP17 is not only influenced by the environment in which the enzyme is expressed, but also by redox partner and/or accessory proteins. The hydroxylation reaction requires molecular oxygen and the input of two electrons from its electron-transfer partner, FAD/FMN-dependent NADPH-cytochrome P450 reductase, while the 17,20 lyase reaction involves a further two rounds of mono oxygenation and is enhanced by the availability of reducing equivalents. Furthermore, the 17,20 lyase activity of CYP17 is enhanced allosterically by cytochrome b_5 and by post-translational phosphorylation (Pandey and Miller, 2005).

The aim of this study was to investigate the catalytic properties of Angora CYP17 and to determine to what extent CYP17 is responsible for the low cortisol output by the adrenal cortex. Two CYP17 alleles were identified in the South African Angora goat. The hydroxylase and 17,20 lyase activity of the two isoforms was investigated away from the influence of other steroidogenic enzymes. Both CYP17 isoforms were also expressed in the presence of both 3βHSD and cytochrome b_5 to determine to what extent the competition for mutual substrates and the modulating effect of cytochrome b_5 influence the catalytic output of the adrenal cortex.

MATERIALS AND METHODS

Isolation and Cloning of CYP17, 3βHSD, and Cytochrome b_5

Angora goat adrenal glands and liver were obtained from the abattoir, flash frozen and stored in liquid N_2 until use. Polyadenylated (poly A^+) RNA was prepared from Angora adrenal cortex using a mRNA Capture kit (Roche Applied Science, Mannheim, Germany). RT-PCR was subsequently carried out using the Titan™ One Tube RT-PCR system (Roche Applied Science, Mannheim, Germany). The reverse transcription reaction was performed at 50°C for 30 min followed immediately by thermocycling. The CYP17 and 3βHSD RT-PCR products were subsequently cloned into the pcDNA3.2/V5/GW/D-TOPO® mammalian expression vector (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Similarly, cytochrome b_5 *c*DNA was prepared from Angora liver and cloned as above. In addition, the RT-PCR products were amplified by PWO polymerase (Roche Applied Science, Mannheim, Germany) and subjected to direct sequence analysis. The primers used for the amplification of CYP17, 3βHSD and cytochrome b_5 are listed in Table 1. Plasmid constructs were screened by restriction digestion analysis and positive clones were subsequently subjected to sequence analysis. All DNA sequencing was preformed on an ABI Prism 3100 Genectic Analyzer (Applied Biosystems, Foster city, California).

Isolation and Genotyping of Genomic DNA

Genomic DNA was isolated from fresh Angora goat blood using the Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin). Genomic DNA was amplified using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, California) with the primers shown in Table 1. The resulting 6 kbp fragment was gel purified prior to direct sequence analysis. Furthermore, genomic DNA was amplified using PWO DNA Polymerase High Fidelity (Roche Applied Science, Mannheim, Germany) with the primers shown in Table 1 prior to genotyping. The resulting 1.2 kbp fragment was gel purified and subjected to restriction digest analysis using ACS1 (Roche Applied Science, Mannheim, Germany).

Table 1 Primer sequences used in the amplification of Angora goat CYP17, 3βHSD and cytochrome b_5. The primers used for the genotyping and mutagenesis of CYP17 are shown, with the codons for the changed amino acid indicated in bold, underlined text in P41L LP and L41P LP.

Primer	Oligonucleotide sequence
CYP17TOPO (Sense)	5′-CACCGTCGACGCCACTCCACAGCTCTTTGT-3′
CYP17RP (Antisense)	5′-GGAGGAAGAAGGAATGGTGG-3′
3βHSDTOPO (Sense)	5′-CACCTGGCTTGCCACAATC-3′
3βHSDRP (Antisense)	5′-CGGAAGGCAGATAGTAAGG-3′
CYB5TOPO (Sense)	5′-CACCCTCGCTGAGTTAAGAAATG-3′
CYB5RP (Antisense)	5′-CTCCCTGGACCAAAGCAG-3′
ACS LP (Sense)	5′-GAGATCCTGTCAGACAACCA-3′
ACS RP (Antisense)	5′-TTTCAACACAACCCTTCATC-3′
P41L LP (Sense)	5′-CTGGTGGGCAGCCTG**CTG**TTCCTCCCC-3′
P41L RP (Antisense)	5′-GCAGGCTGCCCACCAGGGGCAGGGATG-3′
L41P LP (Sense)	5′-CTGGTGGGCAGCCTG**CCG**TTCCTCCCC-3′
L41P RP (Antisense)	5′-GCAGGCTGCCCACCAGGGGCAGGGATG-3′

Site-Directed Mutagenesis

Site-directed mutagenesis of Angora CYP17 was carried out with the Gene Tailor™ site-directed mutagenesis system (Invitrogen, Carlsbad, California) according to the manufacturer's instructions, using the mutagenic primers listed in Table 1. The mammalian expression constructs, containing the cloned Angora CYP17 *c*DNA, were used as templates. The mutations L41P and P41L were confirmed by direct sequence analysis.

Enzymatic Assays in Transiently Transfected COS-1 Cells

COS-1 cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 4 mM L-glutamine, and 25 mM glucose. Cells were plated in 12 well dishes at 1×10^5 cells/ml, 24 h prior to transfection. CYP17 plasmid constructs (0.5 μg) were transiently transfected into COS-1 cells using Genejuice transfection reagent (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. Control transfection reactions were preformed using the mammalian expression vector pCI-neo (Promega, Madison, Wisconsin) containing no insert. Cotransfections of CYP17 and cytochrome b_5, and of CYP17, 3βHSD, and cytochrome b_5, were preformed using an equal amount of each construct up to a total of 0.5 μg of plasmid DNA. When cytochrome b_5 was excluded, the cytochrome b_5 plasmid was replaced by pCI neo (Promega, Madison, Wisconsin) containing no insert. After 72 h, enzymatic activities were assayed using PREG (1 μM), 17-OHPREG (1 μM), and PROG (1 μM) as substrates. Aliquots of either 50 μl, for HPLC analysis, or 500 μl, for UPLC-APCI-MS analysis, were removed at specific time intervals. On completion of each experiment, the cells were washed with and collected in 0.1 M phosphate buffer, pH 7.4. The cells were subsequently homogenised with a small glass homogeniser and the protein content of the homogenate determined by the BCA method (Pierce Chemical, Rockford, Illinois) according to the manufacturer's instructions.

Extraction of Steroids

Steroids were extracted from the incubation media by liquid-liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 2 min, centrifuged at 500 g for 5 min, after which the water phase was aspirated off. The organic phase was transferred to a clean extraction glass tube and the samples were dried under a stream of nitrogen. The dried steroids were dissolved in 100 μl methanol prior to analysis.

HPLC Separation and Quantification of Steroids

Chromatography was preformed on a high performance liquid chromatograph (P4000, Thermo Separation Products, San Jose, California) attached to a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, Florida). The ratio of scintillant to column effluent was 3:1. PREG and PROG, and their respective metabolites were separated on a Phenomenex C12 (60 mm×150 mm, 4 μm) column at a flow rate of 1 ml/min. The mobile phases consisted of solvent A (3:1 methanol:water) and solvent B (methanol). The column was eluted isocratically with 100% A for 4 min, followed by a 5-min gradient elution from 100% A to 100% B and an isocratic elution for a further 2 min. A linear

gradient returned the column to 100% A in 3 min. Kinetic constants (K_m and V values) were determined by non-linear regression using GraphPad Prism (version 4) software (GraphPad Software, San Diego, California).

UPLC-APCI-MS Separation and Quantification of Steroids

Steroids were separated by UPLC (ACQUITY UPLC, Waters, Milford, Massachusetts) using a Waters UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm) column at 50°C. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (3:1 acetonitrile:methanol with 1% isopropanol). The column was eluted isocratically with 56% A and 44% B for 6 min, followed by a linear gradient from 44% B to 80% B in 0.01 min. A linear gradient was subsequently followed from 80% B to 100% B in 2.49 min after which a linear gradient returned the column to 56% A and 44% B in 0.5 min. The total run time per sample was 11 min at a flow rate of 0.3 ml/min. The injection volume of standards and samples was 5 μl.

An API Quattro Micro tandem mass spectrometer (Waters, Milford, Massachusetts), was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters, Milford, Massachusetts) was used for the atmospheric pressure-chemical ionization interface in positive mode. The corona pin was set to 7 μA, the cone voltage 30V and APCI probe temperature was 450°C. All other settings were optimised to obtain the strongest possible signal. Calibration curves were constructed by using weighted linear least squares regression. Data was collected with the MassLynx (version 4) software program (Waters, Milford, Massachusetts).

RESULTS

Characterization of Angora CYP17, 3βHSD, and Cytochrome b_5 *c*DNA

The RT-PCR amplification of Angora goat CYP17 mRNA from multiple adrenal glands consistently yielded single 1.6 kbp products which were subsequently cloned. Sequence analysis of the RT-PCR products revealed four nucleotide base changes indicated by the double nucleotide base pair peaks in the coding region at positions 17, 122, 637, and 1065, suggesting the presence of more than one CYP17 isoform. Genomic DNA was subsequently investigated and sequencing confirmed the presence of two CYP17 alleles. Analysis of the cloned RT-PCR products identified two CYP17 *c*DNAs—the first *c*DNA (GenBank accession no. **EF524064**) is 100% homologous with Boer goat (*Capra hircus*) CYP17 *c*DNA (GenBank accession no. **AF251387**) and was named CYP17 ACS+ due to the presence of an ACS1 restriction site at position 635–640; the second *c*DNA (GenBank accession no. **EF524063**) was named CYP17 ACS- due to the absence of the ASC1 restriction site at position 635–640. In addition this ACS1 restriction site was used for genotyping. Of the 83 goats genotyped, 24 were homozygous for CYP17 ACS– and 59 were heterozygous. No goats homozygous for CYP17 ACS+ were detected.

CYP17 ACS+ and ACS– share 99.74% homology, with the three nucleotide differences at positions 17, 122, and 637, resulting in amino acid substitutions at positions 6, 41, and 213. CYP17 ACS+ has glycine, leucine, and isoleucine residues at these positions, while in CYP17 ACS– these residues are replaced by alanine, proline, and valine residues, respectively. The substitutions at positions 6 and 213 are both conservative and not expected to affect the three-dimensional structure of the enzyme. The non-conservative

amino acid substitution P41L, lies in the highly conservative proline rich sequence (PR) which is critical for the correct folding of all cytochromes P450 (Yamazaki et al., 1993; Kusano et al., 2001a; Kusano et al., 2001b). The absence of a proline residue at this position in CYP17 ACS+ may therefore influence the folding of the enzyme, resulting in a change in the three-dimensional structure and enzymatic activity of the protein. Both CYP17 isoforms were therefore mutated at this position to the corresponding proline or leucine residue in order to investigate the possible influence of this amino acid substitution on the enzymatic activity of CYP17. The sequence of the mutated constructs, CYP17 ACS– P41L and CYP17 ACS+ L41P, were subsequently confirmed by direct sequence analysis.

The RT-PCR amplification of Angora goat cytochrome b_5 mRNA yielded a single 0.45 kbp product which was subsequently cloned and sequenced. Angora cytochrome b_5 *c*DNA (GenBank accession no. **EF524066**) encodes for a predicted 134 amino acid protein and shares 96.27% with Bovine cytochrome b_5 (GenBank accession no. **X13617**), the most closely related species available on Genbank. Amplification of Angora goat 3βHSD mRNA by RT-PCR yielded a single 1.2 kbp product. Angora goat 3βHSD *c*DNA (GenBank accession no. **EF524065**) encodes for a predicted 373 amino acid protein and shares 97.32% with Bovine 3βHSD (GenBank accession no. **NM_174343**).

Kinetic Analysis of Angora CYP17 Isoforms

Both CYP17 isoforms were expressed in COS-1 cells and assayed for activity with PREG and PROG as substrates. The transfected COS-1 cells converted PREG to 17-OHPREG and DHEA, while PROG metabolism yielded 17-OHPROG and low, but detectable, levels of 16-OHPROG. No A4 was detected after 8 h of incubation. The K_m and V values were determined for PREG and PROG by non-linear regression (Table 2). No significant difference in K_m values were observed between the two CYP17 isoforms with PREG as substrate. In contrast, CYP17 ACS+ has a K_m value that is significantly greater than that of CYP17 ACS– for PROG. Unlike the K_m values, which are not dependent on enzyme concentration and can be directly compared to one another if obtained under identical experimental conditions, the V values are influenced by enzyme concentration. The observed variations in V values obtained for the two expressed CYP17 isoforms may therefore result from differing transfection efficiencies. It is, however, interesting to note that the V values obtained for the CYP17 ACS– are significantly greater than those obtained for CYP17 ACS+ expressed under identical conditions for both PREG and PROG.

Table 2 Summary of kinetics of PREG and PROG metabolism by Angora goat CYP17 ACS– and ACS+ expressed in COS-1 cells. The initial reaction rates were determined by linear regression for each substrate concentration. At least four time points were used for each rate determination. The R-squared value for all initial rate regressions were always higher than 0.95. K_m values are expressed as the mean ± SEM of three replicate experiments. K_m values were compared for each substrate with an unpaired *t* test. *p < 0.05.

	Pregnenolone		Progesterone	
	K_m (μM)	V †	K_m (μM)	V †
CYP17 ACS–	2.6 ± 0.4	95.2 ± 5.0	1.2 ± 0.2	31.1 ± 1.1
CYP17 ACS+	1.8 ± 0.4	54.3 ± 3.7*	2.2 ± 0.2*	25.4 ± 0.7*

†(nmol·h^{-1}·mg total protein^{-1}).

The enzymatic activity of the CYP17 isoforms and mutants were further investigated by expressing the constructs in COS-1 cells. After 8 h, CYP17 ACS–, ACS+ and ACS+ L41P metabolized ≈90% of the PREG substrate to ≈80% 17-OHPREG and ≈10% DHEA, while only ≈58% PREG was metabolized to 17-OHPREG by ACS– P41L with no detectable DHEA. In cotransfections carried out with CYP17 and cytochrome b_5, the addition of cytochrome b_5 resulted in a substantial increase in 17,20 lyase activity for all CYP17 constructs. However, the effect was significantly greater for CYP17 ACS– and ACS+ L41P, which both completely metabolized PREG to DHEA in 8 h, while CYP17 ACS+ and ACS– P41L only produced ≈69% and 79% DHEA, respectively (Fig. 1B).

CYP17 ACS– and ACS+ L41P demonstrated a greater 17,20 lyase activity than CYP17 ACS+ and ACS– P41L when 17-OHPREG was used as substrate, both in the presence and absence of cytochrome b_5 (Fig. 2).

Similarly to PREG metabolism, ACS–, ACS+ and ACS+ L41P shared comparable steroid profiles when metabolizing PROG, each metabolizing more than 95% PROG to ≈92% 17-OHPROG and ≈6% 16-OHPROG, whereas CYP17 ACS– P41L metabolized ≈83% PROG to ≈79% 17-OHPROG and ≈4% 16-OHPROG (Fig. 3A). The addition of cytochrome b_5 resulted in a significant increase in the formation of 16-OHPROG for all CYP17 constructs. Although no A4 was detected in the absence of cytochrome b_5, A4 was detected for both CYP17 ACS– and ACS+ L41P, albeit less than 5% (Fig. 3B), upon addition of cytochrome b_5.

Cotransfection of CYP17 and 3βHSD

In the adrenal CYP17 and 3βHSD compete for the same substrates, with the ratio and the substrate specificities of these two enzymes determining the steroidogenic output of the adrenal cortex. Both isoforms were therefore cotransfected in COS-1 cells with 3βHSD to determine if the differing activities observed for the two CYP17 isoforms could

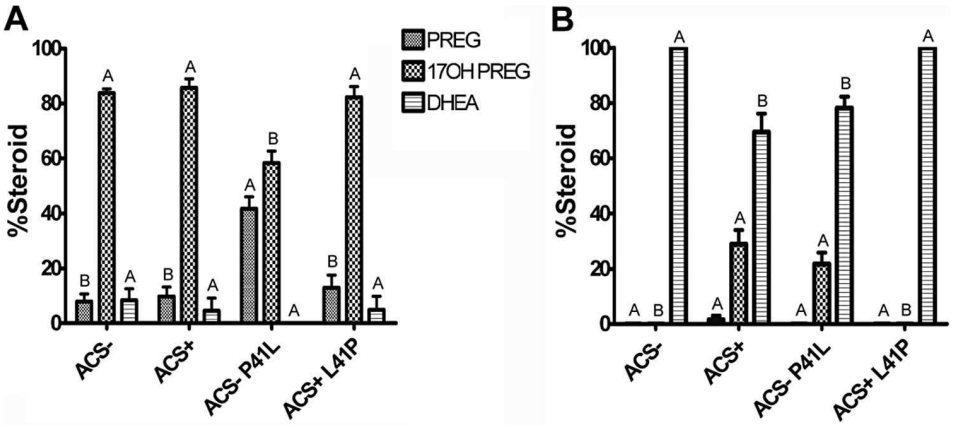

Figure 1 Steroid profile of PREG (1 μM) metabolism after 8 h by Angora goat CYP17 ACS–, ACS+ and mutant constructs CYP17 ACS-P41L and ACS+L41P expressed in COS-1 cells. Substrate conversion was assayed without cytochrome b_5 (A) and in the presence of cytochrome b_5. (B). Individual steroids were compared for each construct by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p < 0.05$), Results are representative of at least three independent experiments.

