THE METABOLISM OF ADRENAL 11β-HYDROXYPROGESTERONE, 11KETO-PROGESTERONE AND 16α-HYDROXYPROGESTERONE BY STEROIDOGENIC ENZYMES

by

Desmaré van Rooyen

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Supervisor: Prof Amanda C. Swart
Co-supervisor: Prof Pieter Swart

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Declaration

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D. van Rooyen

March 2017

ABSTRACT

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This study describes

- Cytochrome P450 11β-hydroxylase (CYP11B1) and cytochrome P450 aldosterone synthase (CYP11B2) both catalyse the 11β-hydroxylation of progesterone (P4) to 11β-hydroxyprogesterone (11OHP4).

- the 11β-hydroxylation of 16α-hydroxyprogesterone (16OHP4) catalysed by CYP11B2 only yielding 4-pregnen-11β,16α-diol-3,20-dione (11,16diOHP4).

- Both 5α-reductase isozymes 1 and 2 (SRD5A1 and SRD5A2) catalyse the reduction of 11OHP4, 11keto-progesterone (11KP4), 16OHP4 and 11,16diOHP4 to 5α-pregnan-11β-ol,3,20-dione (11OH-DHP4), 11keto-dihydroprogesterone (11K-DHP4), 5α-pregnan-16α-ol,3,20-dione (16OH-DHP4) and 5α-pregnan-11β,16α-diol-3,20-dione (11,16diOH-DHP4).

- AKR1C2 catalyses the 3α-reduction of 11OH-DHP4, 11K-DHP4 and 16OH-DHP4 2 to 3α,11β-dihydroxy-5α-pregnan-20-one (3,11diOH-DHP4), 5α-pregnan-3α-ol-11,20-dione (alfaxalone) and to 3α,16α-dihydroxy-5α-pregnan-20-one (3,16diOH-DHP4).

- Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) catalyses the metabolism of alfaxalone to 11keto-androsterone (11KAST) –converting a C_{21} steroid to a C_{19} androgenic steroid.

- 11OHP4 and 11KP4 are metabolised in the LNCaP cell model. 11OHP4 was metabolised to 11KP4, 11K-DHP4, alfaxalone and 11keto-dihydrotestosterone (11KDHT) while 11KP4 was metabolised to 11K-DHP4, alfaxalone and 11KDHT.

- the activation of the androgen- and progesterone receptors by 11OHP4, 11KP4, 16OHP4 and their 5α-reduced metabolites.

OPSOMMING

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Hierdie studie beskryf

- Beide sitochroom P450 11β-hidroksilase (CYP11B1) en sitochroom P450 aldosteroon sintase (CYP11B2) kataliseer die 11β-hidroksilasie van progesteroon (P4) na 11β-hidroksieprogesteroon (11OHP4).

- die 11β-hidroksilasie van 16α-hidroksieprogesteroon (16OHP4) gekataliseer deur slegs CYP11B2 na 4-pregnen-11β,16α-diol-3,20-dioon (11,16diOHP4).

- Beide 5α-reduktase isosieme 1 en 2 (SRD5A1 en SRD5A2) kataliseer die reduksie van 11OHP4, 11ketoprogesteroon (11KP4), 16OHP4 en 11,16diOHP4 na 5α-pregnan-11β-ol,3,20-dioon (11OH-DHP4), 11keto-dihidroprogesteroon (11K-DHP4), 5α-pregnan-16α-ol,3,20-dioon (16OH-DHP4) en 5α-pregnan-11β,16α-diol-3,20-dioon (11,16diOH-DHP4).

- AKR1C2 kataliseer die 3α-reduksie van 11OH-DHP4, 11K-DHP4 en 16OH-DHP4 na 3α,11β-dihidroksi-5α-pregnan-20-oon, 5α-pregnan-3α-ol-11,20-dioon (alfaksaloon) en na 3α,16α-dihydroksi-5α-pregnan-20-oon.

- Sitochroom P450 17α-hidroksilase/17,20-liase (CYP17A1) kataliseer die metabolisme van alfaksaloon na 11ketoandrosteroon (11KAST) -die omskakeling van 'n C_{21} steroïed na 'n C_{19} androgeniese steroïed.

- 11OHP4 en 11KP4 is gemetaboliseer in die LNCaP-sel model. 11OHP4 was gemetaboliseer na 11KP4, 11K-DHP4, alfaksaloon en 11keto-dihydrotestosterone (11KDHT) terwyl 11KP4 gemetaboliseer was na 11K-DHP4, alfaksaloon en 11KDHT., as bewys vir die metabolisme van C11-oksie C_{21} steroïede in die "agterdeur" padweg tot aktiewe androgene.

- die aktivering van die androgeen- en progesteroon reseptore deur 11OHP4, 11KP4, 16OHP4 sowel as hul 5α-gereduseerde metaboliete.

Dedicated to my role models and parents, Desmond and Petro, who taught me to always stay inquisitive, appreciate hard work and to just keep going.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

General

21OHD	21-hydroxylase deficiency congenital adrenal hyperplasia
ACTH	adrenocorticotropin
ADT	androgen deprivation therapy
Ang	angiotensinogen
AngI	angiotensin I
AngII	angiotensin II
AR	androgen receptor
BPH	benign prostatic hyperplasia
CAH	congenital adrenal hyperplasia
cAMP	adenosine-3',5'-monophosphate
CRH	corticotropin-releasing hormone
CRPC	castration-resistant prostate cancer
DMEM	Dulbecco's modified eagle's medium
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	estrogen receptor
ESI+	electrospray ionisation in positive mode
FBS	fetal bovine serum
FSH	follicle-stimulation hormone
GR	glucocorticoid receptor
HEK-293	human embryonic kidney cell line
HPA	hypothalamus-pituitary-adrenal
HSP	heat shock protein
LB	Luria-Bertani
LBD	ligand-binding domain
LC-MS/MS	liquid chromatography tandem mass spectrometry
LH	luteinizing hormone
LNCaP	androgen-dependent human prostate cancer

LOD	limit of detection
LOQ	limit of quantification
MR	mineralocorticoid receptor
MRM	multiple reaction monitoring
MTBE	methyl tert-butyl ether
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
p	probability
PBS	phosphate buffered saline
PCa	prostate cancer
PCOS	polycystic ovarian syndrome
PKA	protein kinase A
PR	progesterone receptor
PRA	plasma renin activity
PR-A	progesterone receptor isoform A
PR-B	progesterone receptor isoform B
PRE	progesterone response element
Q-TOF MS	quadrupole/quantitative time-of-flight mass spectrometry
RAAS	renin-angiotensin aldosterone system
RPMI-1640	Roswell Park Memorial Institute 1640 media
TART	testicular adrenal rest tumour
UHPLC-MS/MS	ultra-high performance liquid chromatography tandem mass spectrometry
UPC^2-MS/MS	ultra-performance convergence chromatography tandem mass spectrometry
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticularis

Enzymes

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11βHSD	11β-hydroxysteroid dehydrogenase
11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
17βHSD	17β-hydroxysteroid dehydrogenase
17βHSD2	17β-hydroxysteroid dehydrogenase type 2
17βHSD3	17β-hydroxysteroid dehydrogenase type 3
17βHSD5	17β-hydroxysteroid dehydrogenase type 5
17βHSD6	17β-hydroxysteroid dehydrogenase type 6
3αHSD	3α-hydroxysteroid dehydrogenase
3βHSD2	3β-hydroxysteroid dehydrogenase type 2
ADX	adrenodoxin
AKR	aldo-keto reductase
AKR1C2	3α-hydroxysteroid dehydrogenase type 3
CYP11A1	cytochrome P450 cholesterol side-chain cleavage
CYP11B1	cytochrome P450 11β-hydroxylase
CYP11B2	cytochrome P450 aldosterone synthase
CYP17A1	cytochrome P450 17α-hydroxylase/17,20-lyase
CYP19A1	cytochrome P450 aromatase
CYP21A2	cytochrome P450 21-hydroxylase
cyt b_5	cytochrome b_5
FAD	flavin adenine dinucleotide
Fedx	ferredoxin
FeRed	ferredoxin reductase
FMN	flavin mononucleotide
HSD	hydroxysteroid dehydrogenase
P450	cytochrome P450
POR	P450 oxidoreductase
SRD	short chain dehydrogenases/reductases
SRD5A	5α-reductase
StAR	steroidogenic acute regulatory protein