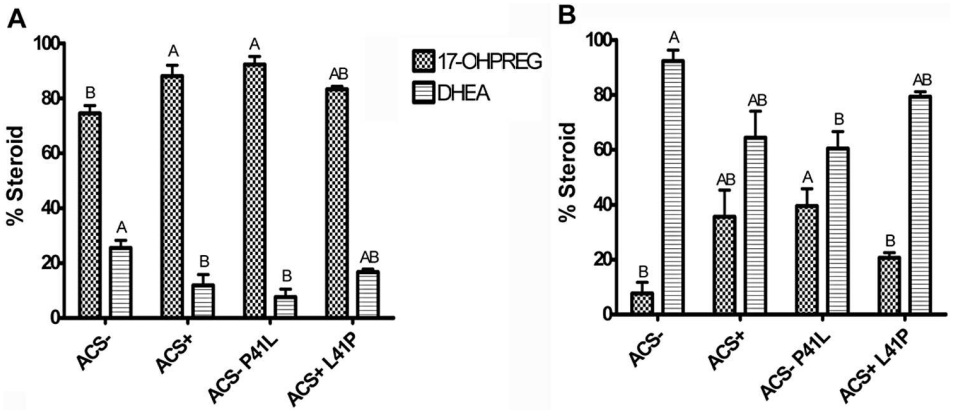

Figure 2 Steroid profile of 17-OHPREG (1 μM) metabolism after 8 h by Angora goat CYP17 ACS–, ACS+ and mutant constructs CYP17 ACS– P41L and ACS+ L41P expressed in COS-1 cells. Substrate conversion was assayed without cytochrome b_5 (A) and in the presence of cytochrome b_5. (B). Individual steroids were compared for each construct by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p < 0.05$). Results are representative of at least three independent experiments.

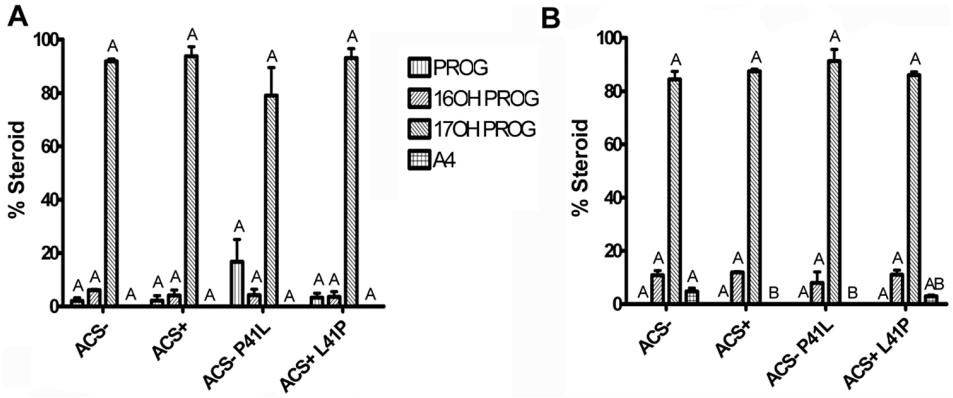

Figure 3 Steroid profile of PROG (1 μM) metabolism after 8 h by Angora goat CYP17 ACS–, ACS+, and mutant constructs CYP17 ACS– P41L and ACS+ L41P expressed in COS-1 cells. Substrate conversion was assayed without cytochrome b_5 (A) and in the presence of cytochrome b_5. (B). Individual steroids were compared for each construct by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p < 0.05$), Results are representative of at least three independent experiments.

contribute to the observed hypoadrenocorticoidism in the Angora goat. In addition, cotransfections were carried out in the presence of cytochrome b_5 to determine if the catalytic outcome would be influenced by an effector of CYP17. The enzymatic activity of 3βHSD for PREG, 17-OHPREG and DHEA was confirmed prior to cotransfections with CYP17 (data not shown). After 8 h COS-1 cells cotransfected with CYP17 ACS– and 3βHSD metabolized PREG to 17-OHPROG (≈61%) and A4 (≈21%). The steroid output of COS-1 cells cotransfected with CYP17 ACS+ and 3βHSD was distinctly different, with PREG metabolized to PROG (≈16%), 17-OHPROG (≈54%) and A4 (≈9%). Furthermore, the difference in 17,20 lyase activity between the two CYP17 isoforms expressed in

COS-1 cells, was even more pronounced during cotransfections with 3βHSD due to the competition of both enzymes for 17-OHPREG (Fig. 4). As Angora CYP17 is unable to metabolize 17-OHPROG to A4 (Fig. 3), this difference is indicated by A4 derived from the conversion of DHEA by 3βHSD. The inclusion of cytochrome b_5 in the cotransfections resulted in an even greater difference in the steroid profiles of PREG metabolism, with CYP17 ACS– transfected COS-1 cells producing predominantly A4 (≈62%), while the CYP17 ACS+ transfected COS-1 cells produced predominantly 17-OHPROG (≈50%) (Fig. 4B). The difference in A4 production in both the presence and absence of cytochrome b_5 can be attributed to the greater 17,20 lyase activity of CYP17 ACS–, which causes a greater flux through the Δ^5 pathway, with a resulting decrease of cortisol precursors (Fig. 5).

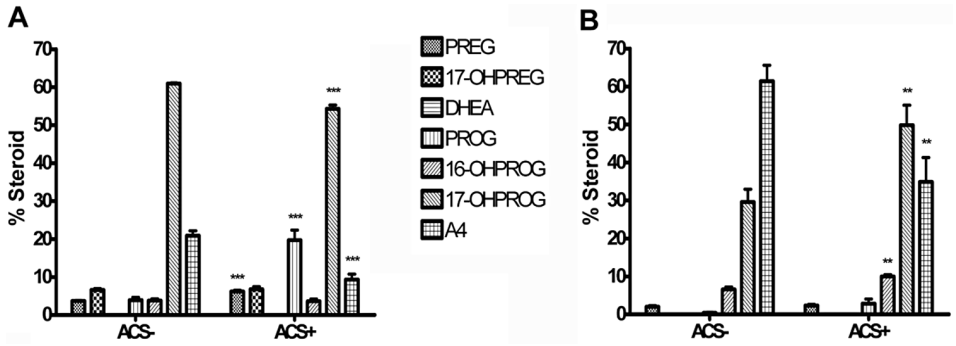

Figure 4 Steroid profile of PREG (1 μM) metabolism after 8 h by Angora goat CYP17 and 3βHSD coexpressed in COS-1 cells, without cytochrome b_5 (A) and in the presence of cytochrome b_5. (B). Individual steroids were compared for each construct by unpaired *t* test (**p < 0.01, ***p <0.001). Results are representative of at least three independent experiments.

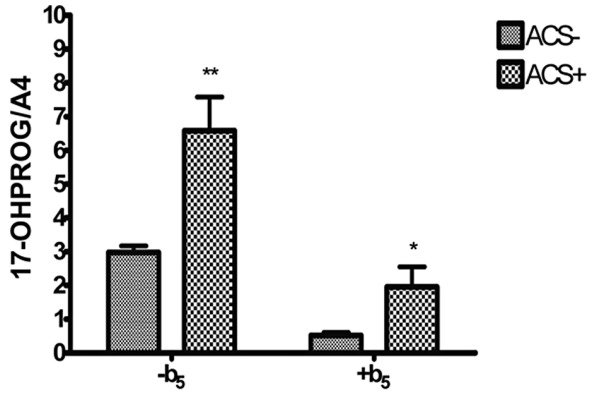

Figure 5 Ratio of end products, 17-OHPROG and A4, of the metabolism of PREG (1 μM) after 8 h by Angora CYP17 ACS– and ACS+ coexpressed with 3βHSD in COS-1 cells without and in the presence of Angora cytochrome b_5. The ratio for CYP17 ACS+ was compared to that of ACS- both in the presence and absence of cytochrome b_5 by an unpaired *t* test (*p < 0.05, **p < 0.01). Results are representative of at least three independent experiments.

DISCUSSION

Two CYP17 alleles have been identified in the South African Angora goat, which differ by four nucleotides resulting in three amino acid substitutions, viz. G6A, L41P, and I213V. The cloned angora *c*DNA encoding the two isoforms, CYP17 ACS– and CYP17 ACS+, exhibited similar, but also distinct differences in catalytic activity when expressed in COS-1 cells. The 17α-hydroxylase activity was similar for PREG as reflected by the K_m values (Table 2). The hydroxylase activity for PROG was, however, different as seen by the 1.8 fold increase in K_m of CYP17 ACS+. In addition, CYP17 ACS– demonstrated a significantly greater 17,20 lyase activity when compared to CYP17 ACS+ (Fig 2A).

Since the P41L is a nonconservative amino acid substitution, site-directed mutagenesis was employed to investigate the influence of this substitution on the catalytic activity of the enzyme and to identify the possible effect on the steroidogenic output of the Angora goat adrenal cortex. The P41L substitution lies in the highly conservative proline rich sequence (PR), critical for the correct folding of all cytochromes P450, specifically in stabilizing the conformation prior to heme binding. The sequence is, however, not critical for maintaining the functional form of the protein (Yamazaki et al., 1993; Kusano et al., 2001a, 2001b). Furthermore, P41 is a highly conserved residue amongst CYP17 of all species cloned to date. Similar substitutions in the PR in other cytochromes P450 result in reduced activities towards some, but not all substrates. In human CYP21, a P30L substitution reduced the activity of this enzyme for 17-OHPROG and PROG to 60 and 30% of the wild type, respectively (Tusie-Luna et al., 1991). Similarly, a naturally occurring mutation P34S in human CYP2D6 caused a significant reduction in activity towards certain drugs (Kagimoto et al., 1990; Johansson et al., 1994; Fukuda et al., 2000).

In this study, CYP17 ACS– demonstrated a greater 17,20 lyase activity when compared to CYP17 ACS+. The difference in 17,20 lyase activity of the two isoforms was clearly demonstrated to be primarily due to the nonconservative amino acid substitution at position 41 (Fig. 2A). The substitution of the proline residue in CYP17 ACS– for a leucine residue resulted in a significant decrease in 17,20 lyase activity for 17-OHPREG. The hydroxylase activity for PREG was also significantly lower for this substitution. Conversely, introducing the L41P substitution into CYP17 ACS+ partially restored the basal 17,20 lyase activity (Fig. 2A), indicating that although the P41L substitution is primarily responsible for the difference in activity between the two isoforms, its effect may be augmented by the conservative substitutions G6A and I213V.

Cytochrome b_5 expression in the adrenal cortex has been shown to alter the adrenals steroidogenic output in other species (Kominami et al., 1992). In addition, cytochrome b_5 allosterically enhances the 17,20 lyase reaction, increasing adrenal androgen production. The addition of cytochrome b_5 enhanced the 17,20 lyase reaction of all the CYP17 constructs. However, the 17,20 lyase activity of CYP17 ACS– and ACS+ L41P remained significantly greater than that of CYP17 ACS+ and ACS– P41L (Figs. 1B and 2B). In addition, both constructs with a proline at position 41 (CYP17 ACS- and ACS+ L41P) were able to metabolize low levels of A4 from PROG upon the addition of cytochrome b_5 (Fig. 3B). In CYP17, the 17,20 lyase reaction requires the C_{20} of the steroid substrate, rather than the C_{17}, to align with the iron-oxygen complex. As the P41L substitution lies within the PR, which is important for correct protein folding, this substitution almost certainly causes a change in the three-dimensional structure of the folded enzyme (Kusano et al., 2001a, 2001b; Yamazaki et al., 1993), which does not favour the optimal repositioning of the substrate.

Low levels of 16-OHPROG were detected for all Angora CYP17 constructs (Fig. 3A). To date, 16α-hydroxylase activity towards PROG has only been identified in human CYP17 and that of other primate species (Swart et al., 1993; Arlt et al., 2002). The 16α-hydroxylase reaction, as in the 17,20 lyase reaction, requires the repositioning of the steroid substrate with the C_{16} aligned with the iron-oxygen complex. Cytochrome b_5 has been shown to promote the 17,20 lyase reaction through an allosteric mechanism, which promotes the association of CYP17 with P450 oxidoreductase increasing the efficiency of electron transfer required for the 17,20 lyase reaction (Auchus et al., 1998; Geller et al., 1999; Miller 2005). Furthermore, cytochrome b_5 has been shown to have no influence on the 17α-hydroxylase activity of CYP17. However, the cotransfection of all Angora CYP17 constructs with cytochrome b_5 resulted in a significant increase in the 16α-hydroxylase activity (Fig. 3). As the 16α-hydroxylase reaction does not require additional electrons from P450 oxidoreductase, this effect must be exclusively due to the allosteric interaction between CYP17 and cytochrome b_5, which we propose alters the three-dimensional structure of CYP17 in such a way to promote the repositioning of the substrate in an orientation that is more favourable for the 16α-hydroxylase reaction. It follows that a cytochrome b_5 induced change in the three-dimensional structure of CYP17 can result in the optimal repositioning of the steroid substrate for the 17,20 lyase reaction and that the repositioning is partially responsible for the allosteric effects of cytochrome b_5 on CYP17. This is further supported by the observation that both CYP17 ACS+ and ACS– P41L, which have different three-dimensional structures to ACS– and ACS+ L41P, are less responsive to cytochrome b_5, with only the latter two expressed enzymes being able catalyse the 17,20 lyase of PROG (Fig 3B). The possibility that the reduced 17,20 lyase activity is due to a reduced interaction with cytochrome b_5 has been ruled out as there is no significant difference in the increased 16α-hydroxylase activity caused by cytochrome b_5 (Fig. 3). It is, however, possible that although the interaction of CYP17 and cytochrome b_5 is unchanged, the interaction of CYP17/cytochrome b_5 complex with P450 oxidoreductase could be affected.

Engelbrecht and Swart (2000) have previously demonstrated that the Angora goat produces significantly more DHEA and less glucocorticoid precursors than Boer goats and Merino sheep do as a result of an increased 17,20 lyase activity in the Angora CYP17. They concluded that CYP17 may be a contributing factor to the hypoadrenocorticoidism observed in Angora goats. We propose that the gene encoding CYP17 ACS– is the wild type. CYP17 ACS+, which is 100% homologous to the Boer goat CYP17, was possibly introduced through breeding programs in an attempt to breed more hardy Angora goats. To date, the majority of the goats genotyped (59/83) were heterozygous. The remaining goats (24/83) were homozygous for CYP17 ACS–, with no goats homozygous for CYP17 ACS+ detected. It is possible that the third genotype may be lethal, however, a larger number of goats will have to be genotyped.

Our study has shown that of the two CYP17 isoforms identified in the Angora goat, CYP17ACS– has a significantly greater 17,20 lyase activity than CYP17 ACS+, which is in agreement with the data obtained by Engelbrecht and Swart (2000). The difference between the enzymatic activities of the two CYP17 isoforms was even more pronounced when the enzymes were competing with 3βHSD for substrates (Fig. 4). It is apparent that a goat homozygous for CYP17 ACS– would produce significantly more A4 than a goat homozygous for CYP17 ACS+ (Fig. 4). This results from an increased flux through the Δ^5 pathway as CYP17 ACS– has a greater 17,20 lyase activity for 17-OHPREG than ACS+, yielding higher levels of DHEA. DHEA is subsequently metabolized to A4 by 3βHSD. As

Angora CYP17 is unable to produce significant levels of A4 from 17-OHPROG, it can be assumed that the Δ^5 pathway is the major source of A4 in the Angora adrenal (Fig. 3). The increased flux through the Δ^5 pathway therefore diminishes the capacity of the Angora adrenal to produce cortisol due to the reduced availability of cortisol precursors (Fig. 5). Although cotransfections with 3βHSD revealed that COS-1 cells transfected with CYP17 ACS– produced more cortisol precursor, 17-OHPROG, than ACS+ transfected cells, significantly more PROG is available to ACS+ for the formation of 17-OHPROG as a result of the lower flux through the Δ^5 pathway. In addition, as CYP17 ACS– is significantly more responsive to cytochrome b_5, the expression of cytochrome b_5 in the Angora adrenal cortex would increase the flux through the Δ^5 pathway, further reducing the availability of cortisol precursors (Fig. 4). This is substantiated by inspection of the ratio of 17-OHPROG/A4, which demonstrates that the greater flux through the Δ^5 pathway, augmented by CYP17 ACS–, resulted in significantly more A4 than 17-OHPROG being produced. The increased A4 production is further attenuated by the presence of cytochrome b_5 (Fig. 5).

In conclusion, this study has identified two CYP17 alleles in the South African Angora goat. While the 17α-hydroxylase activity of both CYP17 isoforms was similar for PREG, the hydroxylase activity was significantly different for PROG. Furthermore, CYP17 ACS– had a significantly greater 17,20 lyase activity than ACS+ in both the presence and absence of cytochrome b_5. Site-directed mutagenesis clearly revealed that the difference in activities resulted from the P41L substitution in the PR. In addition, COS-1 cells transfected with either clone demonstrated low 16α-hydroxylase activity towards PROG, which could be enhanced by cotransfection with cytochrome b_5. Cotransfections with 3βHSD revealed that one CYP17 isoform strongly favoured steroid metabolism through the Δ^5 pathway both in the presence and in the absence of cytochrome b_5. Our data supports that of Engelbrecht and Swart (2000) and further implicates CYP17 as the primary cause of the observed hypoadrenocorticoidism in the South African Angora goat.

ACKNOWLEDGMENTS

The authors wish to thank our colleagues, Marietjie Stander, Mariliza Oosthuizen and Anne Louw, for technical support. Angora goat blood samples were provided by Gretha Snyman (Grootfontein Agricultural Development Centre, Middleburg, South Africa). This work was financially supported by the South African Mohair Council, NRF, University of Stellenbosch and Wilhelm Frank Bursary Fund.

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6.2 Conclusion

The data presented in this chapter clearly demonstrated the presence of two CYP17 isoforms in the South African Angora goat population. The *in vitro* studies suggested that the increased 17,20-lyase activity of CYP17 ACS- may be the primary cause of the observed hypocortisolism. However, further research was required to determine the effect of the two CYP17 isoforms *in vivo* and to determine why the ACS+/ACS+ genotype was not detected by this study. This question together with the physiological implication of the two isoforms are addressed in the following chapter.

CHAPTER 7
TWO CYP17 GENES IN THE SOUTH AFRICAN ANGORA GOAT (*CAPRA HIRCUS*). THE IDENTIFICATION OF THREE GENOTYPES THAT DIFFER IN COPY NUMBER AND STEROIDOGENIC OUTPUT.

7.1 Introduction

It is evident from the data presented in the preceding chapter that there are two unique CYP17 isoforms in the South African Angora goat and that these isoforms have significantly different enzymatic activities. This data was, however, obtained in model cell culture systems and the implication of CYP17 as a primary cause of the observed hypocortisolism remained to be demonstrated *in vivo*. Furthermore, the ACS+/ACS+ genotype was not detected in Angora goats using the restriction digest genotyping method developed by Slabbert (2003). For this reason a more accurate method was developed — rapid real-time PCR genotyping. The results obtained by the new method indicated that the two CYP17 isoforms were not two alleles of the same gene, but rather two separate genes. This novel finding was confirmed by quantitative real-time PCR. Furthermore, this resulted in the identification of three unique genotypes, which differ not only by the CYP17 genes expressed, but also by CYP17 copy number.

A number of Angora goats were subsequently divided into three groups according to the indentified genotypes and each group was investigated for cortisol production in response to intravenous insulin injection. The results of this study were published in the FEBS journal and are presented in the article included in this chapter (Storbeck et al., 2008b).

the FEBS Journal

Two CYP17 genes in the South African Angora goat (*Capra hircus*) – the identification of three genotypes that differ in copy number and steroidogenic output

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Keywords
Angora goat; copy number; cortisol; CYP17; cytochrome P450 17α-hydroxylase/17–20 lyase

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(Received 31 March 2008, revised 3 June 2008, accepted 4 June 2008)

doi:10.1111/j.1742-4658.2008.06539.x

In mammals, cytochrome P450 17α-hydroxylase/17–20 lyase (CYP17), which is encoded by a single gene, plays a critical role in the production of mineralocorticoids, glucocorticoids and androgens by the adrenal cortex. Two CYP17 isoforms with unique catalytic properties have been identified in the South African Angora goat (*Capra hircus*), a subspecies that is susceptible to cold stress because of the inability of the adrenal cortex to produce sufficient levels of cortisol. A real-time-based genotyping assay was used in this study to identify the distribution of the two CYP17 alleles in the South African Angora population. These data revealed that the two CYP17 isoforms were not the product of two alleles of the same gene, but two separate CYP17 genes encoding the two unique CYP17 isoforms. This novel finding was subsequently confirmed by quantitative real-time PCR. Goats were divided into three unique genotypes which differed not only in the genes encoding CYP17, but also in copy number. Furthermore, *in vivo* assays revealed that the identified genotypes differed in their ability to produce cortisol in response to intravenous insulin injection. This study clearly demonstrates the presence of two CYP17 genes in the South African Angora goat, and further implicates CYP17 as the primary cause of the observed hypocortisolism in this subspecies.

In mammals, steroid hormones are derived from the parent compound cholesterol through a sequence of hydroxylation, C–C bond scission (lyase) and dehydrogenase–isomerase reactions. Cytochrome P450-dependent enzymes catalyse the hydroxylase and lyase activities, whereas a specific hydroxysteroid dehydrogenase is responsible for the dehydrogenase–isomerase action. The adrenal, testes and ovaries are the most important steroidogenic tissues in the body in which these enzymes are expressed. The mineralocorticoids, glucocorticoids and androgens, produced in the adrenal cortex, are vital for the control of water and mineral balance, stress management and reproduction, respectively, whereas androgens and oestrogens are the main steroids produced by the gonads. Of all the steroidogenic cytochromes P450 only one, cytochrome P450 17α-hydroxylase/17–20 lyase (CYP17), catalyses two distinct reactions, namely a 17α-hydroxylation and a C17–C20 lyase reaction. The dual enzymatic activity of CYP17 places this enzyme at a key branch point in the biosynthesis of adrenal steroid hormones.

Abbreviations
17-OHPREG, 17-hydroxypregnenolone; 17-OHPROG, 17-hydroxyprogesterone; 3βHSD, 3β-hydroxysteroid dehydrogenase; A4, androstenedione; CYP17, cytochrome P450 17α-hydroxylase/17–20 lyase; DHEA, dehydroepiandrosterone; HPA, hypothalamic–pituitary–adrenal; PREG, pregnenolone; PROG, progesterone; UPLC-APCI-MS, ultra-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry.

In adrenal steroidogenesis, the 17α-hydroxylation of the Δ^5 and Δ^4 steroid precursors pregnenolone (PREG) and progesterone (PROG) by CYP17 yields 17-hydroxypregnenolone (17-OHPREG) and 17-hydroxyprogesterone (17-OHPROG), respectively. The 17,20-lyase action of CYP17 produces the cleavage of the C17,20 bond of 17-OHPREG and 17-OHPROG to yield the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively [1–3]. In addition, PREG, 17-OHPREG and DHEA are substrates for 3β-hydroxysteroid dehydrogenase (3βHSD), which metabolizes them to the corresponding Δ^4 3-ketosteroids: PROG, 17-OHPROG and A4 [4]. The substrate specificities, enzymatic activities and expression levels of these two enzymes, which compete for the same substrates, therefore ultimately play an important role in determining the steroidogenic output of the adrenal.

In all mammalian species reported to date, CYP17 is the product of a single gene [2,5–10]. In mice, the deletion of *CYP17* causes early embryonic lethality [11]. In humans, 17α-hydroxylase/17,20-lyase deficiency, an autosomal recessive disease, causes congenital adrenal hyperplasia. This condition is characterized by hypertension, hypokalaemia, low cortisol and suppressed plasma renin activity [12]. In addition, 17α-hydroxylase/17,20-lyase deficiency is characterized by sexual infantilism and primary amenorrhoea in genotypic females (46,XX), whereas genotypic males (46,XY) demonstrate impaired virilization and pseudohermaphroditism [13–16]. Partial deficiencies in CYP17 can cause milder or intermediate phenotypes [13,17]. In rare instances, mutations only significantly impair the 17,20-lyase reaction, causing isolated 17,20-lyase deficiency, which can result in male pseudohermaphroditism and a lack of progression into puberty in females [18,19]. As a result of its role as a branch point enzyme in adrenal steroidogenesis, it is apparent that even small changes in either the 17α-hydroxylation or lyase activity of CYP17 may have profound physiological effects.

In an investigation into the impaired stress tolerance displayed by the South African Angora goat (*Capra hircus*), two CYP17 isoforms, which differ by three amino acid residues (A6G, P41L and V213I), were identified in the population. The isoforms were named CYP17 ACS+ (GenBank accession no. EF524064) and CYP17 ACS−, respectively, which was attributed to a nucleotide change at position 637 within an ACS1 recognition site, which results in the V213I substitution [20]. The expression of both isoforms in COS-1 cells revealed that CYP17 ACS− has a significantly enhanced lyase activity and strongly favours androgen production by the Δ^5 steroid pathway. Although the hydroxylase activities of these isoforms are similar, the lyase activity of CYP17 ACS+ results in the production of significantly more glucocorticoid precursors, essential for cortisol production. Site-directed mutagenesis revealed that the difference in lyase activity was primarily a result of the substitution of a highly conserved proline residue at position 41 with a lysine residue in CYP17 ACS+ [20].

An abrupt decrease in glucose concentration has previously been implicated as the critical factor responsible for the inability of the South African Angora goat to produce the metabolic heat required during cold spells, resulting in large stock losses during the winter [21,22]. In mammals, physiological stress stimulates the release of glucocorticoids from the adrenal cortex via the hypothalamic–pituitary–adrenal (HPA) axis, which favours glucose production at the expense of glycolysis [23]. Previous studies have shown that the *in vivo* stimulation of the HPA axis with insulin and adrenocorticotropic hormone results in less cortisol being produced in Angora goats when compared with Boer goats (*C. hircus*) and Merino sheep (*Ovis aries*) [24]. In addition, using subcellular fractions prepared from adrenocortical tissue, Engelbrecht and Swart [25] found that Angora goats produced significantly more androgens and less glucocorticoid precursors when compared with Boer goats and Merino sheep. Taken together, these studies indicate that the increased lyase activity of CYP17 ACS− is the primary cause of the observed hypocortisolism in the South African Angora goat, as it produces significantly less glucocorticoid precursors than does the ACS+ isoform [20].

In order to investigate the distribution of the two CYP17 isoforms in the South African Angora population, goats were genotyped on the basis of a restriction digest assay. It was determined that 29% of the goats genotyped were homozygous for *CYP17* ACS−, whereas the remaining 71% were heterozygous. No goats homozygous for *CYP17* ACS+ were detected [20]. There are two possible explanations for this observation: either this genotype is lethal, or genotyping by restriction analysis was not sufficiently sensitive for the detection of goats homozygous for *CYP17* ACS+.

The aim of this study was to search for the missing *CYP17* genotype in the South African Angora population. A more sensitive real-time PCR method yielded unexpected results, which suggested that the two CYP17 isoforms were not two alleles of the same gene, but rather two individual genes. This finding, the first for any mammalian species reported to date, was

confirmed by quantitative real-time PCR. Goats were subsequently divided into their respective genotypes based on the difference observed in their *CYP17* composition and copy number. The physiological effect of this novel finding was investigated by testing goats of each genotype for their ability to produce cortisol in response to intravenous insulin injection. The results of this study clearly demonstrate the existence of two CYP17 genes in the South African Angora goat, and further implicate CYP17 as a primary cause of the observed hypocortisolism.

Results and Discussion

Genotyping *CYP17*

Subsequent to the identification of two unique CYP17 isoforms (ACS− and ACS+) in the South African Angora goat population, a number of goats were genotyped using a restriction digest assay. Eighty three goats were genotyped, 24 (29%) of which were homozygous for *CYP17* ACS− and 59 (71%) of which were heterozygous. No goats homozygous for *CYP17* ACS+ were detected [20]. The absence of the ACS+/ACS+ genotype was investigated by real-time PCR using hybridization probes that were developed specifically for this study. The sensor probe was designed to be a perfect match for the *CYP17* ACS+ sequence, and dissociated at 57 °C when bound to a mismatched sequence (*CYP17* ACS−) and at 63 °C when bound to the perfectly matched *CYP17* ACS+ sequence.

In addition, the sensor probe was able to bind to ovine *CYP17*, as the sequences are homologous. Although ovine CYP17 is encoded by a single gene, two sequences, which differ by two nucleotides, have been deposited in GenBank. To date it is unknown whether these sequences are two alleles of *CYP17* or the result of a PCR artefact. The sensor probe used in this study binds to an area which includes one of the two nucleotide substitutions. It contains only one mismatched nucleotide when bound to the first ovine *CYP17* (GenBank accession no. L40335) and dissociates at 57 °C. There is an additional mismatched nucleotide when the probe is bound to the second ovine *CYP17* (GenBank accession no. AF251388), resulting in a lower melting temperature of 55 °C. A number of heterozygous sheep were detected in this study, revealing that there are two CYP17 alleles in sheep. The design of the probes is shown in Fig. 1, with the resulting melting curves in Fig. 2A.

This method was subsequently used to genotype 576 Angora goats from two separate populations. The

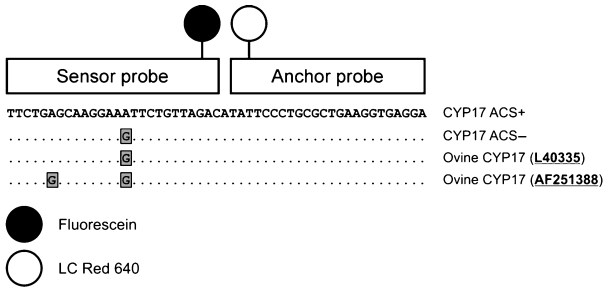

Fig. 1. Hybridization probe design. The sequence to which the sensor and anchor probes bind is shown for *CYP17* ACS+. Mismatched base pairs (position 637) are highlighted for *CYP17* ACS− and the two ovine CYP17 alleles (positions 628 and 631).

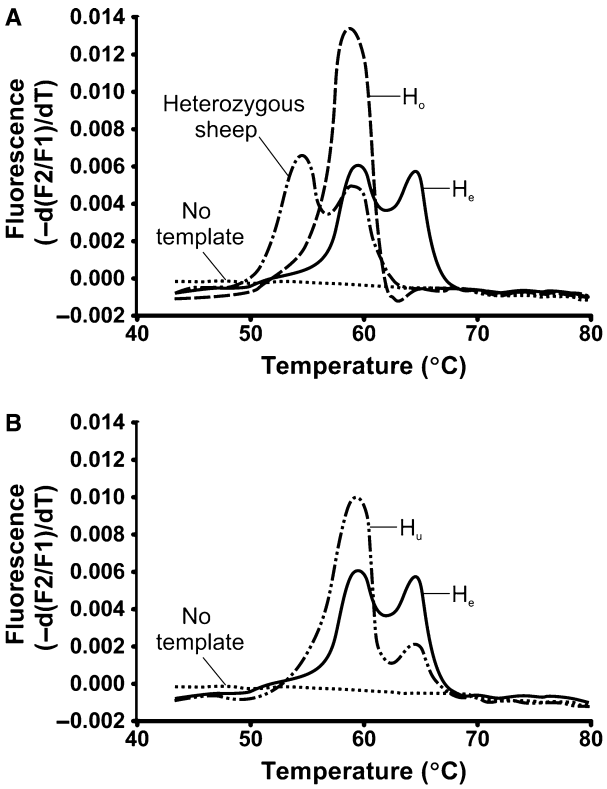

Fig. 2. Melting curves of *CYP17* ACS− and ACS+. (A) Typical melting curves for the H_o and H_e genotypes, as well as heterozygous Merino sheep. (B) Typical peak distortion obtained for the H_u genotype, shown with the H_e genotype for comparison.

ACS+/ACS+ genotype remained undetected, but an interesting observation was made. Genotyping of heterozygous samples with hybridization probes typically yields two melting peaks of similar peak area [26]. This was the case in 42.9% of the heterozygous animals investigated in this study. However, 40.6% of the heterozygous animals consistently yielded melting

profiles with unequal peak areas, in which the peak representative of *CYP17* ACS+ had a substantially smaller area than that representative of *CYP17* ACS– (Fig. 2B). Furthermore, this pattern was consistently observed for the same samples, even when tested using different DNA isolations and blood samples (data not shown). As a control, 107 Boer goats were also genotyped using the same method. These animals were all heterozygous and showed no distortion in peak area. Similarly, all the sheep that were genotyped as heterozygotes demonstrated no peak distortion.

As the copy number of individual alleles has a direct influence on the respective peak areas when genotyping with hybridization probes [26], the difference in peak areas observed in this study may be the result of differences in *CYP17* copy number. Based on the melting peak profiles, the goats were subsequently divided into three groups, namely homozygotes for ACS– (H_o), heterozygotes (H_e) and heterozygotes (H_u) in which the observed unequal peak area ratio may indicate a lower abundance of *CYP17* ACS+ (Table 1).