SULT	sulfotransferase
SULT2A1	sulfotransferase family 2A member 1
UGT	uridine diphosphate glucuronosyltransferase

Steroids

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11,16diOH-DHP4	5α-pregnan-11β,16α-diol-3,20-dione
11,16diOHP4	4-pregnen-11β,16α-diol-3,20-dione
11K-3α-Adiol	11-keto-5α-androstane-3α,17β-diol
11K-5α-dione	11-keto-5α-androstanedione
11KA4	11keto-androstenedione
11KAST	11keto-androsterone
11K-DHP4	11keto-dihydroprogesterone
11KDHT	11keto-dihydrotestosterone
11KP4	11keto-progesterone
11KT	11keto-testosterone
11OH-5α-dione	11β-hydroxy-5α-androstanedione
11OHA4	11β-hydroxyandrostenedione
11OHAST	11β-hydroxyandrosterone
11OH-DHP4	5α-pregnan-11β-ol,3,20-dione
11OHDHT	11β-hydroxydihydrotestosterone
11OHP4	11β-hydroxyprogesterone
11OHT	11β-hydroxytestosterone
16OH-DHP4	5α-pregnan-16α-ol,3,20-dione
16OHP4	16α-hydroxyprogesterone
17OHP4	17α-hydroxyprogesterone
17OHP5	17α-hydroxypregnenolone
18OH-CORT	18-hydroxycorticosterone
3,11diOH-DHP4	3α,11β-dihydroxy-5α-pregnan-20-one
3,16diOH-DHP4	3α,16α-dihydroxy-5α-pregnan-20-one
3α-Adiol	5α-androstane-3α, 17β-diol
5α-dione	5α-androstanedione
A4	androstenedione
Adiol	androstenediol
ALDO	aldosterone
Alfaxalone	5α-pregnan-3α-ol-11,20-dione

allopregnanolone	5α-pregnane-3α-ol-20-one
AST	androsterone
CORT	corticosterone
D2-T	deuterated testosterone
D4-cortisol	deuterated cortisol
D7-11OHA4	deuterated 11β-hydroxyandrostenedione
D7-A4	deuterated androstenedione
D8-17OHP4	deuterated 17α-hydroxyprogesterone
D9-P4	deuterated progesterone
deoxycortisol	11-deoxycortisol
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulphate
DHP4	5α-pregnan-3,20-dione
DHT	dihydrotestosterone
DHT-G	dihydrotestosterone glucuronide
DOC	deoxycorticosterone
DOF	21-desoxycortisol
GnRH	gonadotropin-releasing hormone
Mib	mibolerone
P4	progesterone
P5	pregnenolone
Pdiol	3α-hydroxy-5β,17α-pregnan-20-one
Pdione	5α-pregnane 17α-ol-3, 20-dione
pregnanetriol	5α-Pregnane-3α,17α,20α-triol
R5020	promegestone
T	testosterone

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CHAPTER 1

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Introduction

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In adrenal disorders such as 21-hydroxylase deficiency congenital adrenal hyperplasia (21OHD), polycystic ovarian syndrome (PCOS), and androgen-dependent prostate cancer (PCa), steroids such as 16α-hydroxyprogesterone (16OHP4), 11β-hydroxyprogesterone (11OHP4) and 11keto-progesterone (11KP4) were generally less frequently reported to be associated with these clinical conditions. However, in recent years, active research into steroid hormone biosynthesis, together with advances in liquid chromatography tandem mass spectrometry (LC-MS/MS) based technologies, has led to the detection and quantification of these less conventional steroids in hormone profiles in normal physiological and pathological conditions (Oshima *et al.*, 1967; Turcu *et al.*, 2015; Warren and Salhanick, 1961). The aforementioned conditions are associated with abnormal androgen levels and although these steroids had been detected by the mid-1900s, studies were hampered by low sensitivity and high crosstalk associated with immuno-based analytical techniques. Recent advances in LC-MS/MS, however, have once more placed the focus on the aforementioned steroid hormones with interest in these steroids re-emerging in investigations into hyperandrogenic diseases. As such, the relevance of these steroids in the progression of androgen-associated disease conditions has been brought to the fore.

In adrenal steroidogenesis, pregnenolone (P5) is metabolised in the Δ^5-pathway to dehydroepiandrosterone (DHEA), androstenedione (A4) and subsequently 11β-hydroxyandrostenedione (11OHA4), which serve as androgen precursors in peripheral androgen steroidogenic pathways. These pathways yield potent androgens such as testosterone (T), dihydrotestosterone (DHT) and their C11-ketone derivatives, 11keto-testosterone (11KT) and 11keto-dihydrotestosterone (11KDHT), which are important in normal male differentiation and sexual development. In addition, P5 and 17α-hydroxypregnenolone (17OHP5) are metabolised by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) to progesterone (P4) and 17α-hydroxyprogesterone (17OHP4) – precursors to the biosynthesis of mineralocorticoids and glucocorticoids, aldosterone (ALDO) and cortisol, respectively. P4 is the direct precursor for the mineralocorticoids. Metabolism by cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) yields the 17α-hydroxylated product, 17OHP4, which is utilized in the biosynthesis of glucocorticoids. In addition to the conventional metabolism of P4 by CYP17A1 and cytochrome P450 21-hydroxylase (CYP21A2), the human CYP17A1 catalyses a third reaction in which P4 is hydroxylated at carbon 16 (C16) to form 16OHP4 (Swart *et al.*, 1993). P4 may also be converted to 11OHP4 by the adrenal CYP11B isozymes and subsequently metabolised by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) to 11KP4 (Van Rooyen et al., 2017).

PCa is an androgen-dependent disease, and treatment is aimed at reducing androgens through chemical or surgical castration. The research group of A.C. Swart (Biochemistry, Stellenbosch

University) recently investigated levels of 16OHP4, 11OHP4 and 11KP4 in tissue and plasma of PCa patients. Higher levels of C11-oxy C_{21} steroids were detected in those patients receiving an androgen antagonist as treatment (unpublished data).

Although therapy initially succeeds in lowering circulating T, the effectiveness of treatment in lowering DHT levels is unsuccessful in the long term since the cancer re-emerges as the androgen-driven castration-resistant prostate cancer (CRPC). Further investigations demonstrated an alternative pathway able to biosynthesize DHT in which this potent androgen is produced from A4 via 5α-androstanedione (5α-dione) without T as intermediate (Chang *et al.*, 2011; Luu-The *et al.*, 2008). In addition, adrenal 11OHA4 is metabolised in the 11OHA4 pathway yielding 11KT and 11KDHT, which have androgenic potency equal to DHT (Storbeck *et al.*, 2013). These multiple pathways play an intricate role in the progression of PCa and steroidogenesis in PCa, and can therefore not be attributed to a single pathway, nor can the progression be attributed to T and DHT alone. The elucidation of the 11OHA4 pathway, and the contribution of the C11-oxy C_{19} steroids to the PCa, has emphasized the importance of exploring alternative steroidogenic pathways fully. In this regard other downstream pathways, such as the backdoor pathway, in which 17OHP4 is converted by 5α-reductase (SRD5A), 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2) and CYP17A1 to DHT indicated that this pathway is not only vital for male sexual differentiation, but also prevails in 21OHD (Auchus, 2004).