The relative melting peak areas of polymorphic samples have been used previously to detect gene duplications and deletions. For heterozygous samples, a melting peak area ratio of 2 : 1 is indicative of gene duplication [26]. An example of gene quantification using hybridization probes is the detection of the autosomal dominant demyelinating peripheral neuropathy Charcot–Marie–Tooth disease type 1A, which is associated with the duplication of a specific 1.5 Mb region at chromosome 17p11.2-p12. The ratio obtained between the areas under the melting peak of each allele for heterozygous Charcot–Marie–Tooth disease type 1A samples was successfully used to determine whether or not the sequence was duplicated [27]. Similarly, melting curve analysis has been used in the clinical diagnosis of α^+-thalassaemias and trisomy 21, as well as in the detection of gene duplications in the HER2/*neu* gene, which is amplified in 25–30% of primary breast cancers [28–30].

It should be noted, however, that unequal melting peaks may not always be the result of a change in gene frequency. Fluorescence decreases with increasing temperature, resulting in melting peaks that may have larger areas at lower temperatures than at higher temperatures. Probes melted from the less stable allele may re-anneal to the excess templates of the more stable allele. Preferential binding may also occur when probe concentrations are limiting [26]. Quantitative real-time PCR was therefore employed to determine whether the unequal peak areas observed in this study were an artefact of the genotyping assay or a result of unequal allele distribution.

Table 1. *CYP17* genotyping by real-time PCR using hybridization probes. Goats were divided into three genotypes (H_o, H_u and H_e) based on the melting peak areas, as shown in Fig. 2. Values in parentheses are percentages.

	H_o	H_u	H_e	Total
Population 1	30 (12.9)	93 (39.9)	110 (47.2)	233
Population 2	65 (19.0)	141 (41.1)	137 (39.9)	343
Angora goat totals	95 (16.5)	234 (40.6)	247 (42.9)	576
F2 generation G1 goats[a]	1 (1.4)	21 (29.6)	49 (69.0)	71
Boer goats	0 (0)	0 (0)	107 (100)	107

[a] F2 generation of the 75% Angora goat : 25% Boer goat line (G1) established by Snyman [36].

CYP17 copy number determination

Relative copy number determinations were performed for each of the three putative genotypes identified above using quantitative real-time PCR. Fold change values for the samples were calculated relative to an H_o calibrator using the $\Delta\Delta C_t$ method [31]. The H_e genotype demonstrated a significantly ($P < 0.05$) greater (1.7-fold) copy number than the H_o group (Fig. 3). In addition, all Boer goats (all Boer goats genotyped were H_e, Table 1) demonstrated the same 1.7-fold greater copy number. Although the H_u genotype yielded a copy number 1.4-fold greater than that of the H_o group, this genotype was not significantly

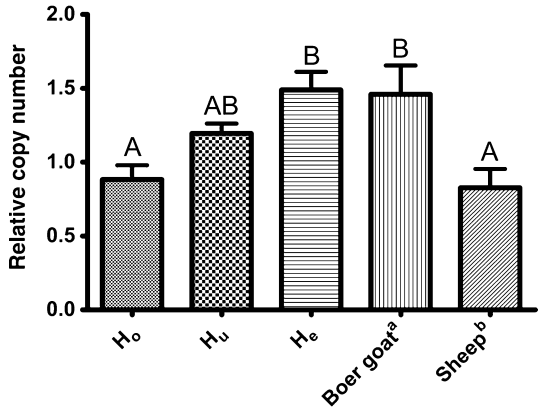

Fig. 3. *CYP17* copy number for the three Angora genotypes (H_o, H_u and H_e), Boer goat and heterozygous Merino sheep relative to an H_o calibrator. Error bars indicate the standard deviation for six unique samples per group. Each group was compared with every other group by a one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Columns labelled with different letters are significantly different ($P < 0.05$). [a]All Boer goats genotyped in this study belong to the H_e genotype. [b]Only heterozygous Merino sheep were used for copy number determinations.

different from either the H_o or H_e genotypes (Fig. 3). Furthermore, all heterozygous sheep showed no significant difference in copy number, confirming that the two ovine *CYP17* sequences in GenBank (GenBank accession nos. L40335 and AF251388) are two alleles of the same gene (Fig. 3).

These data reveal the novel finding that, in both the South African Angora goat and the Boer goat, *CYP17* ACS− and ACS+ are not two alleles of a single CYP17 gene [20], but, instead, two separate genes. To date, no other mammal has been reported to possess two CYP17 genes encoding two CYP17 isoforms [2,5–10].

The data indicate that the H_o genotype has only one CYP17 gene, namely ACS−. Conversely, the H_e genotype has both CYP17 genes (ACS+ and ACS−) at two different loci, and therefore twice the copy number of H_o (Fig. 3). Furthermore, ACS− is always present with ACS+, and therefore the homozygote for ACS+ is never detected. Crossing H_o and H_e goats would yield the proposed intermediate genotype H_u. This genotype would receive both ACS− and ACS+ from the H_e parent, but only ACS− from the H_o parent (Fig. 4). Therefore, in this genotype, the ACS− : ACS+ ratio would be 2 : 1, which corresponds to the distortion in peak area obtained during genotyping with hybridization probes. This is further supported by the copy number determination, where the H_u genotype yielded a 1.4-fold greater copy number than the H_o group, but was not significantly different from either the H_o or H_e genotypes (Fig. 3). Furthermore, data obtained from preliminary breeding studies have confirmed the existence of the three genotypes (data not shown).

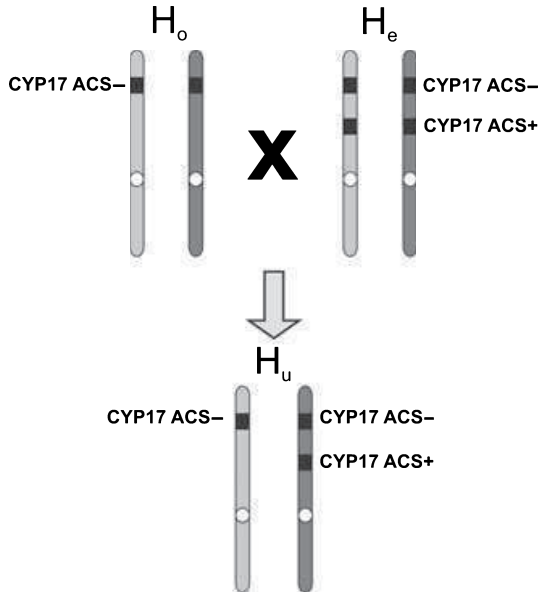

Fig. 4. Schematic representation of a proposed cross between the H_o and H_e genotypes, yielding the H_u genotype. The difference in copy number, shown in Fig. 3, is clearly demonstrated in this diagram. Both ACS− and ACS+ are shown on the same chromosome in order to simplify the diagram, although the genes are yet to be mapped.

The observation that all Boer goats, but not all Angora goats, genotyped to date are H_e suggests that this genotype originated in the Boer goat and not the Angora goat. The individual CYP17 genes probably originated from two of the subspecies that were used in the breeding of the Boer goat, probably through nonhomologous recombination, although it remains to be determined whether both genes are located nearby on the same chromosome [32,33]. It is unlikely that a gene duplication event occurred followed by subsequent diversion [34], as *CYP17* available on GenBank for the domestic goat *C. hircus* (GenBank accession no. AF251387) is 100% identical to that of ACS+, whereas the ACS− gene alone is found in H_o Angora goats. We suggest that it was early breeding practices in South Africa, in which Angora goats were crossed with the native goat (which fits the documented description of the early Boer goat) that led to the introduction of the second CYP17 gene (ACS+) into the South African Angora population [35].

Recently, a breeding programme was carried out in which South African Angora goats were crossed with Boer goats in order to establish a more hardy mohair-producing goat with a relatively high reproductive ability and good carcass characteristics. Crossbred does (50% Angora goat : 50% Boer goat) were mated with Angora bucks in order to obtain 75% Angora goat : 25% Boer goat progeny. These were subsequently mated with each other to establish a 75% Angora goat : 25% Boer goat line (G1) [36]. A number of F2 generation G1 goats have subsequently been genotyped (Table 1). These results confirm that crosses with Boer goats significantly increase the frequency of the H_e genotype in the Angora population, whilst decreasing the H_o and H_u genotypes as expected.

In vitro and *in vivo* CYP17 activity assays

We have previously demonstrated that ACS− and ACS+ have distinctly different catalytic properties *in vitro*. In comparison with CYP17 ACS+, CYP17 ACS− expressed in COS-1 cells has a significantly enhanced lyase activity which strongly favours androgen production by the Δ^5 steroid pathway, with a resulting decrease in glucocorticoid precursor production. In the adrenal, CYP17 and 3βHSD compete for the same substrates, with the ratio and substrate

specificities of these two enzymes determining the steroidogenic output of the adrenal cortex. The effect of the difference in CYP17 activity was clearly demonstrated when each CYP17 isoform was coexpressed with 3βHSD in COS-1 cells [20]. In addition, cotransfections were carried out in the presence of cytochrome b_5, which allosterically enhances the 17,20-lyase activity of CYP17, and is expressed in the adrenal of similar species [37,38]. Eight hours after the addition of the PREG substrate to COS-1 cells expressing CYP17 ACS− and 3βHSD, significantly more adrenal androgens and less glucocorticoid precursors were produced ($P < 0.001$) than were produced by cells expressing CYP17 ACS+ and 3βHSD (Fig. 5A). The inclusion of cytochrome b_5 in the cotransfections resulted in an increased difference in the steroid profiles of PREG metabolism, with CYP17 ACS−-expressing COS-1 cells predominantly producing adrenal androgens (~ 68%), whereas glucocorticoid precursor production was predominant in CYP17 ACS+-expressing cells (~ 71%) (Fig. 5B). The difference in androgen production in both the presence and absence of cytochrome b_5 can be attributed to the greater 17,20-lyase activity of CYP17 ACS−, which results in a greater flux through the Δ^5 pathway, and a concomitant decrease in glucocorticoid precursors [20].

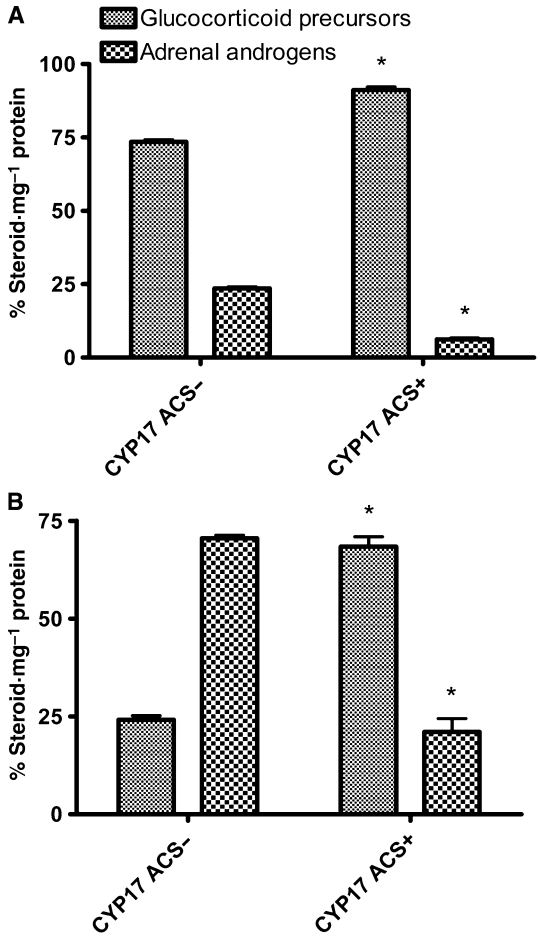

Fig. 5. Steroid profile of PREG (1 μM) metabolism after 8 h by Angora goat CYP17 and 3βHSD coexpressed in COS-1 cells in the absence (A) and presence (B) of cytochrome b_5. Glucocorticoid precursors (PREG, 17-OHPREG, PROG and 17-OHPROG) and adrenal androgens (DHEA and A4) were compared for each construct by unpaired *t*-test (*$P < 0.001$). Results are derived from the data obtained from three independent experiments.

The *in vitro* study gave a clear indication that the difference in activities observed for the CYP17 isoforms should have a significant effect on the steroid output of the adrenal. The discovery that the two CYP17 isoforms are two genes and that the genotypes differ not only by the genes present, but also by the copy number, suggests that the physiological effects may be more complex than previously believed. Therefore, in order to establish the effect of the three novel genotypes, an *in vivo* assay for cortisol production was performed.

Ten goats from each group (H_o, H_u and H_e) were tested for their ability to produce cortisol in response to intravenous insulin injection. There was no significant difference in the basal cortisol levels for the three groups, and each group demonstrated a decrease in plasma glucose and an increase in plasma cortisol levels in response to insulin injection (Fig. 6). However, although the decrease in plasma glucose was similar for all groups, the amplitude of the response in cortisol production was significantly greater in the H_e group ($P < 0.05$) than in the H_o group. After 120 min, the mean plasma cortisol concentration of the H_e group (155.5 ± 66.8 nmol·L^{-1}) was 1.4-fold greater than that of the H_o group (114.6 ± 42.1 nmol·L^{-1}). The cortisol response in the H_u group was not significantly different from either the H_o or H_e group, with a mean plasma cortisol level (134.6 nmol·L^{-1}) at 120 min postinjection between the values of the H_o and H_e groups. The greater capacity of CYP17 ACS+ to produce glucocorticoid precursors, as demonstrated previously in COS-1 cells, suggests that it is the expression of this gene in the H_e genotype that is responsible for the increased cortisol production when compared with H_o [20]. However, relative expression levels of CYP17 in the different genotypes have yet to be determined in the adrenal. Johansson *et al.* [39] have demonstrated previously that CYP2D6 gene duplication results in an increased metabolic capacity for drugs such as debrisoquine. The influence of copy number can therefore not be ignored, and may be a contributing factor towards the increased cortisol production in H_e and H_u goats.

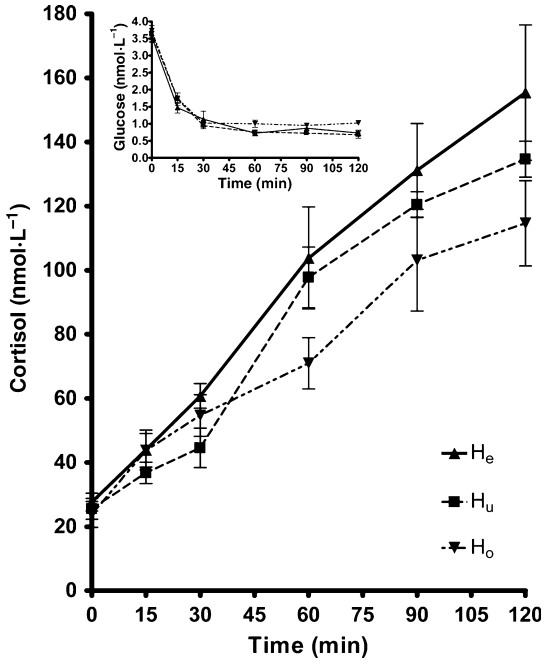

Fig. 6. Plasma cortisol levels in the three Angora genotypes (n = 10 per group) following intravenous insulin injection. Plasma glucose levels are shown in the inset. The groups were compared by one-way analysis of variance (ANOVA) with repeated measures test, followed by Dunnett's repeated measures post-test. The H_o and H_e groups demonstrated a significantly ($P < 0.05$) different response in cortisol production.

This study has clearly shown that the unique *CYP17* genotypes identified differ significantly in their ability to produce cortisol, unequivocally identifying CYP17 as a cause of hypocortisolism in the South African Angora goat. In addition, the difference in cytochrome b_5-stimulated androgen production by the two CYP17 isoforms (ACS− and ACS+) provides a model to study the interaction of cytochrome b_5 with steroidogenic cytochromes P450.

Conclusions

This investigation clearly identifies, for the first time, two distinctive genes encoding two CYP17 isoforms in both the South African Angora goat and Boer goat. The unique genotypes in the South African Angora goat have been shown to differ not only in terms of the genes encoding CYP17, but also in copy number. Furthermore, we have demonstrated that the identified genotypes have a significantly different capacity to produce cortisol. This study therefore confirms CYP17 as a primary cause of the observed hypocortisolism in the South African Angora goat.

Materials and methods

Isolation of genomic DNA

Genomic DNA was isolated from blood using either the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) or the DNA Isolation Kit for Mammalian Blood (Roche Applied Science, Mannheim, Germany).

Genotyping by real-time PCR

Primers and hybridization probes (Tib-Molbiol, Berlin, Germany), designed to amplify a 200 bp fragment of the CYP17 gene, are listed in Table 2. Real-time PCR was carried out using a LightCycler® 1.5 instrument. Amplification reactions (20 μL) contained 2 μL LightCycler® FastStart DNA Master HybProbe Master Mix (Roche Applied Science), 3 mM $MgCl_2$, 0.5 μM of each *CYP17* primer, 0.2 μM fluorescein-labelled *CYP17* sensor probe, 0.2 μM LC640-labelled *CYP17* anchor probe and 10–100 ng genomic DNA. Following an initial denaturation at 95 °C for 10 min to activate the FastStart *Taq* DNA polymerase, the 35 cycle amplification profile consisted of heating to 95 °C with an 8 s hold, cooling to 52 °C with an 8 s hold and heating to 72 °C with a 10 s hold. The transition rate between all steps was 20 °C·s^{-1}. Data were acquired in single mode during the 52 °C phase using LIGHTCYCLER® software (version 3.5). Following amplification, melting curve analysis was performed as follows: denaturation at 95 °C with a 20 s hold, cooling to 40 °C with a 20 s hold and heating at 0.2 °C·s^{-1} to 85 °C with continuous data acquisition. The sensor probe was designed to be a perfect match for the *CYP17* ACS+ sequence (Fig. 1), and dissociates at 63 °C when bound to the perfectly matched *CYP17* ACS+ sequence. However, when bound to the mismatched sequence (*CYP17* ACS−), dissociation occurs at 57 °C (Fig. 2). A no-template control (negative control) was also included in each assay.

Table 2. Nucleotide sequences of the primers and probes used in genotyping and relative copy number determination.

Primer	Oligonucleotide sequence (5′- to 3′)
Real-time *CYP17* LP (sense)	CAATGATGGCATCCTGGAG
Real-time *CYP17* RP (antisense)	GAGGCAGAGGTCACAGTAAT
CYP17 sensor probe	TTCTGAGCAAGGAAATTCTGTTAGAC-FL
CYP17 anchor probe	640-TATTCCCTGCGCTGAAGGTGAGGA-p
Real-time *3βHSD* LP (sense)	CTGCAAGTTCTCCAGAGTC
Real-time *3βHSD* RP (antisense)	ATTGGACTGAGCAGGAAGC

CYP17 copy number determination

Primers for *CYP17* and a reference gene, *3βHSD*, were designed to have similar melting temperature and product sizes (Tib-Molbiol), and are listed in Table 2. Real-time PCR was carried out using a LightCycler® 1.5 instrument. Amplification reactions (20 μL) contained 4 μL LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Master Mix (Roche Applied Science), 0.5 μM of either *CYP17* or *3βHSD* primer and 50 ng genomic DNA. Following an initial denaturation at 95 °C for 10 min to activate the FastStart *Taq* DNA polymerase, the 35 cycle amplification profile consisted of heating to 95 °C with an 8 s hold, cooling to 52 °C with an 8 s hold and heating to 72 °C with a 10 s hold. The transition rate between all steps was 20 °C·s^{-1}. Data were acquired in single mode during the 52 °C phase using LIGHTCYCLER® software (version 3.5). Following amplification, melting curve analysis was performed as follows: denaturation at 95 °C with a 20 s hold, cooling to 65 °C with a 60 s hold and heating at 0.1 °C·s^{-1} to 95 °C with continuous data acquisition. Both the target and reference genes were always independently amplified for each DNA sample in the same experimental run. A calibrator was included in duplicate for each experimental run. A no-template control (negative control) was also included in each assay. The melting curve analysis showed that all reactions were free of primer dimers and other nonspecific products.

Two-fold serial dilutions were performed in triplicate and used to determine the PCR efficiencies for both the target and reference genes. The PCR efficiencies were calculated from the slopes of the standard curves generated by LIGHTCYCLER® software (version 3.5) over two orders of magnitude, and were always > 95%. C_t values were generated for both the target and reference genes for each sample using the second-derivative maximum mode of analysis. The ΔC_t value for the calibrator was calculated on the basis of the mean C_t values from the two technical replicates in each run for both the target and reference genes. Fold change values for the samples relative to the calibrator were calculated using the $\Delta\Delta C_t$ method [31].

Enzyme assays in transiently transfected COS-1 cells

COS-1 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, 4 mM L-glutamine and 25 mM glucose. Cells were plated in 12-well dishes at 1×10^5 cells·mL^{-1}, 24 h prior to transfection. Angora CYP17, 3βHSD and cytochrome b_5 had all been cloned previously into the pcDNA/V5/GW/D-TOPO® mammalian expression vector (Invitrogen, Carlsbad, CA, USA) [20]. Cotransfections of CYP17 and 3βHSD, with and without cytochrome b_5, were performed with an equal amount of each construct up to a total of 0.5 μg of plasmid DNA using Genejuice transfection reagent (Novagen, Darmstadt, Germany), according to the manufacturer's instructions. Control transfection reactions were performed using the mammalian expression vector pCI-neo (Promega) containing no insert. In transfections without cytochrome b_5, the latter was replaced by the pCI neo vector (Promega). After 72 h, enzymatic activities were assayed using PREG (1 μM) as substrate. Aliquots of 50 μL were removed after 8 h and analysed. On completion of each experiment, the cells were washed with and collected in 0.1 M phosphate buffer, pH 7.4. The cells were subsequently homogenized with a small glass homogenizer, and the protein content of the homogenate was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL, USA), according to the manufacturer's instructions.

Extraction and analysis of steroids

Steroids were extracted from the incubation medium by liquid–liquid extraction using a 10 : 1 volume of dichloromethane to incubation medium. The samples were vortexed for 2 min and centrifuged at 500 ***g*** for 5 min, after which the water phase was aspirated off. The organic phase was transferred to a clean extraction glass tube and the samples were dried under a stream of nitrogen. The dried steroids were dissolved in 100 μL methanol prior to analysis.

Steroids were analysed using the ultra-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (UPLC–APCI–MS) method previously described by Storbeck *et al.* [40]. Briefly, steroids were separated by UPLC (ACQUITY UPLC, Waters, Milford, MA, USA) using a Waters UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at 50 °C. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (3 : 1 acetonitrile : methanol with 1% isopropanol). The column was eluted isocratically with 56% A and 44% B for 6 min, followed by a linear gradient from 44% B to 80% B in 0.01 min. A linear gradient was subsequently followed from 80% B to 100% B in 2.49 min, after which a linear gradient returned the column to 56% A and 44% B in 0.5 min. The total run time per sample was 11 min at a flow rate of 0.3 mL·min^{-1}. The injection volume of standards and samples was 5 μL.

An API Quattro Micro tandem mass spectrometer (Waters) was used for quantitative mass spectrometric detection. An Ion Sabre probe (Waters) was used for the APCI interface in positive mode. The corona pin was set to 7 μA, the cone voltage to 30 V and the APCI probe temperature to 450 °C. All other settings were optimized to obtain the strongest possible signal. Calibration curves were constructed using weighted (1/×2) linear least-squares regression. Data were collected using the MASSLYNX (version 4) software program (Waters).

In vivo cortisol test

Ten Angora goats of each CYP17 genotype were randomly selected from the same flock. Each group of 10 contained five ewes and five rams. The animals were all born during the same kidding season, and were approximately 14 months of age. A single dose of insulin (Humalin R, Eli Lilly, Bryanston, South Africa) was administered intravenously (0.1 $U \cdot kg^{-1}$ body weight). Blood samples were collected prior to insulin injection and subsequently at 15, 30, 60, 90 and 120 min. Blood samples were stored on ice immediately and kept at 4 °C until analyses were carried out by the Pathcare Veterinary Laboratory (Cape Town, South Africa). Ethical approval for experimentation on small stock breeds was not required at the time of the experiment; however, all animals were treated humanely by qualified technical staff.

Acknowledgements

The authors wish to thank Carel van Heerden and Gloudi Agenbag for technical assistance and fruitful discussions; Tino Herselman and the personnel at the Jansenville Experimental Farm for technical assistance; and Patricia Storbeck and Ann Louw for help with the preparation of the manuscript. Blood samples were provided by Ray Hobson and Wynand Kershof. This work was financially supported by the South African Mohair Council, National Research Foundation, University of Stellenbosch and Wilhelm Frank Bursary Fund.

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7.2 Conclusion

The results presented in this chapter clearly implicate CYP17 as the primary cause of hypocortisolism in the South African Angora goat. In addition, the novel finding of a second gene encoding CYP17 in Angora goats is the first such case to be reported for a mammalian species. The genetic variation in the Angora goat which results in different cortisol responses following applied stress by insulin injection, clearly shows the physiological relevance of the expressed isoforms. Furthermore, the Angora goat provides an excellent model for studies on the relationship between relative hydroxylase/lyase activities. A summary of the results obtained in this study and their physiological implications will be presented in the following chapter. Furthermore, the implementation of a preliminary breeding program to investigate the feasibility of using CYP17 as a genetic marker to breed more hardy Angora goats, without impairing the quality or the production of mohair, will be discussed.

CHAPTER 8

GENERAL DISCUSSION

The aim of this thesis was to investigate the role of the two CYP17 isoforms in cold stress related deaths in the South African Angora goat population. Wentzel et al. (1979) had previously shown that the primary cause of stock loss during cold spells was due to an energy deficiency resulting from a decrease in blood glucose levels, which caused a drop in body temperature and subnormal heart function. In mammals, physiological stress stimulates the HPA axis with the resulting release of glucocorticoids from the adrenal cortex (Munch, 1971). Van Rensburg (1971) hypothesised that the selection for high mohair production by the South African Angora goat had indirectly resulted in reduced adrenal function and as a result reduced cortisol levels, as high plasma cortisol levels were shown to reduce hair production. This was substantiated by Herselman and van Loggerenberg (1995) who demonstrated that cortisol production was significantly smaller in the Angora goat when compared to other small ruminant breeds with varying potentials for fibre production. A comparative study conducted by Engelbrecht et al. (2000) demonstrated that the plasma cortisol levels increased significantly in both the Boer goat and Merino sheep as a result of the hypoglycaemic condition induced by intravenous insulin injection. The Angora plasma cotisol levels did, however, not increase significantly above basal levels, confirming the condition of hypocortisolism suggested by previous studies (Van Rensburg, 1971; Herselman and van Loggerenberg, 1995). Further investigation into adrenal steroidogenesis in the Angora goat by Engelbrecht and Swart (2000),

revealed that the condition of hypocortisolism may be as a result of the unique enzymatic activity of Angora CYP17. While pursuing CYP17 as the possible cause of hypocortisolism in the South African Angora goat, Slabbert (2003) identified two CYP17 isoforms in this species, which differed at four nucleotide positions, including a change in the recognition site for the restriction enzyme ACS I. A restriction digest based genotyping method was subsequently developed and 83 goats genotyped. Twenty four goats were homozygous for CYP17 without the ACS I site and the remaining 59 were heterozygous. No goats homozygous for CYP17 with the ACS I site were detected. Slabbert's attempt to correlate the genotyping results with phenotypical data was, however, unsuccessful as the farmers were unable to identify goats susceptible to cold stress based on phenotypical traits. Nevertheless, Slabbert proposed that a genetic predisposition to stress susceptibility may exist in the Angora goat and that CYP17 may serve as a potential biological marker for such a condition. This proposal could, however, only be investigated once CYP17 was cloned and characterised.

Total mRNA was isolated from the adrenal cortex of a heterozygous Angora goat for this study. RT-PCR yielded the cDNA for CYP17 and 3βHSD, which were subsequently cloned into a mammalian expression vector. In addition, the cDNA for cytochrome b_5 was cloned from mRNA isolated from Angora liver tissue. Sequence analysis confirmed the existence of two CYP17 isoforms, differing at four nucleotide positions — 17, 122, 637 and 1065. The cDNAs were named CYP17 ACS+ due to the presence of an ACS I restriction site at position 635-640 (GenBank accession no. **EF524064**), which is 100% homologous with Boer goat (*Capra hircus*) CYP17 cDNA (GenBank accession

no. **AF251387**); and CYP17 ACS- due to the absence of the ACS I restriction site (GenBank accession no. **EF524063**). Three of the four nucleotide substitutions resulted in amino acid changes in the predicted protein sequence, namely the substitutions at positions 17, 122 and 637, resulting in amino acid substitutions at positions 6, 41 and 213. CYP17 ACS+ has glycine, leucine and isoleucine residues at these positions, while in CYP17 ACS- these residues are replaced by alanine, proline and valine residues, respectively. The G6A and I213V substitutions are both conservative and not expected to affect the three-dimensional structure of the enzyme. However, the non-conservative amino acid substitution P41L, lies in the highly conservative PR, critical for the correct folding of the cytochromes P450 (Yamazaki et al., 1993; Kusano et al., 2001a; Kusano et al., 2001b). The absence of a proline residue at this position in CYP17 ACS+ may therefore influence the folding of the enzyme, resulting in a change in the three-dimensional structure and in the enzymatic activity of the protein. Furthermore, the proline residue at position 41 is highly conserved amongst CYP17 of all species cloned to date.

Both CYP17 isoforms were expressed in COS-1 cells and assayed for activity. The transfected COS-1 cells converted pregnenolone to 17-hydroxypregnenolone and DHEA. Progesterone metabolism yielded 17-hydroxyprogesterone and low, but detectable, levels of 16-hydroxyprogesterone, while no androstenedione was detected. The K_m values of the two isoforms were similar for pregnenolone, while ACS+ had a K_m value that is significantly greater than that of ACS- for progesterone. It was also interesting to note that the *V* values obtained for the ACS- were consistently

significantly greater than those for ACS+ expressed under identical conditions for both substrates. However, as the *V* values are dependent on enzyme concentration, unlike the K_m values, they cannot be compared directly to one another as the variations could be due to differing transfection efficiencies. These values do, however, correspond well to the data obtained by Engelbrecht and Swart (2000).

The enzymatic activity of the CYP17 isoforms were further investigated in a time course study. In addition, the potential influence on enzymatic activity of the P41L substitution was investigated by site directed mutagenesis. Both CYP17 isoforms were mutated at this position to the corresponding proline or leucine residue of the other isoform. After 8 hours of incubation with the pregnenolone, ACS-, ACS+ and ACS+ L41P metabolized ≈ 90% pregnenolone substrate to ≈ 80% 17-hydroxypregnenolone and ≈ 10% DHEA, while only ≈ 58% pregnenolone was, metabolized to 17-hydroxypregnenolone by ACS- P41L, with no detectable DHEA being formed.

The inclusion of cytochrome b_5 resulted in a substantial increase in 17,20-lyase activity of all the CYP17 constructs. However, the effect was significantly greater when the constructs with the proline at position 41 (ACS- and ACS+ L41P) were expressed. Both completely metabolised pregnenolone to DHEA in 8 hours, while ACS+ and ACS- P41L only produced ≈69% and 79% DHEA, respectively. In addition, ACS- and ACS+ L41P demonstrated a greater 17,20-lyase activity than CYP17 ACS+ and ACS- P41L when 17-hydroxypregnenolone was used as substrate, both in the presence and absence of cytochrome b_5. Progesterone metabolism yielded 17-hydroxyprogesterone and small, but detectable amounts of 16-

hydroxyprogesterone. Furthermore, although no androstenedione was detected in the absence of cytochrome b_5, the metabolite was detected for both ACS- and ACS+ L41P upon addition of cytochrome b_5, albeit less than 5%. These data clearly demonstrate that ACS- has a significantly greater 17, 20-lyase activity than ACS+. Furthermore, site directed mutagenesis revealed that the difference in 17,20-lyase activity is primarily due to the non-conservative amino acid substitution at position 41. The substitution of the proline residue for a leucine residue at this position in ACS- resulted in a significant decrease in 17,20-lyase activity for 17-hydroxypregnenolone. Conversely, introducing the L41P substitution into ACS+ partially restored the basal 17,20-lyase activity.

In CYP17 catalysed reactions, the 17,20-lyase requires the C_{20} of the steroid substrate, rather than the C_{17}, to align with the iron-oxygen complex. The P41L substitution lies, as previously mentioned, within a domain important for correct protein folding, and will almost certainly result in a change in the three-dimensional structure of the folded enzyme (Yamazaki et al., 1993; Kusano et al., 2001a; Kusano et al., 2001b) not favouring the optimal repositioning of the substrate.

Similar substitutions in the PR of other cytochromes P450 result in reduced activities towards some, but not all substrates. In human CYP21, a P30L substitution reduced the activity of this enzyme for 17-hydroxyprogesterone and progesterone to 60% and 30% of the wild type, respectively (Tusie-Luna et al., 1991). Similarly, a naturally occurring mutation P34S in human CYP2D6 caused a significant reduction in activity towards

certain drugs (Kagimoto et al., 1990; Johansson et al., 1994; Fukuda et al., 2000).