An inborn metabolic defect in the *CYP21A2* gene, which encodes the CYP21A2 enzyme, prevents the adrenal from producing adequate levels of mineralocorticoids and glucocorticoids. As a result, negative feedback by cortisol in the hypothalamus-pituitary-adrenal (HPA) axis is lost leading to overstimulation of the adrenal by adrenocorticotropin (ACTH) to produce cortisol. This overstimulation subsequently results in elevated levels of P4 and 17OHP4 in the mineralocorticoid and glucocorticoid pathways as well as an increased steroid flux in the androgen precursor pathway. Adrenal androgen precursors are further metabolised in downstream androgen biosynthesis pathways while 17OHP4 is metabolised to DHT in the backdoor pathway increasing active androgen levels. In addition, P4 is metabolised by CYP17A1 and CYP11B to 16OHP4 and 11OHP4 of which elevated levels have been detected in 21OHD, together with elevated C11-oxy androgens such as 11OHA4, 11keto-androstenedione (11KA4), 11β-hydroxytestosterone (11OHT) and 11KT (Turcu *et al.*, 2016, 2015).

Androgen excess in 21OHD presents with symptoms ranging from external genitalia, masculinization, hirsutism, acne, oligomenorrhea and impaired fertility or infertility. In women these symptoms are similar to those exhibited in PCOS, the most common endocrine disorder which affects ±10% of women world-wide (Azziz *et al.*, 2009; Dunaif, 1995). Other symptoms include obesity, insulin resistance, metabolic syndrome and polycystic ovaries. The etiology of PCOS is, however, still unclear. Elevated 17OHP4, 16OHP4 and 11OHP4 levels have also been reported suggesting that PCOS is a secondary complication of 21OHD.

Although there have been studies reporting the detection of 16OHP4, 11OHP4, 11KP4 in hyperandrogenic conditions (Gueux *et al.*, 1987; Turcu *et al.*, 2015), these studies have failed to address the downstream metabolism and potential biological consequence of their production. The

research presented in this study aims to address these aspects by using state-of-the-art liquid chromatography mass spectrometry techniques to analyse the downstream metabolites of these steroids in cell model systems. The physiological implications of 11OHP4, 11KP4, 16OHP4 and their 5α-reduced metabolites, with respect to the activation of the wild type androgen receptor (AR) and the progesterone receptor (PR) isoforms A (PR-A) and B (PR-B), will be assessed in receptor-reporter assays to determine potential cellular effects.

Chapter 2 provides an overview of adrenal-, gonadal- and peripheral androgen steroidogenesis and discusses the hyperandrogenic diseases – congenital adrenal hyperplasia (CAH), highlighting 21OHD, PCOS and PCa.

Chapter 3 and 4 (published manuscript) addressed the aims of this project listed below:

- to investigate the biosynthesis of 11OHP4 and 16OHP4 by CYP11B isozymes;
- to investigate the sequential metabolism of 11OHP4 and 16OHP4 in the backdoor pathway by SRD5A, AKR1C2, CYP17A1;
    - to investigate the metabolism of 16OHP4 and 4-pregnen-11β,16α-diol-3,20-dione (11,16diOHP4), 11OHP4 and 11KP4 by SRD5A isozymes;
    - to investigate the conversion of the 5α-reduced metabolites of 11OHP4, 11KP4 and 16OHP4 by AKR1C2;
    - to investigate the metabolism of the 3α,5α-reduced 11OHP4 and 11KP4 metabolites by CYP17A1;
- to investigate the metabolism of 11OHP4 and 11KP4 in androgen-dependent human prostate cancer (LNCaP) cells, a model expressing all the steroidogenic enzymes endogenously;
- to determine the receptor interaction of 11OHP4, 11KP4, 16OHP4 and their 5α-reduced metabolites with the AR and PR isoforms.

Chapter 3 focuses on the biosynthesis of C11-oxy C_{21} steroids and presents the investigation into the biosynthesis of 11OHP4 by CYP11B isozymes from P4, yielding 11OHP4. The metabolism of 11OHP4 and 11KP4 by SRD5A, AKR1C2 and CYP17A1 is presented and the metabolism of these adrenal steroids is further demonstrated in the LNCaP cell model. Finally, this chapter describes the interaction of 11OHP4, 11KP4 and their respective 5α-reduced metabolites with the AR, PR-A and PR-B.

Chapter 4 presents the published manuscript investigating the metabolism of 16OHP4 by CYP11B and the peripheral steroidogenic enzymes, SRD5A1 and AKR1C2. The receptor interaction of 16OHP4 and 5α-pregnan-16α-ol,3,20-dione (16OH-DHP4) with the AR and PR is also reported in this manuscript.

Chapter 5 provides the experimental detail regarding the protocols followed throughout this project.

Chapter 6 provides a summary of the most important findings to conclude this study.

CHAPTER 2

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Biosynthesis of C_{19} and C_{21} steroids in normal and clinical conditions

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2.1 Introduction

Recent studies, in which steroid panels have been profiled comprehensively due to new and more advanced technologies, have enabled the identification and quantification of novel steroids, both in the normal and clinical physiological settings. In hormone-associated diseases, such as CRPC, CAH and PCOS, elevated concentrations of 16OHP4, 11OHP4 and 11KP4 have been detected. Although these steroids were detected in the early 1900s, the focus of those studies shifted towards steroids in the conventional steroidogenic pathways as they are known and depicted today. With the development of more sensitive and accurate technologies, such as liquid chromatography (LC)- and gas chromatography mass spectrometry, together with studies profiling steroids, 16OHP4, 11OHP4 and 11KP4 are again falling under the spotlight.

This chapter will thus provide an overview of steroid hormones, adrenal steroidogenesis and the enzymes related to the biosynthesis of 16OHP4, 11OHP4 and 11KP4. Gonadal and peripheral steroid production will be discussed as these steroids have been detected in the reproductive organs as well as in circulation. Since the C11-oxy androgens (C_{19} steroids) have been identified in PCa and in CAH with their metabolism leading to potent androgens, the possibility that the C11-oxy C_{21} steroids may act as precursors to potent C11-oxy C_{19} steroids in various androgenic pathways will be discussed. Lastly, an overview of CRPC, CAH and PCOS will be provided.

2.2 Adrenal steroidogenesis

2.2.1 Adrenal physiology

The adrenal gland, also known as the suprarenal gland, is a hormone-producing organ situated atop of the kidneys, and is currently the topic of numerous endocrinology-based studies investigating both normal and diseased states. Investigations characterizing steroids produced in the adrenal are still ongoing, despite decades of research into the gland. The adrenal gland can be anatomically described as consisting of a medulla surrounded by the adrenal cortex and encapsulated by a fibrous capsule (Fig. 2.1). Both the medulla and cortex are endocrine tissue producing important physiological compounds but differ in their embryonic origin – ectodermal and mesoderm, respectively (Baulieu and Kelly, 1990). The adrenal glands are highly vascular tissue receiving a high percentage of cardiac output relative to its size and weight (Nelson, 1980). Blood is supplied to the adrenal directly from the aorta, renal and inferior phrenic aorta to the subcapsular arteriolar plexus which distribute the blood directly to the cortex and medulla via sinusoids and medullary arteries, respectively (Ehrhart-Bornstein *et al.*, 1998). These capillaries drain into the medullary vein leading to the central adrenomedullary vein and

subsequently the left suprarenal vein into the inferior vena cava (Fig. 2.1). The right adrenal drainage differs by the adrenomedullary vein draining directly into the vena cava.