Interestingly, though 16-hydroxyprogesterone production has only previously been reported for human CYP17 and that of other primate species (Swart et al., 1993; Arlt et al., 2002), low levels of 16-hydroxyprogesterone were detected for all Angora CYP17 constructs. This finding can be attributed to the use of mass spectrometry for the quantification of the steroids. A UPLC-APCI-MS method for the analysis of adrenal steroids was developed (chapter 5) as current methods employed in the separation and quantification of these steroids are inadequate for analysing the complex mixture of steroid metabolites produced by cotransfections of CYP17 and 3βHSD. The data presented in chapter 6 indicates that CYP17 of other species may well produce small amounts of 16-hydroxyprogesterone, but that the detection methods used previously were unable to detect these steroids at such low levels.

The 16α-hydroxylase reaction, as in the 17,20-lyase reaction, requires the repositioning of the steroid substrate with the C_{16} aligned with the iron-oxygen complex. Cytochrome b_5 promotes the 17,20-lyase reaction through an allosteric mechanism, which causes a structural change that promotes the association of CYP17 with CPR, increasing the efficiency of electron transfer required for the 17,20-lyase reaction (Auchus et al., 1998; Geller et al., 1999; Miller 2005). The cotransfection of all Angora CYP17 constructs with cytochrome b_5 resulted in a significant increase in the 16α-hydroxylase activity. This is likely due the allosteric interaction between CYP17 and cytochrome b_5, which we propose alters the three-dimensional structure of

CYP17 in a manner which promotes the repositioning of the substrate in an orientation more favourable for the 16α-hydroxylase reaction, as the 16α-hydroxylase reaction does not require additional electrons from CPR. Interestingly, unlike in the case of the 17,20-lyase reaction, no significant difference was observed between the CYP17 isoforms for the increase in 16α-hydroxylase activity caused by cytochrome b_5. This observation ruled out the possibility that the reduced stimulation of the 17,20-lyase activity in ACS+ and ACS- L41P was due to a reduced interaction of these constructs with cytochrome b_5. It is, however, possible that although the interaction of CYP17 and cytochrome b_5 is unchanged, the interaction of CYP17/cytochrome b_5 complex with CPR could be affected.

From the above discussion it is evident that two CYP17 isoforms, with distinct catalytic properties occur in the South African Angora goat. From these data it was possible to speculate that the increased 17,20-lyase activity observed for CYP17 ACS- may be the cause of the observed hypocortisolism as it would result in a greater flux of steroids through the Δ^5-steroid pathway. In the adrenal, CYP17 and 3βHSD compete for the same substrates, with the ratio and the substrate specificities of these two enzymes determining the steroidogenic output of the adrenal cortex. The role of these enzymes on the steroid output of the Angora was therefore investigated by coexpressing the constructs. In comparison to ACS+, ACS- expressed in COS-1 cells, strongly favoured androgen production by the Δ^5 steroid pathway as a result of its significantly enhanced 17,20-lyase activity. As expected, this resulted in a decrease in glucocorticoid precursor production. The inclusion of cytochrome b_5 in the cotransfections resulted in an increased difference in the steroid

profiles of pregnenolone metabolism, with ACS- expressing COS-1 cells predominantly producing adrenal androgens (≈68%), while glucocorticoid precursor production was predominant in ACS+ expressing cells (≈71%). This difference in androgen production in both the presence and absence of cytochrome b_5 was attributed to the greater 17,20-lyase activity of CYP17 ACS-, which as expected, resulted in the greater flux through the Δ^5 pathway, with a concomitant decrease in glucocorticoid precursors. These results are consistent with those obtained by Engelbreght and Swart (2000) who showed that the Angora produced significantly less glucocorticoid precursors, but more androgens, than Boer goat and Merino sheep in microsomal preparations. Furthermore, Angora CYP17 ACS+, which produces significant levels of glucocorticoid precursors, shares 100% sequence identity with Boer goat CYP17, thus implicating ACS- as the primary cause of the observed hypocortisolism. ACS- is also likely the wild type Angora gene, with ACS+ probably being introduced by cross breeding with Boer goats as discussed later in this chapter. These findings, suggest furthermore that CYP17 may be used as a biological marker for stress tolerance in the Angora goat. However, as mentioned above, Slabbert (2003) was unable to correlate stress tolerance and the CYP17 genotype, mainly due to a lack of phenotypic data. It has since become evident that genotyping, using restriction digestion assays is unsatisfactory as this method was unsuccessful in detecting the predicted ACS+/ACS+ genotype.

For this reason a more accurate and efficient real-time PCR based genotyping method was developed and a larger sample size genotyped for this study, as discussed in chapter 7. A sensor probe designed to be 100%

complementary to the ACS+ sequence, dissociated at 57°C when bound to mismatched sequence (ACS-) and at 63°C when bound to ACS+. This method was subsequently used to genotype 576 Angora goats from two separate populations. It is interesting to note that the ACS+/ACS+ genotype remained undetected. However, genotyping by real-time PCR yielded two distinct melting peak profiles for heterozygous samples. Melting peaks of similar peak area, which is normally expected for heterozygous samples (Lyon, 2001), were observed in 42.9% of the animals investigated in this study. However, although 40.6% of the animals were genotyped as heterozygotes, they consistently yielded melting profiles with unequal peak areas, where the peak representative of ACS+ had a substantially smaller area than that of ACS-. Furthermore, this pattern was consistently observed for the same samples even when tested using different DNA isolations and blood samples. As a control, 107 Boer goats were genotyped and were all heterozygous showing no distortion in peak area. Similarly all the sheep that were genotyped as heterozygotes demonstrated no peak distortion.

Since the copy number of individual alleles has a direct influence on respective peak areas when genotyping with hybridization probes (Lyon, 2001), the difference in peak areas observed in this study may thus have been the result of differences in CYP17 copy number. Based on the melting peak profiles, goats were subsequently divided into three genotypes, namely: homozygotes for ACS- (H_o); heterozygotes yielding unequal peaks areas (H_u); and heterozygotes yielding equal peak sizes (H_e). Since the relative melting peak areas of polymorphic samples are frequently used in the detection of gene duplications and deletions (Ruiz-Ponte et al., 2000; Lyon, 2001; Lyon et

al., 2001; Pont-Kingdon and Lyon, 2003; Timmann et al., 2005), it was quite possible that the observed unequal peak area ratio of the H_u group indicated a lower abundance of ACS+ in these goats. However, unequal melting peaks may not always be the result of a change in gene frequency as fluorescence decreases with increasing temperature, resulting in melting peaks that may have greater areas at lower temperatures than at higher temperatures. Furthermore, probes melted from the less stable allele may re-anneal to the excess templates of the more stable allele and preferential binding may also occur when probe concentrations are limiting (Lyon, 2001).

Quantitative real-time PCR was therefore employed to determine if the observed unequal peak areas were an artefact of the genotyping assay or due to unequal allele distribution. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative copy number of H_e and H_u genotypes relative to an H_o calibrator. The H_e genotype revealed a significant 1.7-fold increase in copy number when compared to the H_o group. In addition, all Boer goats (all Boer goats genotyped were H_e) demonstrated the same 1.7-fold increase in copy number. Although the H_u genotype yielded a 1.4-fold increase when compared to H_o, this genotype was, however, not significantly different from either the H_o or H_e genotypes. This data indicated that, in both the South African Angora goat and the Boer Goat, CYP17 ACS- and ACS+ are not two alleles of a single CYP17 gene as first thought, but instead two separate genes. To date no other mammal has been reported to have two CYP17 genes encoding two CYP17 isoforms (Nakajin et al., 1981; Chung et al., 1987; Picado-Leonard and Miller, 1987; Sparkes et al., 1991; Youngblood et al., 1991; Fan et al., 1992). The only other report of two CYP17 genes has

been in Tilapia (*Oreochromis mossambicus*) and Medaka Fish (*Oryzias latipus*) (Zhou et al., 2007).

The data indicates that the H_o genotype has only one CYP17 gene, namely ACS-. Conversely, the H_e genotype has both CYP17 genes (ACS+ and ACS-) at two different loci and therefore twice the copy number of H_o. This data also explains why the ACS+/ACS+ genotype was not detected — ACS- is always present together with ACS+.

Crossing H_o and H_e goats would yield the intermediate genotype, H_u, which receives both ACS- and ACS+ from its H_e parent, but only ACS- from the H_o parent resulting in the ratio of ACS-:ACS+ being 2:1. This is supported by the distortion in peak areas obtained during genotyping with hybridization probes as well as by the relative copy number determination, where H_u genotype yielded a 1.4-fold increase when compared to H_o, but was not significantly different from either the H_o or H_e genotypes.

The observation that all Boer goats, but not all Angoras, genotyped to date are H_e, suggests that this genotype originated in the Boer goat and not the Angora. Although the origins of the Boer goat are vague, it is commonly accepted that this breed was developed by farmers in South Africa from indigenous African goats from as early as 1800, with the emergence of a distinct breed by the beginning of the 20th century (Van Rensburg, 1938, Casey and Van Niekerk, 1988). It is feasible that a gene duplication, resulting from non-homologous recombination, occurred during the development of this subspecies (Ohno 1970). In other species, including humans, unequal crossing over events have resulted in gene duplications of other cytochromes P450, such as CYP21 and CYP2D6 (Amor et al., 1985; Chung et al., 1985;

Skow et al., 1988; Gitelman et al 1992; Johansson et al 1993). The individual CYP17 genes likely originated from two of the subspecies that were used in the breeding of the Boer goat through non-homologous recombination, though it remains to be determined if both genes are located in close proximity on the same chromosome. The maintenance of both genes in the Boer goat may be a result of the selective pressure put on this subspecies during its development. It is, however, unlikely that a gene duplication event occurred followed by subsequent diversion (Hurles, 2004), as the CYP17 available on GenBank for the domestic goat, *Capra hircus* (GenBank accession no. **AF251387**) is 100% homologous to that of ACS+, while the ACS- gene alone is found in H_o Angora goats.

The Angora goat was first imported into South Africa from Turkey in 1838 (Uys, 1988). Many of the imported Angoras were crossed with the native goats during the early development of the mohair industry in South Africa. Some pure Angoras were, however, maintained for stud purposes (Hayes, 1882). The Boer goat fits the description of the native goats used in these breeding practices, albeit in the early stage of its development (Hayes, 1882). We therefore suggest that it was these early breeding practices that led to the introduction of the second CYP17 gene (ACS+) into the South African Angora population.

Recently, a breeding program was carried out, where South African Angora goats were crossed with Boer goats in order to establish a more hardy mohair producing goat with a relatively high reproductive ability and good carcass characteristics (Snyman, 2004). Crossbred does (50% Angora goat:50% Boer goat) were mated to Angora bucks in order to obtain 75%

Angora goat: 25% Boer goat-progeny. These were then mated to each other to establish a 75% Angora goat: 25% Boer goat line (G1). A number of F2 generation G1 goats have subsequently been genotyped for this study and the results confirm that crosses with Boer goats significantly increase the frequency of the H_e genotype in the Angora population, while decreasing the H_o and H_u genotypes as expected. In order to confirm that CYP17 ACS+ originated in the South African Angora population through cross breeding practices, goats from outside the South African population will have to be genotyped. Future research will also aim at identifying the origin of the ACS+ gene by genotyping native African goats. If a goat with the elusive ACS+/ACS+ genotype is identified, the *in vitro* results obtained in this study suggest that such a goat would have a high capacity for cortisol production. Crossing such a goat with Angora goats may help to further improve the hardiness of the Angora goat. However, increased cortisol production may affect favourable fibre characteristics negatively as will be discussed below (Van Rensburg, 1971; Herselman and Pieterse, 1992).

Subsequent to the identification of the three genotypes in the South African Angora population, the question arose whether the genotypes differed in their ability to cope with cold stress. The results obtained in the *in vitro* studies discussed earlier demonstrated the significant difference in the 17,20-lyase activity of the two CYP17 isoforms and cotransfections with 3βHSD suggested that the ACS- isoform was responsible for the observed hypocortisolism as it favoured androgen production via the Δ^5 pathway at the expense of glucocorticoid synthesis. At this stage, however, it was assumed that the two isoforms were two alleles of the same gene. The subsequent

discovery that the two isoforms are two separate CYP17 genes complicated any deductions made from the *in vitro* studies. For this reason the *in vivo* ability of each genotype to produce cortisol in response to intravenous insulin injection was investigated. Insulin injection induces a state of hypoglycemia which activates the HPA axis (Plotsky et al., 1985; Suda et al., 1992).

No significant difference in the basal cortisol levels for the three genotypes were observed prior to injection and the three groups all demonstrated a similar decrease in plasma glucose concentration post injection. Similarly, the three groups all demonstrated an increase in plasma cortisol in response to the induced hypoglycemic state. However, the amplitude of the response was significantly greater in the H_e ($p < 0.05$) group than in the H_o group. After 120 minutes, the mean plasma cortisol concentration (155.5 ± 66.8 nmol/L) of the H_e group was 1.4-fold greater than that of the H_o group (114.6 ± 42.1 nmol/L). The cortisol response in the H_u group was not significantly different from either the H_o or the H_e group as with the mean plasma cortisol levels (134.6 nmol/L) lying between the values of the H_o and H_e group, 120 minutes post injection. This data correlates well with the *in vitro* studies, and confirms the greater capacity of the ACS+ gene to produce glucocorticoid precursors, as both genotypes containing this gene produced more cortisol than the H_o genotype in which this gene is absent. Furthermore, the H_e genotype, which has two copies of ACS+, produced more cortisol than the H_u, which has only copy of ACS+. However, the relative expression levels of CYP17 in the adrenal of the different genotypes have yet to be determined. Johansson et al. (1993) have previously demonstrated that CYP2D6 gene duplication results in an increased metabolic capacity for drugs

such as debrisoquine. The influence of copy number can therefore not be ignored and may be a contributing factor towards the increased cortisol production in H_e and H_u goats.

The data presented in this study, as discussed above, has clearly demonstrated, for the first time, two distinctive genes encoding two CYP17 isoforms in both the South African Angora goat and Boer goat. Three unique genotypes in the South African Angora goat have been shown to differ not only by the genes encoding CYP17, but also in copy number. Furthermore, the three identified genotypes differ significantly in their capacity to produce cortisol, thereby confirming CYP17 as a primary cause of the observed hypocortisolism in the South African Angora goat. In addition, an accurate and rapid genotyping method has been developed with the aim of identifying goats susceptible to cold stress. Employing quantitative real-time PCR, the Angora CYP17 can be used as a genetic marker for cold stress tolerance. The ultimate goal is to use this data to advise farmers on future breeding practices in an effort to breed more hardy Angora goats and minimise losses due to cold spells.

It has been suggested by van Rensburg (1971) that the low level of adrenal function may be advantageous for hair production, as it would remove the inhibitory effects of cortisol. Furthermore, Herselman and Pieterse (1992) demonstrated that regular intravenous injection of cortisol in Angora goats significantly reduced the greasy hair mass produced and concurrently resulted in a greater internal fat mass and back fat depth. Therefore to investigate the feasibility of using the CYP17 genotype as a genetic marker for more hardy Angora goats, a breeding program has been initiated at the experimental farm

in Jansenville. All possible pairings have been performed with a total of 107 ewes and six sires used in this study. The ewes are expected to kid in September of 2008. The kids will subsequently be genotyped and the pedigree data used to investigate the pattern of inheritance as suggested by this study. Data such as birth weight; weaning weight and age; eighth month body weight; fleece weight from the first shearing (shearing occurs at 8 months of age for ram kids and 12 months of age for the ewe kids); and fleece quality traits such as fibre diameter and staple length will be recorded for each kid born in this study. The fleece quality traits will again be determined for the young ewes at their third shearing at 16-18 months of age. The body weight of the young ewes will be recorded before their first mating and the ewes will be scanned for pregnancy. The reproductive performance of each ewe will also be determined. In addition, the ability of each kid born in this study to cope with cold spells, which are likely to occur during winter, will be monitored. *In vivo* insulin induced stress experiments may also be preformed. The data obtained during this breeding trial will be used to determine if more hardy goats can be bred using the CYP17 genetic marker and also to determine whether such breeding would have any negative effects on the reproductive characteristics and fleece quality and quantity. This data will allow farmers to ascertain if these breeding strategies will be viable for large scale implementation within the mohair industry. Unfortunately, due to the long duration of the breeding program, these result could not be included in the present study. It is, however, unlikely that future selection for the most hardy genotype, H_e, (based on cortisol production) would affect the mohair industry

negatively, as a large number of these goats (42.9%) are already present in the populations genotyped for this study.

In conclusion, this study has clearly implicated CYP17 as the primary cause of hypocortisolism in the South African Angora goat and has laid the foundation for future research into breeding a more hardy Angora goat.

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APPENDIX A

CYP17 causes hypocortisolism in the South African Angora goat

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(Accepted for publication in Molecular and Cellular Endocrinology)

Abstract

Two cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) isoforms have been identified in the South African Angora goat (*Capra hircus*) and have been implicated as the primary cause of hypocortisolism in this subspecies. These goats are the most efficient fibre producing, but least hardy, small stock breed in Southern Africa. Their inability to cope with prolonged exposure to cold and the resulting stock loss which occurs during winter have been the subject of numerous studies. The two isoforms are encoded for by two separate genes, a novel finding for a mammalian species. The enzymes have unique catalytic properties and differ with respect to their 17,20-lyase activities towards 17-hydroxypregnenolone and subsequent androgen production. *In vivo* assays confirmed that the three resulting genotypes differed in their ability to produce cortisol in response to intravenous insulin injection implicating CYP17 as the primary cause of the observed hypocortisolism.

Keywords: cytochrome P450 17α-hydroxylase/17,20-lyase; CYP17; Angora goat; hypocortisolism
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Contents

1. Introduction

South Africa is the world leader in mohair production, supplying approximately 55% of the global demand. South African Angora goats (*Capra hircus*) are the most efficient fibre producing, but least hardy small stock breed in Southern Africa. The industry is hampered by the severe loss of young, newly shorn Angora goats, which occur during cold spells. Investigations into cold-stress related deaths at first implicated the adrenal cortex and subsequently a single steroidogenic enzyme, cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17), as the cause of the problem.

CYP17 catalyzes two distinct mixed-function oxidase reactions, namely the 17α-hydroxylation of the C21 steroids, pregnenolone (PREG, Δ^5-steroid) and progesterone (PROG, Δ^4-steroid), followed by the cleavage of the C17–20 bond to produce the C19 steroids, Dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively (Fig 1). The 17,20 lyase activity of CYP17 is enhanced by allosteric interactions with cytochrome b_5 and by post-translational phosphorylation (Pandey and Miller, 2005). The dual activity of

CYP17 places this enzyme at a key branch point in the synthesis of mineralocorticoids, glucocorticoids and adrenal androgens. Changes in either the hydroxylase or lyase activity of the enzyme will influence the steroidogenic output of the adrenal and thus have significant physiological effects as demonstrated in recent studies.

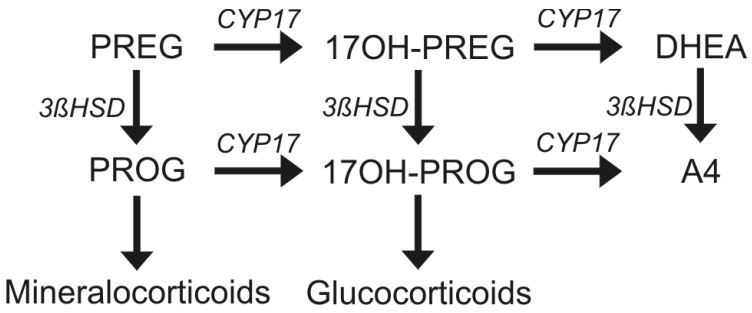

Fig. 1. Schematic representation of the reactions catalysed by CYP17 and 3βHSD.

2. Early investigations

Preliminary investigations found that the primary cause of stock loss during cold spells was due to an energy deficiency resulting from a decrease in blood glucose levels, which causes a drop in body temperature and subnormal heart function (Wentzel et al., 1979). It was subsequently demonstrated that Angora goats, on a low feeding level, could withstand cold wet and windy conditions for 1.5 hours, while goats on high feeding levels could survive for up to three hours. In contrast, Boer goats, on a low feeding level, could withstand the conditions for three hours and those on the high feeding level were still comfortable after six hours (Fourie, 1984). This demonstrated that supplementary feeding practices, previously proposed by Wentzel (1982, 1983), could increase the Angora goat's tolerance for cold, but not eliminate the problem of cold-stress related deaths. It was therefore concluded that the Angora goat does not have the metabolic capacity to

produce enough heat, a problem further compounded by the weak insulation of the short hair found in shorn goats (Fourie, 1984).

In 1992, Cronje demonstrated that Angora does have a lower blood glucose concentration and a slower response of glucose synthesis rate to dietary energy increments, than Boer goat does, providing further evidence of the inability of the Angora goat to mobilize glucose precursors.

As early as 1971 Van Rensburg had suggested that selection for high mohair production indirectly resulted in reduced adrenal function and as a result reduced cortisol levels, as a negative relationship between plasma cortisol levels and hair production was identified. Herselman (1990) confirmed that the high hair production in Angora goats resulted in the total energy metabolism being less effective when compared to other goat species, and suggested that hair production might be at the expense of other production functions. Intravenous injection of dihydroxycortisolacetate over a six month period was found to change the metabolism of a test group of Angora goats to resemble that of more hardy breeds with a concomitant decrease in fibre production (Herselman and Pieterse, 1992). Cortisol production was subsequently compared in a number of small ruminant breeds with varying potentials for fibre production. While intravenous insulin injection caused a drop in blood glucose concentration in all breeds with a resulting increase in the plasma cortisol concentration, the peak plasma cortisol concentration was three to five times smaller in the Angora when compared to that of the other breeds. Similarly, the response in plasma cortisol to intravenous corticotropin releasing factor (CRF) was three to four times smaller in the Angora than in the other breeds. This study concluded that a form of hypocortisolism

contributes significantly to the carbohydrate metabolism disorders observed in the Angora (Herselman and van Loggerenberg, 1995).

3. Implicating CYP17

Engelbrecht et al. (2000) compared the adrenal response of Angora goats, Boer goats and Merino sheep to insulin-induced stress as well as adrenocorticotropic hormone (ACTH) and CRH stimulation. Insulin induced a hypoglycaemic condition in all three species. Plasma cortisol levels increased significantly in both the Boer goat and sheep, but not in the Angora (Fig. 2A), confirming the hypocortisolism reported by previous studies (Van Rensburg, 1971; Herselman and Pieterse, 1992; Herselman and van Loggerenberg, 1995). Sheep CRH was unable to elicit a response in either of the two goat species, while ACTH stimulation caused a rise plasma cortisol concentration in all three species indicating that the hypothalamic-pituitary-adrenal-axis was functional in all three species. The response was, however, significantly lower in the Angora goat than in the other two species, indicating that the Angora adrenal might have a reduced ability to produce cortisol.

Microsomal and mitochondrial subcellular adrenal fractions were subsequently investigated to compare adrenal steroidogenesis in the three aforementioned species (Engelbrecht and Swart, 2000). The production of the glucocorticosteroid precursors (deoxycorticosterone and deoxycortisol) and androgens, (DHEA and A4) were compared between species using PREG as substrate. Angora goat microsomes produced significantly less glucocorticosteroid precursors (36%) than Boer goat (79%) and Merino sheep (82%) microsomes. In contrast, Angora goat produced significantly more

17OH-PREG and DHEA (35%) than did the other two species (9% and 0%, respectively). Unfortunately, 17OH-PREG and DHEA were not quantified individually in this study. When PROG was used as a substrate, Angora microsomes produced significantly more deoxycorticosterone and significantly less deoxycortisol than the other species, while A4 and 17OH-PROG production was less than 5% in all three species.

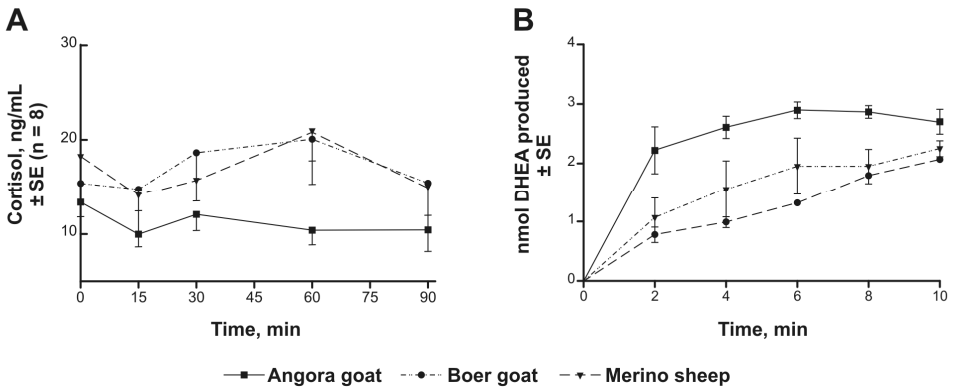

Fig. 2. Comparative study of (A) cortisol production and (B) CYP17 activity in Angora goats, Boer goats and merino sheep. Reproduced from (A) Engelbreght et al., 2000 and (B) Engelbreght and Swart, 2000.

The differences in steroid output in the microsomal preparations from the three species suggested that there was a difference in activity of one or more of the steroidogenic enzymes. The activity of specific enzymes in the adrenal steroidogenic pathway of the three species were subsequently studied by the selective addition of cofactors. There was no significant difference in the activity of the microsomal enzymes, 3β-hydroxysteroid dehydrogenase (3βHSD) and cytochrome P450 21-hydroxylase (CYP21), or the mitochondrial enzymes cytochrome P450 side-chain cleavage (CYP11A1) and cytochrome P450 11β-hydroxylase (CYP11B1). CYP17 activity was studied by measuring the conversion of PREG to DHEA using NADPH as cofactor in the absence of

NAD^+. CYP17 in the Angora goat adrenal microsomes converted PREG to DHEA significantly faster than in the adrenal microsomes of the other two species (Fig 2B). CYP17 therefore demonstrated the only significant difference in enzymatic activity between species. Engelbrecht and Swart (2000) concluded that the preference exhibited by Angora CYP17 for the Δ5-steroid pathway during adrenal steroidogenesis would likely result in an increased production of adrenal androgens *in vivo* with a concomitant decrease in the production of glucocorticoids when compared to the other species.

4. Characterisation of Angora CYP17

Angora CYP17, 3βHSD and cytochrome b_5, which alostericaly affects CYP17 activity (Pandey and Miller, 2005) were subsequently cloned by Storbeck et al. (2007). Two CYP17 isoforms were identified, namely ACS- (GenBank accession no. **AF251387**) and ACS+ (GenBank accession no. **EF524064**). The isoforms differed by three amino acid residues of which two of the substitutions, A6G and V213I are conservative. The single non-conservative substitution, P41L, lies in the highly conservative proline rich sequence (PR) which is critical for the correct folding of all cytochromes P450, specifically in stabilizing the conformation of the protein prior to heme binding (Yamazaki et al., 1993; Kusano et al., 2001a; Kusano et al., 2001b).

The CYP17 isoforms were subsequently expressed in COS-1 cells and assayed for activity. PREG was converted to 17-OHPREG and DHEA, while PROG metabolism yielded 17-OHPROG and low, but detectable, levels of 16-OHPROG. No A4 was detected. While the 17α-hydroxylase activity was

similar for both constructs, ACS- demonstrated a significantly increased lyase activity towards 17OH-PREG in both the presence and absence of cytochrome b_5. Site-directed mutagenesis revealed that the difference in 17,20 lyase activity was primarily due to the non-conservative P41L substitution (Fig. 3), which was proposed to alter the three-dimensional structure of the enzyme.

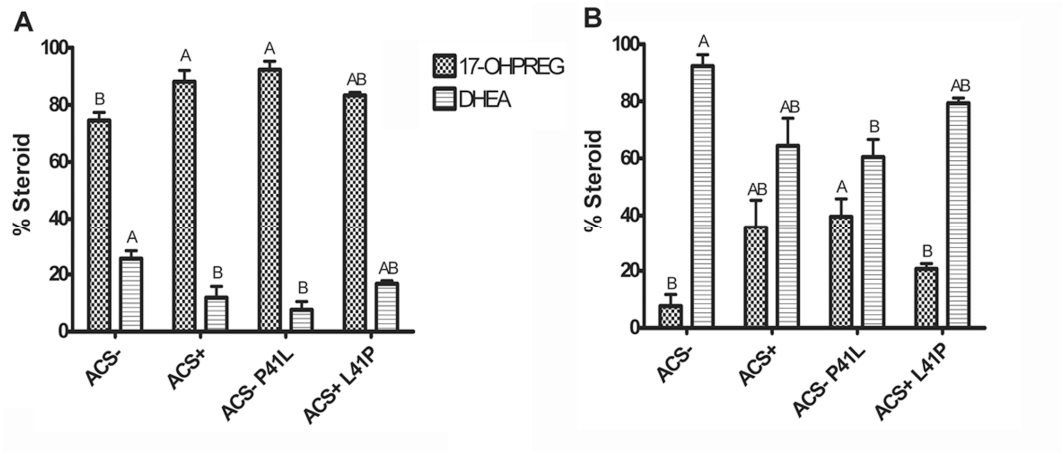

Fig. 3. Steroid profile of 17-OHPREG (1μM) metabolism after 8 hours by Angora goat CYP17 constructs expressed in COS-1 cells, (A) without cytochrome b_5 and (B) in the presence of cytochrome b_5. Individual steroids were compared for each construct by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p< 0.05$), Results are representative of at least three independent experiments.

In the adrenal, CYP17 and 3βHSD compete for the same substrates, with the ratio and the substrate specificities of these two enzymes determining the steroidogenic output of the adrenal cortex (Fig. 1). The effect of the difference in CYP17 activity was therefore investigated by coexpressing each CYP17 isoform with 3βHSD in COS-1 cells using PREG as substrate. COS-1 cells expressing ACS- and 3βHSD, produced significantly more adrenal androgens and less glucocorticoid precursors than cells expressing ACS+ and 3βHSD (Fig. 4A). The inclusion of cytochrome b_5 in the cotransfections resulted in a more pronounced difference in the steroid profiles of PREG

metabolism, with COS-1 cells expressing CYP17 ACS- cells predominantly producing adrenal androgens (≈68%), while glucocorticoid precursors production was predominant in CYP17 ACS+ expressing cells (≈71%) (Fig. 4B). The difference in androgen production in both the presence and absence of cytochrome b_5 was attributed to the greater 17,20 lyase activity of CYP17 ACS-, which results in a greater flux through the Δ^5 pathway, and a concomitant decrease in glucocorticoid precursors.

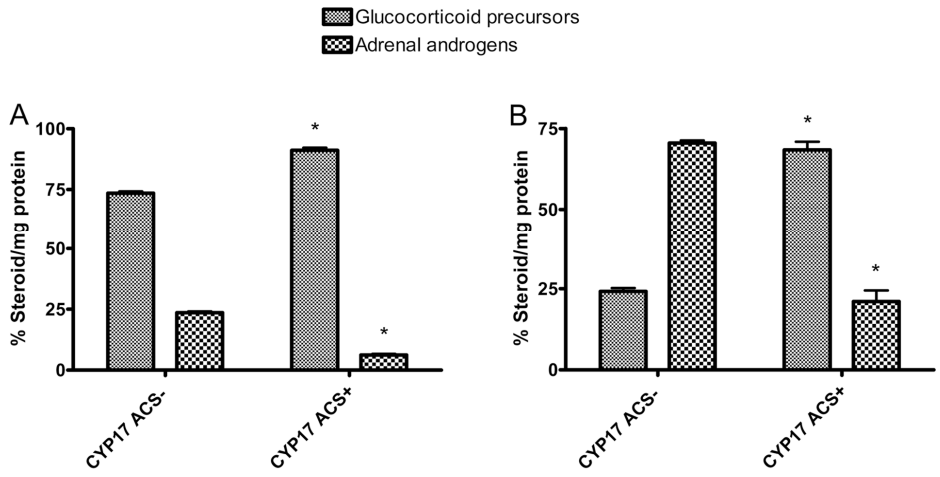

Fig. 4. Steroid profile of PREG (1μM) metabolism after 8 hours by Angora goat CYP17 and 3βHSD coexpressed in COS-1 cells, (A) without cytochrome b_5 (B) and in the presence of cytochrome b_5. Glucocorticoid precursors (PREG, 17OH-PREG, PROG and 17OH-PROG) and adrenal androgens (DHEA and A4) were compared for each construct by unpaired *t* test (* $p < 0.001$). Results are representative of three independent experiments.

During this study it was assumed that the two CYP17 isoforms were two alleles of the same gene. Goats were genotyped based on a restriction digest assay. The majority of the goats genotyped (59/83) were heterozygous while the remaining goats (24/83) were homozygous for ACS-. No goats homozygous for ACS+ were detected.

5. The identification of two CYP17 genes

A real time PCR based genotyping assay was developed in order to determine why the ACS+ homozygote was not detected and 576 Angora goats from two separate populations were subsequently genotyped. Although the ACS+/ACS+ genotype remained undetected, heterozygous samples could be divided into two distinct groups based on their melting profiles. The first heterozygous group, H_e had two melting peaks of similar peak area as was expected when using hybridisation probes. The second heterozygous group, H_u, consistently yielded melting profiles with unequal peak areas, where the peak representative of ACS+ had a substantially smaller area than that of ACS- (Fig. 5A). This pattern was consistently observed for the same samples even when tested using different DNA isolations and blood samples. A third genotype of homozygous goats homozygous for ACS- was named H_o. As a control, 107 Boer goats were genotyped and found to all belong to the H_e genotype (Storbeck et al., 2008).