The medulla produces neuropeptides and catecholamines, epinephrine and norepinephrine, which are important in the autonomic (sympathetic) nervous system (Ehrhart-Bornstein *et al.*, 1998). The adrenal cortex is more complex in terms of morphology and function and comprises about 80% of the adrenal (Nelson, 1980). The adrenal cortex consists of three layers, or zonae, which differ histologically from each other and in terms of the steroid hormones produced.
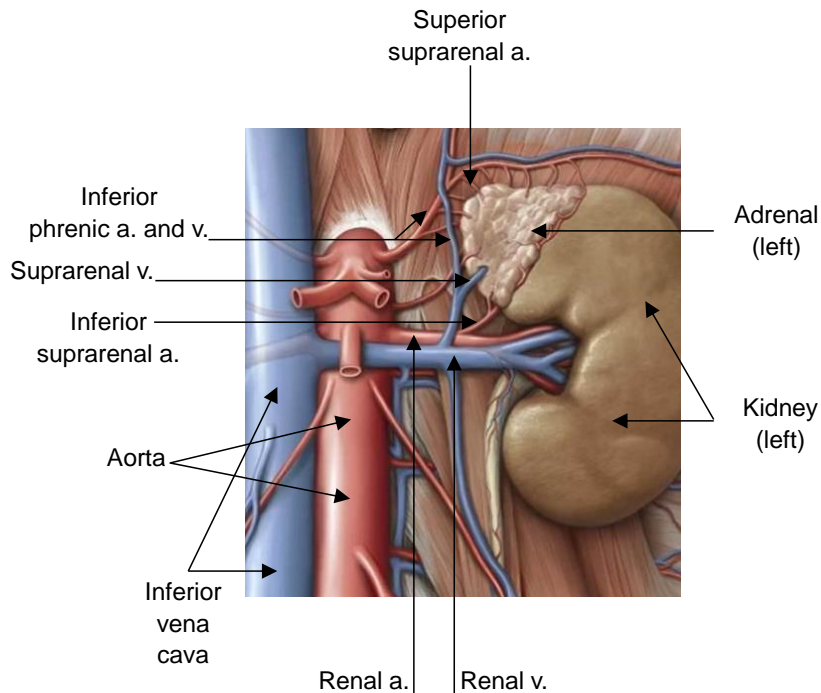
Figure 2.1   Blood supply and drainage to and from the left adrenal gland. The adrenal is situated above the kidney and receives blood directly from the aorta via the superior and inferior suprarenal arteries of which the prior originate from the inferior phrenic artery. Blood is removed from the adrenal via the suprarenal vein draining into the inferior vena cava back to the heart. Reproduced and modified from Schuenke *et al.* (2012).

2.2.2   *Zonae of the adrenal cortex*

Steroidogenic enzyme expression and distribution in cells determine the steroid hormone biosynthesis. The adrenal cortex is histologically subdivided into three physiologically distinct zones, each producing different steroid hormones. The zonae glomerulosa (ZG), zonae fasciculata (ZF) and zonae reticularis (ZR) are believed to function as independent units (Fig. 2.2). However, rat studies have shown cells of the ZG to migrate into the ZF (Ogishima *et al.*, 1992). Steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side-chain cleavage (CYP11A1) are expressed in all of the three zonae, allowing the independent biosynthesis of the specific steroids. Lipid bodies are also present in the cytosol of the adrenal cortex cells. The lipid bodies act as cholesterol ester reservoirs. Upon ACTH

stimulation cholesterol esterase is activated via adenosine-3',5'-monophosphate (cAMP) increasing free cholesterol levels. (Nelson, 1980). Upon completion of steroid biosynthesis, steroids are released into the blood to be transported to target tissue.

2.2.2.1   Zona glomerulosa and mineralocorticoids

The outer layer of the adrenal, closest to the capsule, is the ZG which produces the mineralocorticoids (Fig. 2.2), comprising ±15% of the adrenal cortex (Neville and O'Hare, 1985). The expression of cytochrome P450 aldosterone synthase (CYP11B2) is exclusively associated with this zone (Waterman and Bischof, 1997) which lacks CYP17A1 thus preventing steroidogenesis towards androgen precursors. 3βHSD2 catalyses the oxidation of P5 to P4 at C3. In the absence of CYP17A1, P4 is converted by CYP21A2 to deoxycorticosterone (DOC). The latter is 11β-hydroxylated by CYP11B2 to corticosterone (CORT) and in a two-step reaction via the intermediate 18-hydroxycorticosterone (18OH-CORT) finally yielding ALDO (Miller and Auchus, 2011).

Mineralocorticoids and glucocorticoids are 21 carbon (C_{21}) corticosteroids. The name, mineralocorticoid, is derived from the steroid property to regulate mineral levels such as sodium and potassium and thus maintain the homeostasis of electrolytes and regulate blood pressure. ALDO is the most potent natural mineralocorticoid and possesses salt-retaining properties and exerts its effect on the kidneys to retain sodium and to indirectly reabsorb water. It should be noted that while the mineralocorticoid function of the ALDO precursors, DOC and CORT (Nelson, 1980) is reduced, cortisol, a glucocorticoid which is present in significantly higher levels than ALDO, can also bind to the MR with the same affinity as ALDO. Thus, in mineralocorticoid target tissue, 11βHSD2 is expressed to inactivate cortisol by conversion to cortisone, preventing overstimulation of the MR. This ZG is regulated by numerous regulatory systems including the renin-angiotensin aldosterone system (RAAS), as well as sodium-, potassium- and fluid levels (Miller and Auchus, 2011; New and Levine, 1984).

2.2.2.2   Zona fasciculata and glucocorticoids

The ZF is the middle and largest zone of the cortex (Fig. 2.2) and is characterised by the expression of 3βHSD2, CYP21A2, cytochrome P450 11β-hydroxylase (CYP11B1) and CYP17A1 and negligible amounts of cytochrome b_5 (cyt b_5). Although the 17, 20-lyase activity of CYP17A1 is augmented by cyt b_5 (Auchus *et al.*, 1998; Katagiri *et al.*, 1995; Lee-Robichaud *et al.*, 1995) the low expression levels in the ZF account for the negligible production of DHEA in the zone while the 17α-hydroxylase remains unchanged resulting primarily in the biosynthesis of 17OHP4 and glucocorticoids (Suzuki *et al.*, 2000b). With the expression of 3βHSD and CYP17A1, P5 can be either oxidized at C3 to yield P4 which in turn can be hydroxylated on C17 to yield 17OHP4 or P5 can be hydroxylated to yield 17OHP5 prior to the conversion to 17OHP4, catalysed by 3βHSD2. P4 and 17OHP4 are both substrates for CYP21A2 leading to the production of both DOC and 11-deoxycortisol (deoxycortisol) in the ZF, respectively. CYP11B1 accounts for the final hydroxylation in glucocorticoid biosynthesis converting deoxycortisol to cortisol as well as DOC to CORT. This zone is specifically regulated by ACTH which stimulates cell proliferation (increase in ZF size) or results in the decrease of the ZF when absent.

The term "glucocorticoid" is derived from the steroid's ability to regulate glucose metabolism. These steroids are involved in the immune system and suppress inflammation, the metabolism of carbohydrates, proteins and lipids, and play a role in fetal development, in the central nervous system and circadian rhythm.

2.2.2.3 Zona reticularis and adrenal androgens

The innermost layer of the cortex (Fig. 2.2) is ZR comprised of "compacts cells" rich in mitochondria with a pronounced endoplasmic reticulum (Nelson, 1980). This zone is associated with higher CYP17A1 and cyt b_5 expression levels but lacks CYP21A2 and CYP11B1. As a result, high levels of DHEA are biosynthesized (Miller and Auchus, 2011). P5 is converted to P4 as in the ZF followed by the 17-hydroxylation of P5 and P4 to their respective metabolites, 17OHP4 and 17OHP5. The latter is subsequently converted to DHEA in a reaction catalysed by 17,20 lyase (CYP17A1) augmented by cyt b_5. Most of the DHEA produced in the adrenal is sulfonated by steroid sulfotransferase family 2A member 1 (SULT2A1) yielding the inactivated product, DHEA sulfate (DHEA-S), which is in reversible equilibrium with DHEA. DHEA also serves as substrate for 3βHSD2 yielding A4. On the other hand, the lyase reaction catalysing the conversion of 17OHP4 to A4 is not characteristic of human steroidogenesis. 11OHA4 is also produced in the adrenal (Rege and Rainey, 2012; Swart *et al.*, 2013; Xing *et al.*, 2011). A4 is subsequently reduced at C17 to T by 17β-hydroxysteroid dehydrogenase (17βHSD) but due to the low expression of the enzyme in the adrenal, adrenal T levels are low.
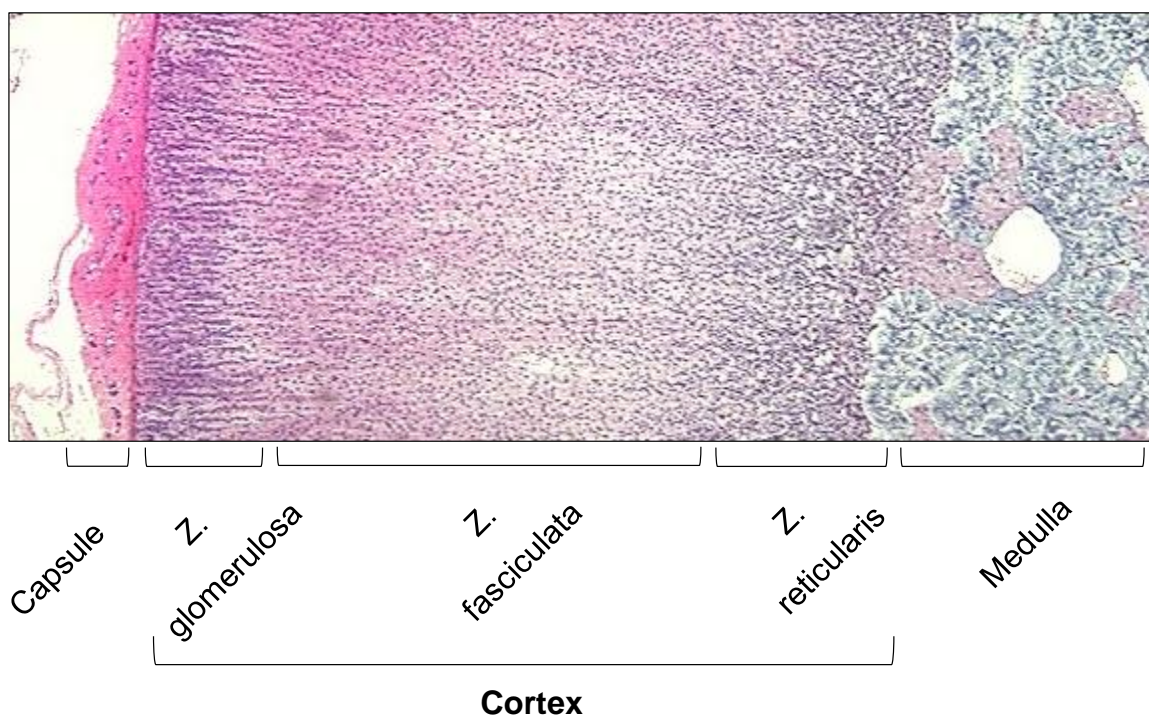
Figure 2.2 Schematic histological representation of a cross section of the adrenal cortex. The capsule is adjacent of the Z. glomerulosa (outer zone), followed by the Z. fasciculata and the Z. reticularis. The medulla is found in the inner section of the adrenal. Reproduced and modified from Bowen (n.d.).

Androgens are crucial in male sexual differentiation as well as male sexual development and homeostasis. High levels of DHEA are produced in the adrenal compared to other adrenal androgens, but the steroid possesses low androgenic activity (Nelson, 1980). Nevertheless, DHEA is metabolised to A4 and subsequently to T thus increasing androgenic activity. The production of 11OHA4 has in recent studies shown to be an important adrenal androgen which is metabolised to potent androgens such as 11KT and 11KDHT (Storbeck *et al.*, 2013).

2.2.3 *Adrenal steroidogenic pathway*

Steroid biosynthesis occurs in steroidogenic tissue which include the adrenal, gonadal- and other peripheral tissue (Norman and Litwack, 1987). Hormone production is specific to the steroid enzymes expressed and the substrate delivered to the tissue. Adrenal steroids are released into circulation to be transported to the numerous tissue sites where it will impact associated cellular mechanisms such as regulating hormonal homeostasis. All steroid hormones are biosynthesized from cholesterol by the steroidogenic enzymes consisting of P450 and HSD enzyme (Fig. 2.3).

This conversion of cholesterol to P5 is catalysed by the mitochondrial CYP11A1. These pathways are characterised by the collection of enzymes specific to the zonae of the adrenal cortex and the end-product produced. P5 is metabolised in the Δ^5-pathway or androgen pathway in a two-step manner by CYP17A1 to yield 17OHP5 and DHEA, respectively. In the ZR, the CYP17A1 cofactor, cyt b_5 is highly expressed and both undergo an allosteric interaction to enhance the lyase activity of CYP17A1, thus promoting the androgen pathway in this zone (Auchus *et al.*, 1998). DHEA is inactivated by SULT2A1 in the adrenal and, as in the gonads, also converted to A4. DHEA and A4 are subsequently converted to androstenediol (Adiol) and T respectively, albeit at low levels due to the negligible expression of 17βHSD in the adrenal. However, in the adrenal the Δ^5-steroids (C5/C6 double bond) are substrates for 3βHSD2 yielding the Δ^4-steroids (C4/C5 double bond) namely P4, 17OHP4 and A4. In the Δ^4-pathway the conversion of 17OHP4 to A4 is absent/negligible in humans. P4 acts as a substrate for the human CYP17A1 resulting in the biosynthesis of largely 17OHP4 and to a lesser degree, 16OHP4 in humans (Swart *et al.*, 2010).

As mentioned previously P4 is metabolised by CYP21A2 to produce substrates for cytochrome CYP11B2 in the mineralocorticoid pathway yielding DOC, CORT and ALDO, while in the glucocorticoid pathway P4 is metabolised by both CYP17A1 and CYP21A2, presenting CYP11B1 substrates, DOC and deoxycortisol, in the production of CORT and cortisol by respectively (Miller and Auchus, 2011).