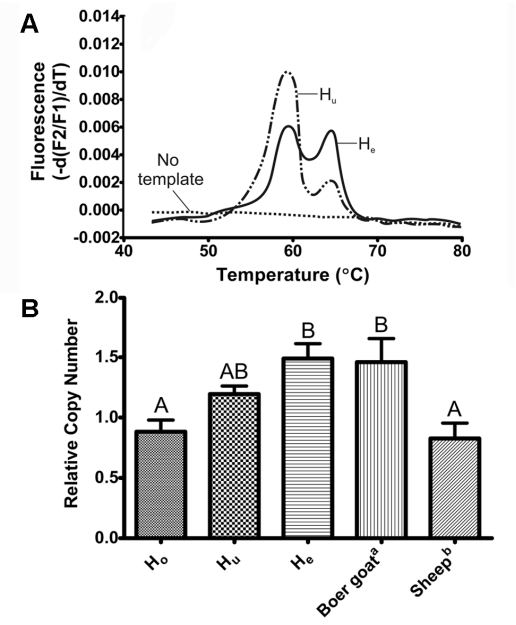

Fig. 5. (A) Typical melting curves for the H_u and H_e genotypes. (B) CYP17 copy number determinations. Each group (n=6 per group) was compared to every other group by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p< 0.05$).
[a]All Boer goats genotyped in this study belong to the H_e genotype.
[b]Only heterozygous Merino sheep were used for copy number determinations.

As the copy number of individual alleles has a direct influence on respective peak areas when genotyping with hybridization probes (Lyon, 2001) Storbeck et al. (2008) subsequently investigated if the distorted peak area found in the H_u group could be due to differences in CYP17 copy number.

Relative copy number determinations were performed for each of the three putative genotypes using quantitative real-time PCR. The H_e genotype revealed a significant 1.7-fold increase in copy number when compared to the H_o group (Fig. 5B). In addition, all Boer goats demonstrated the same 1.7-fold increase in copy number. The H_u genotype yielded a 1.4-fold increase when compared to H_o. Furthermore, as expected for two alleles of the same gene, all heterozygous sheep showed no significant change in copy number.

This data reveals the novel finding that, in both the South African Angora goat and the Boer Goat, ACS- and ACS+ are not two alleles of a single CYP17 gene, but instead two separate genes, which is the first report of a mammalian organism with two CYP17 genes (Nakajin et al., 1981; Chung et al., 1987; Picado-Leonard and Miller, 1987; Sparkes et al., 1991; Youngblood et al., 1991; Fan el al., 1992; Givens et al., 1994). The H_o genotype therefore has only one CYP17 gene, namely ACS-. Conversely, the H_e genotype has both CYP17 genes (ACS+ and ACS-) at two different loci and therefore twice the copy number of H_o. This explained why the ACS+ homozygote was never detected, as ACS- is always present with ACS+. It was proposed that crossing H_o and H_e goats would yield the intermediate genotype H_u, which receives both ACS- and ACS+ from its H_e parent, but only ACS- from the H_o parent and therefore has an ACS-:ACS+ ratio of 2:1, which corresponds to the data obtained by genotyping (Fig. 5A) and the copy number determination (Fig. 5B). Subsequently a breeding trial has been initiated to investigate the inheritance of CYP17. Preliminary data (not shown) supports the proposed mechanism. It was hypothesised that the H_e genotype originated in the Boer goat as all Boer goats, but not all Angora's genotyped were H_e, though further investigation is required.

6. *In vivo* study

Storbeck et al. (2008) subsequently preformed an *in vivo* assay for cortisol production in order to establish the physiological effect of the three novel genotypes. Ten goats from each CYP17 genotype were tested for their ability to produce cortisol in response to intravenous insulin injection. There

was no significant difference in the basal cortisol levels of the three genotypes and the three groups demonstrated a decrease in plasma glucose and an increase in plasma cortisol levels in response to insulin injection (Fig. 6). While the decrease in plasma glucose was similar for all groups, the amplitude of the response in cortisol production was significantly greater in the H_e ($p < 0.05$) group than in the H_o group. The cortisol response in the H_u group was greater than in the H_o group, but not significantly different from either the H_o or the H_e groups. This data confirmed that goats with the ACS+ were able to produce more cortisol as was hypothesised from results obtained *in vitro* (Storbeck et al., 2007).

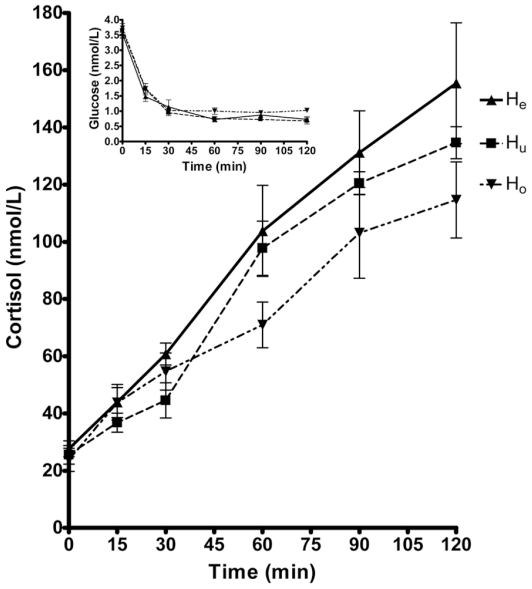

Fig. 6 Plasma cortisol levels in the three Angora genotypes (n=10 per group) following intravenous insulin injection. Plasma glucose levels are shown in the insert. The groups were compared by one-way ANOVA with repeated measures test, followed by Dunnett's repeated measures post test. The H_o and H_e groups demonstrated a significantly ($p<0.05$) different response in cortisol production.

7. Conclusions

CYP17 has clearly been implicated as the cause of the observed hypocortisolism in the South African Angora goat. Storbeck et al. (2007) showed that when expressing Angora CYP17 in COS-1 cells one of the two CYP17 isoforms, ACS-, has an enhanced 17,20 lyase activity which results in increased androgen production at the expense of glucocorticoid synthesis. This is in agreement with the results obtained by Engelbrecht and Swart (2000) using microsomal fractions. Furthermore, the effect of this CYP17 isoform on cortisol production was subsequently confirmed by *in vivo* studies. A genotyping assay developed for the two CYP17 isoforms was, however, complicated by the discovery that the two CYP17 isoforms are not two alleles of the same gene, but rather two separate CYP17 genes (Storbeck et al., 2008). Breeding trials based on this genetic information have subsequently been initiated to determine if more hardy Angora goats can be bred without the loss of favourable mohair producing characteristics.

Acknowledgements

This work was financially supported by Mohair SA, the NRF, the University of Stellenbosch and the Wilhelm Frank Bursary Fund.

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APPENDIX B

Nucleotide and deduced amino acid sequences of the two Angora CYP17 isoforms: ACS- (GenBank accession no. **EF524063**) and ACS+ (GenBank accession no. **EF524064**).

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ACS+                            G
ACS-   1     ATG TGG GTG CTC TTG GCT GTC TTT CTG CTC ACC CTC GCC TAT TTA   45
ACS-   1     Met Trp Val Leu Leu Ala Val Phe Leu Leu Thr Leu Ala Tyr Leu   15
ACS+                           Gly

ACS+
ACS-   46    TTT TGG CCC AAG ACC AAG CAC TCT GCT GCC AAG TAC CCC AGA AGC   90
ACS-   16    Phe Trp Pro Lys Thr Lys His Ser Ala Ala Lys Tyr Pro Arg Ser   30
ACS+

ACS+                                                    T
ACS-   91    CTC CCA TCC CTG CCC CTG GTG GGC AGC CTG CCG TTC CTC CCC AGA   135
ACS-   31    Leu Pro Ser Leu Pro Leu Val Gly Ser Leu Pro Phe Leu Pro Arg   45
ACS+                                               Leu

ACS+
ACS-   136   CGT GGC CAG CAA CAC GAG AAC TTC TTC AAG CTG CAG GAA AAA TAT   180
ACS-   46    Arg Gly Gln Gln His Glu Asn Phe Phe Lys Leu Gln Glu Lys Tyr   60
ACS+

ACS+
ACS-   181   GGC CCC ATC TAT TCC TTT CGT TTG GGT TCC AAG ACT ACT GTG ATG   225
ACS-   61    Gly Pro Ile Tyr Ser Phe Arg Leu Gly Ser Lys Thr Thr Val Met   75
ACS+

ACS+
ACS-   226   ATT GGA CAC CAC CAG TTG GCC AGG GAG GTG CTT CTC AAG AAG GGC   270
ACS-   76    Ile Gly His His Gln Leu Ala Arg Glu Val Leu Leu Lys Lys Gly   90
ACS+

ACS+
ACS-   271   AAG GAA TTC TCT GGG CGT CCC AAA GTG GCC ACT CTA GAC ATC CTG   315
ACS-   91    Lys Glu Phe Ser Gly Arg Pro Lys Val Ala Thr Leu Asp Ile Leu   105
ACS+

ACS+
ACS-   316   TCA GAC AAC CAA AAG GGC ATT GCC TTT GCC GAC CAT GGT GCC CAC   360
ACS-   106   Ser Asp Asn Gln Lys Gly Ile Ala Phe Ala Asp His Gly Ala His   120
ACS+

ACS+
ACS-   361   TGG CAG CTG CAT CGG AAG CTG GTA CTG AAT GCC TTT GCC CTG TTC   405
ACS-   121   Trp Gln Leu His Arg Lys Leu Val Leu Asn Ala Phe Ala Leu Phe   135
ACS+

ACS+
ACS-   406   AAG GAT GGC AAC CTG AAG TTA GAG AAG ATC ATT AAT CAG GAA GCC   450
ACS-   136   Lys Asp Gly Asn Leu Lys Leu Glu Lys Ile Ile Asn Gln Glu Ala   150
ACS+

ACS+
ACS-   451   AAC GTG CTG TGT GAT TTC CTG GCT ACC CAG CAT GGA CAG TCC ATA   495
ACS-   151   Asn Val Leu Cys Asp Phe Leu Ala Thr Gln His Gly Gln Ser Ile   165
ACS+

ACS+
ACS-   496   GAT CTG TCC GAG CCT CTC TCT CTG GCG GTC ACC AAC ATA ATC AGC   540
ACS-   166   Asp Leu Ser Glu Pro Leu Ser Leu Ala Val Thr Asn Ile Ile Ser   180
ACS+

ACS+
ACS-   541   TTT ATC TGC TTC AAC TTC TCC TTC AAG AAT GAG GAT CCT GCC CTG   585
ACS-   181   Phe Ile Cys Phe Asn Phe Ser Phe Lys Asn Glu Asp Pro Ala Leu   195
ACS+
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ACS+
ACS-   586   AAG GCC ATA CAA AAT GTC AAT GAT GGC ATC CTG GAG GTT CTG AGC   630
ACS-   196   Lys Ala Ile Gln Asn Val Asn Asp Gly Ile Leu Glu Val Leu Ser   210
ACS+

ACS+               A
ACS-   631   AAG GAA GTT CTG TTA GAC ATA TTC CCT GCG CTG AAG ATT TTC CCC   675
ACS-   211   Lys Glu Val Leu Leu Asp Ile Phe Pro Ala Leu Lys Ile Phe Pro   225
ACS+               Ile

ACS+
ACS-   676   AGC AAA GCC ATG GAA AAG ATG AAG GGT TGT GTT GAA ACG CGA AAT   720
ACS-   226   Ser Lys Ala Met Glu Lys Met Lys Gly Cys Val Glu Thr Arg Asn   240
ACS+

ACS+
ACS-   721   GAA TTG CTG AAT GAA ATC CTT GAA AAA TGT CAG GAG AAC TTC ACC   765
ACS-   241   Glu Leu Leu Asn Glu Ile Leu Glu Lys Cys Gln Glu Asn Phe Thr   255
ACS+

ACS+
ACS-   766   AGC GAC TCC ATC ACT AAC TTG CTG CAC ATA CTG ATG CAA GCC AAG   810
ACS-   256   Ser Asp Ser Ile Thr Asn Leu Leu His Ile Leu Met Gln Ala Lys   270
ACS+

ACS+
ACS-   811   GTG AAT GCA GAC AAT AAC AAC GCT GGC CCA GAC CAG GAT TCA AAG   855
ACS-   271   Val Asn Ala Asp Asn Asn Asn Ala Gly Pro Asp Gln Asp Ser Lys   285
ACS+

ACS+
ACS-   856   CTG CTT TCA AAC AGA CAC ATG CTC GCT ACC ATA GCG GAC ATC TTC   900
ACS-   286   Leu Leu Ser Asn Arg His Met Leu Ala Thr Ile Ala Asp Ile Phe   300
ACS+

ACS+
ACS-   901   GGG GCT GGT GTG GAG ACC ACC ACC TCT GTG ATA AAG TGG ATC GTG   945
ACS-   301   Gly Ala Gly Val Glu Thr Thr Thr Ser Val Ile Lys Trp Ile Val   315
ACS+

ACS+
ACS-   946   GCC TAC CTG CTA CAC CAT CCT TCG TTG AAG AAG AGG ATC CAG GAT   990
ACS-   316   Ala Tyr Leu Leu His His Pro Ser Leu Lys Lys Arg Ile Gln Asp   330
ACS+

ACS+
ACS-   991   AGC ATT GAC CAG AAT ATA GGT TTC AAT CGC ACC CCA ACC ATC AGT   1035
ACS-   331   Ser Ile Asp Gln Asn Ile Gly Phe Asn Arg Thr Pro Thr Ile Ser   345
ACS+

ACS+                                             G
ACS-   1036  GAC CGG AAC TGC CTT GTC CTG CTG GAG GCA ACC ATC CGA GAG GTG   1080
ACS-   346   Asp Arg Asn Cys Leu Val Leu Leu Glu Ala Thr Ile Arg Glu Val   360
ACS+

ACS+
ACS-   1081  CTC CGA ATC CGG CCT GTG GCC CCT ATG CTG ATC CCC CAC AAG GCT   1125
ACS-   361   Leu Arg Ile Arg Pro Val Ala Pro Met Leu Ile Pro His Lys Ala   375
ACS+

ACS+
ACS-   1126  ATC ATT GAC TCC AGC ATT GGC GAC CTT ACC ATT GAC AAG GGC ACA   1170
ACS-   376   Ile Ile Asp Ser Ser Ile Gly Asp Leu Thr Ile Asp Lys Gly Thr   390
ACS+

ACS+
ACS-   1171  GAC GTT GTG GTC AAC CTG TGG GCA CTG CAT CAC AAT GAG AAG GAG   1215
ACS-   391   Asp Val Val Val Asn Leu Trp Ala Leu His His Asn Glu Lys Glu   405
ACS+

ACS+
ACS-   1216  TGG CAG CAG CCC GAC CTG TTC ATG CCC GAG CGC TTC TTG GAC CCC   1260
ACS-   406   Trp Gln Gln Pro Asp Leu Phe Met Pro Glu Arg Phe Leu Asp Pro   420
ACS+
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ACS+
ACS-   1261  ACG GGG ACA CAA CTC ATC TCG CCA TCG TTA AGC TAC TTG CCC TTT   1305
ACS-   421   Thr Gly Thr Gln Leu Ile Ser Pro Ser Leu Ser Tyr Leu Pro Phe   435
ACS+

ACS+
ACS-   1306  GGA GCT GGA CCC CGC TCC TGT GTA GGT GAG ATG CTA GCC CGC CAG   1350
ACS-   436   Gly Ala Gly Pro Arg Ser Cys Val Gly Glu Met Leu Ala Arg Gln   450
ACS+

ACS+
ACS-   1351  GAG CTC TTC CTC TTC ATG TCC CGG CTG CTG CAG AGG TTC AAC CTG   1395
ACS-   451   Glu Leu Phe Leu Phe Met Ser Arg Leu Leu Gln Arg Phe Asn Leu   465
ACS+

ACS+
ACS-   1396  GAG ATC CCG GAT GAT GGG AAG CTA CCC TCT CTG GAG GGC AAT CCC   1440
ACS-   466   Glu Ile Pro Asp Asp Gly Lys Leu Pro Ser Leu Glu Gly Asn Pro   480
ACS+

ACS+
ACS-   1441  AGT CTC GTC TTG CAG ATC AAA CCT TTC AAG GTG AAG ATC GAG GTG   1485
ACS-   481   Ser Leu Val Leu Gln Ile Lys Pro Phe Lys Val Lys Ile Glu Val   495
ACS+

ACS+
ACS-   1486  CGC CAG GCC TGG AAG GAA GCC CAG GCT GAG GGT AGC ACC TCA TGA   1530
ACS-   496   Arg Gln Ala Trp Lys Glu Ala Gln Ala Glu Gly Ser Thr Ser End
ACS+
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