11OHP4, a 11β-hydroxylated metabolite of P4, has also been detected in adrenal studies but whether CYP11B1 or CYP11B2 or both catalyse the reaction is unknown. In addition, the aforementioned CYP11B products and the steroid produced in the androgen arm, 11OHA4, a 11β-hydroxylated metabolite of A4, is produced at significant levels in the adrenal by CYP11B1 and to a lesser extent by CYP11B2 (Bloem *et al.*, 2013; Schloms *et al.*, 2012). In contrast, the biosynthesis of 11OHT from T in the hydroxylase reaction catalysed by the CYP11B isozymes is comparable. These C11-hydroxy-C_{19} steroids subsequently serve as substrates to 11βHSD2, which converts the C11-hydroxyl group to a

keto group yielding 11keto-androstenedione (11KA4) and 11KT. The interconversion of the C17 hydroxyl and keto groups of the androgens such as A4 and T, and estrogens (C_{18} steroids) is catalysed by 17βHSD enzymes. Interestingly, although 17β-hydroxysteroid dehydrogenase type 3 and type 5 (17βHSD3 and 17βHSD5) catalyse the conversion of the C17 keto-groups of the C_{19} steroids, 11KA4 and A4 to 11KT and T respectively, these isoforms do not catalyse the conversion of 11OHA4 to 11OHT. On the other hand the reductive 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) readily catalyses the reverse reaction in which all C11-hydroxyl and C11-keto T derivatives are converted to the corresponding A4 derivatives (Bloem *et al.*, 2013).
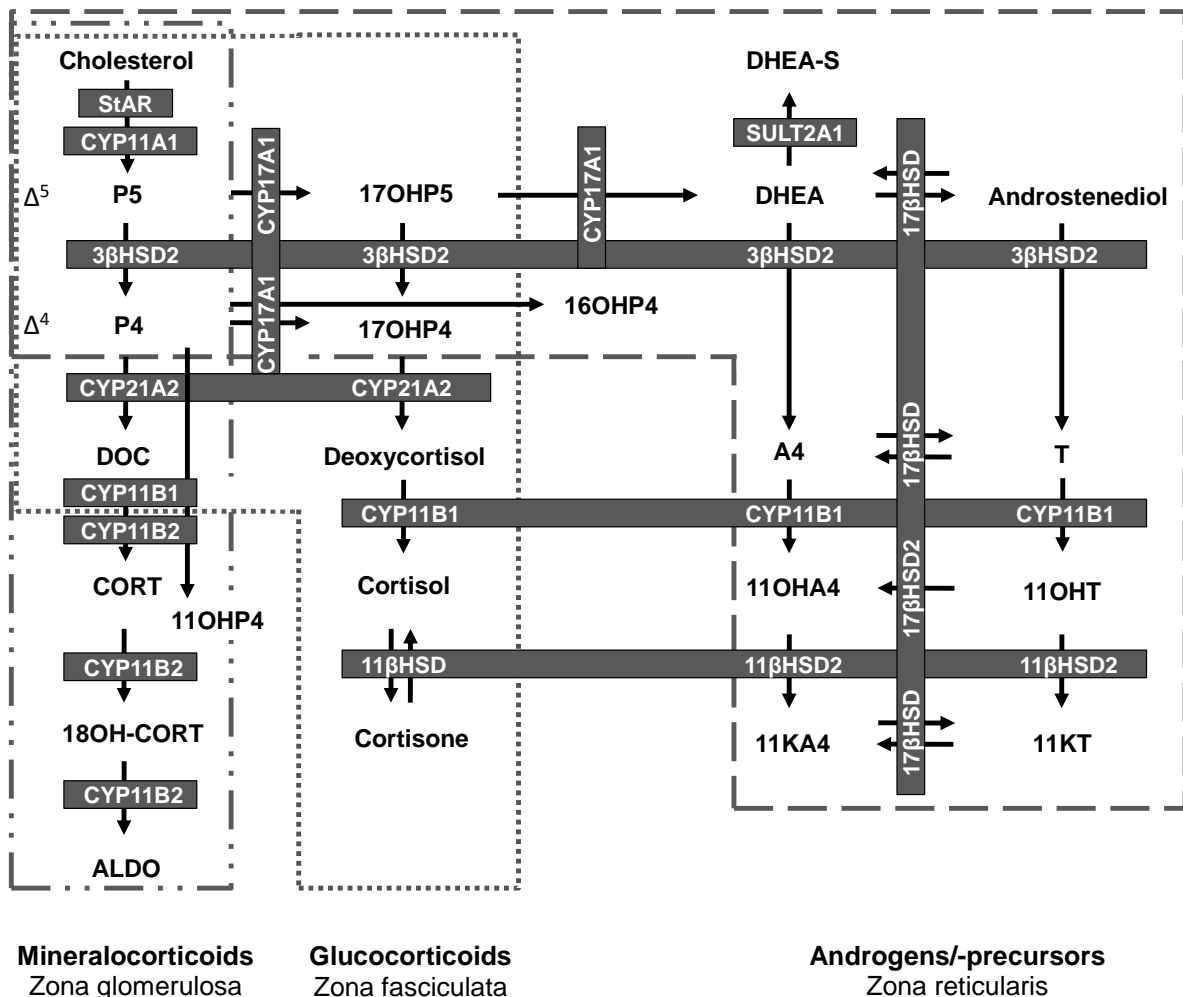
Figure 2.3 Adrenal steroidogenesis showing the classic adrenal pathways and the respective zonae. Adapted from Du Toit (2015).

2.2.4 *Regulation systems of adrenal steroidogenesis*

The central nervous system plays a crucial role in the regulation of steroidogenesis – affecting ACTH levels, regulating the circadian rhythm and cortisol levels in stress conditions via increasing the corticotropin-releasing hormone (CRH) and stimulating the adrenal cortex to regulate electrolyte levels (New and Levine, 1984).

2.2.4.1   Hypothalamus-pituitary-adrenal axis

The HPA axis consists of the hypothalamus, anterior pituitary and the adrenal and regulates cortisol levels. Numerous stimuli, such as stress, trigger a chain reaction via the limbic system signalling the hypothalamus to secrete releasing hormones such as CRH or gonadotropin-releasing hormone (GnRH) in gonadal steroidogenesis (Norman and Litwack, 1987). CRH secretion is determined by either the negative feedback loop stimulated by increase cortisol levels, or by higher nervous systems of the brain controlling the diurnal variations (increase and decrease in ACTH and cortisol levels in the morning and night, respectively). CRH stimulates the anterior pituitary to increase ACTH secretion – a corticotropin important for the regulation of cortisol and adrenal androgen biosynthesis (Ehrhart-Bornstein *et al.*, 1998). ACTH is released from the pituitary and exerts an effect on the adrenal by binding to the G-protein coupled melanocortin receptor type 1 of adrenal cortical cells and upregulating the biosynthesis of cAMP. Induction of adrenal cellular phosphoprotein kinases by cAMP results in the conversion of cholesterol to P5 (Ehrhart-Bornstein *et al.*, 1998; New and Levine, 1984) and a subsequent increase in glucocorticoid and androgen biosynthesis. Elevated free cortisol levels signal the hypothalamus and/or anterior pituitary in a feedback control to lower ACTH secretion (Baulieu and Kelly, 1990; Nelson, 1980).

2.2.4.2   Renin-angiotensin aldosterone system

Under low sodium conditions, due to low arterial blood pressure or the activity of the sympathetic nervous system, the juxtaglomerular apparatus in the kidney releases the enzyme, renin, into the circulation which hydrolyses angiotensinogen (Ang), a α_2-globulin secreted from the liver, to angiotensin I (AngI) (Bongiovanni, 1968). AngI is subsequently converted by angiotensin-converting enzyme (ACE) to angiotensin II (AngII) in the lungs. AngII exerts its effect via vasoconstriction of the arterioles to increase blood pressure and stimulate the pituitary to secrete antidiuretic hormones (ADH) such as vasopressin resulting in water retention. AngII also induces the adrenal gland to increase ALDO production (Fig. 2.4) (Bongiovanni, 1968). In the absence of or in low sodium conditions, ALDO binds to the mineralocorticoid receptor (MR) in the kidney in the distal tubule and collecting duct of the nephron which induces the basolateral sodium/potassium pumps creating a concentration gradient. Sodium is reabsorbed into the cell and potassium released into the interstitial fluid to be excreted in the urine. The increased sodium in the cells results in water diffusing back into the cells, increasing the blood volume and subsequently blood pressure (Aguilera *et al.*, 1978; Aguilera and Catt, 1983; Ehrhart-Bornstein *et al.*, 1998; Mason *et al.*, 1976). High potassium levels in circulation can also directly stimulate the adrenal to increase ALDO secretion.
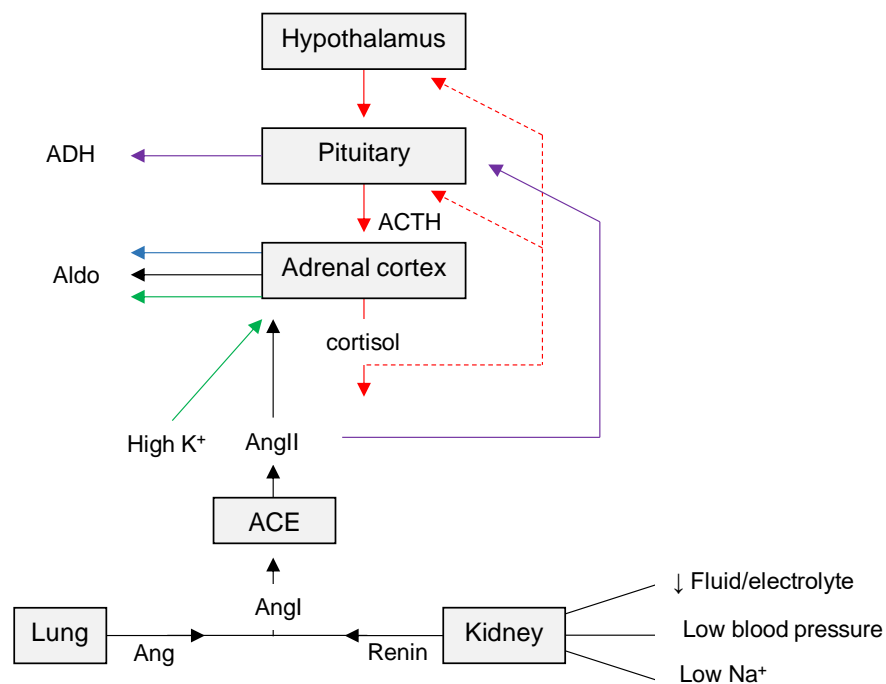
Figure 2.4   Simplified schematic representation of the regulation of adrenal steroidogenesis with respect to the mineralocorticoids, glucocorticoids and adrenal androgens.

2.2.5.   Steroidogenic enzymes

Enzymes involved in steroidogenesis are typically P450s and HSDs. Characteristic of these enzymes is the unidirectional function preventing the flux to be influenced by the build-up of steroid products (Miller and Auchus, 2011). P450 enzymes catalyse the hydroxylation and/or cleavage of carbon-carbon bonds in the steroid molecule. These activities are irreversible in terms of mechanism and physiology. In contrast, HSDs are able to catalyse both the forward or reverse reaction making them mechanistically reversible *in vitro*, whereas *in vivo* the enzyme favours either the oxidation or reduction of the keto- or hydroxysteroid (Miller and Auchus, 2011).

2.2.5.1   Cytochromes P450

P450, or pigment 450, is an umbrella name referring to a group of enzymes named after the ability of the reduced, carbon monoxide complex molecule to absorb light at 450 nm (Omura and Sato, 1964a, 1964b, 1962). These oxidative enzymes mediate phase 1 mono-oxygenation reactions and consist of ±500 amino acid residues with a single heme iron group complexed within a protoporphyrin IX ring structure. Commonly used names for these enzymes are CYP or P450$_{suffix}$ – in the latter case the suffix refers to the principal enzymatic activity associated with the enzyme. The genes encoding these proteins are annotated in italics as *CYP*. P450 enzymes play a crucial part in metabolism of xenobiotics, chemicals and drugs as well as the biosynthesis of steroid hormones (Miller, 2005).

Humans possess 57 genes encoding for these enzymes and can be biochemically sub-divided into mitochondrial (type I) enzymes such as CYP11A1 and CYP11B1 and B2 or microsomal (type II) P450

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enzymes such as CYP17A1 and CYP21A2 (Miller, 2005). These are biochemically distinguished from each other based on the electron receiving mechanism and the intracellular localization of the enzyme. The electron and heme centre serve to activate molecular oxygen in order to catalyse a wide array of oxidation reactions utilizing various steroid substrates (Miller and Auchus, 2011). P450 enzymes are expressed in a number of organs and tissue including liver, kidney, adrenal, reproductive organs and skin, amongst others.

The general P450 catalytic reaction is given by the following equation:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$
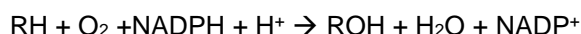
Catalyses commences with the enzyme in the resting state in which the heme iron is in the ferric form (Fig. 2.5). The substrate binds and initiates the displacement of H_2O in the heme environment resulting in an altered spin state equilibrium, as well as a change in the redox potential of the heme group. Consequently, a potential gradient is created which allows the electrons to flow towards the P450 enzyme. During this reaction, the substrate (being steroids hormones, chemical or xenobiotics) once bound to the active site, reduces the ferric iron to a ferrous state by a single electron donated by nicotinamide adenine dinucleotide phosphate (NADPH) (Lewis, 2001). An unstable complex between the ferrous iron and oxygen molecule is then formed resulting in a superoxy anion. A second electron and two protons are subsequently added to the complex, molecular oxygen splits and one oxygen atom remains bound to the iron forming an activated intermediate which is subsequently used in the hydroxylation of the respective substrate. The other oxygen atom contributes to the formation of a water molecule. The hydroxylated substrate is released from the active pocket and the iron is restored to its ferric state (Hasler *et al.*, 1999; Lewis, 2001).
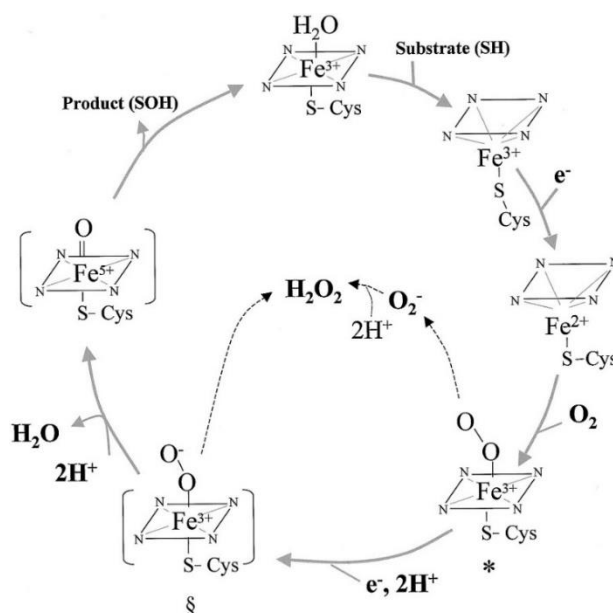
Figure 2.5   General cytochrome P450 catalytic cycle showing the major reactions involved in the hydroxylation of the substrate (SH) to the corresponding product (SOH). Reproduced from Davydov (2001).

In mammals, P450s are associated with the membranes of either the endoplasmic reticulum (microsomal) or the mitochondria and utilize different electron transfer systems to acquire the electrons from NADPH (Hasler *et al.,* 1999). NADPH donates a pair of electrons to the P450 via flavoproteins.

Type I electron transfer system involves electrons transferred from NADPH to Fe^{2+} of ferredoxin reductase (FeRed) which is associated with the inner mitochondrial membrane (Fig. 2.6 A). Electrons are subsequently transferred to ferredoxin (Fedx) which interacts with the redox partner binding site of the P450 enzyme donating the electrons to the P450 heme group.

P450 oxidoreductase (POR) is a membrane bound flavoprotein which delivers electrons to type II P450 enzymes such as CYP17A1, CYP21A2 and cytochrome P450 aromatase (CYP19A1) which consist of two moieties - flavin adenine dinucleotide (FAD) located in the endoplasmic reticulum and flavin mononucleotide (FMN) moiety. Two electrons are donated by NADPH to FAD resulting in conformational change in the protein structure, permitting FAD to hinge closer to FMN. The electrons are subsequently transferred to FMN allowing the protein to return to its original state and thus enabling the interaction of FMN with the P450 redox-partner binding site, donating the electrons required for the catalysis (Fig. 2.6 B). POR is often assisted by cyt b_5 in reactions catalysed by CYP17A1 (Hildebrandt and Estabrook, 1971; Miller, 2005).
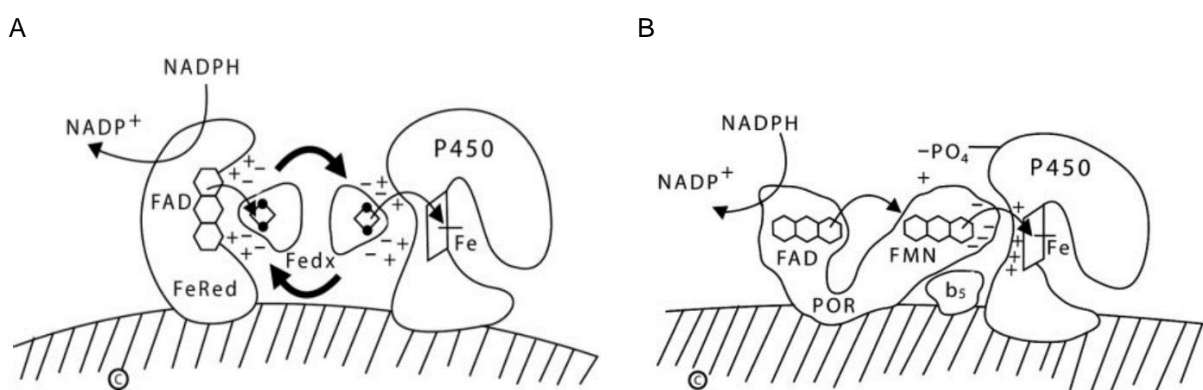
Figure 2.6   Electron transfer systems to type I and type II P450 enzymes. (A) Type I: donated electrons transfer to FeRed, FAD and Fedx to P450. (B) Type II: electrons donated to POR transfer to P450. Electrons are supplied by NADPH and are transferred via FAD and FMN. Reproduced from Miller (2005).

2.2.5.1.1   CYP11A1

Cholesterol is a cyclopentanophenathrene derived molecule (Fig. 2.7) and serves as a precursor for all other steroids in the steroidogenic pathways.

Catalysed by cytochrome P450 enzymes (P450), hydroxysteroid dehydrogenase (HSD) enzymes and co-factors, cholesterol is metabolised to different steroids, each with its own physiological function. Investigations into the steroidogenic enzymes showed that there are more steroid reactions than enzymes, supporting catalysis of numerous reactions per enzyme (Miller and Auchus, 2011).

Cholesterol is mainly derived from intestinal absorption, hepatic biosynthesis and to a lesser degree biosynthesis from acetate by the human adrenal (Gwynne and Strauss, 1982; Mason and Rainey, 1987). Cholesterol translocation from the outer mitochondria membrane to the inner mitochondria membrane is mediated by StAR. Once transported into the cytoplasm, cholesterol is utilized or stored as cholesterol ester in lipid bodies. The first (rate-limiting) step in the adrenal steroid pathways is the conversion of cholesterol to P5 and isocaproaldehyde by the CYP11A1 in the mitochondria. It catalyses the hydroxylation at carbon 22 and 20 prior to the oxidative side chain cleavage of the C20-22 bond (Fig. 2.7). A cell expressing CYP11A1 is described as a steroidogenic cell (Azziz *et al.*, 2000).
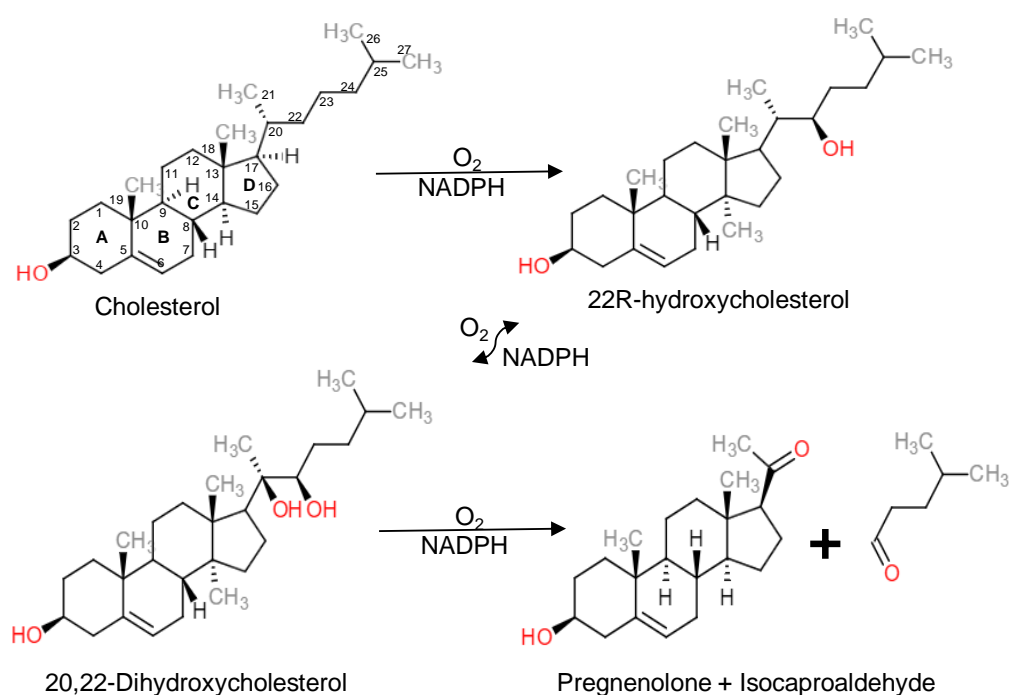
Figure 2.7　　Metabolism of cholesterol to pregnenolone and isocaproaldehyde in a reaction catalysed by CYP11A1.

2.2.5.1.2　　CYP17A1

An enzyme crucial in many steroidogenic pathways, including the adrenal and gonadal pathways, is the microsomal CYP17A1 enzyme due to its bifunctional enzymatic activity to 17-hydroxylate and/or 17, 20-lyase the substrate. Although numerous factors influence the biosynthesis of steroid hormones in the adrenal, CYP17A1 regulates pathways to be engaged as in the adrenal cortex – absence of CYP17A1 account for mineralocorticoid biosynthesis whereas the expression in the ZF and in ZR, together with cyt b_5, allows the production of glucocorticoids and adrenal androgen precursors, respectively.

CYP17A1 catalyses the conversion of P5 to androgen precursors by catalysing the hydroxylation of C17 prior to scission of the 17,20-carbon bond in numerous tissue including the adrenal, ovaries, testes,

and to a lesser extent skin (Miller and Auchus, 2011). The 17-hydroxylation efficiency of P5 and P4 by CYP17A1 is roughly equivalent to one another but differ in the downstream lyase reaction (Δ^4- and Δ^5-pathways). The conversion rate of 17OHP4 to A4 is only 3% of the rate observed in the production of DHEA in humans, thus, designating DHEA as the adrenal androgen precursor rather than 17OHP4 (Auchus *et al.*, 1998; Azziz *et al.*, 2000). The co-expression of cyt b_5 and CYP17A1 in the adrenal and gonads result in a 10-fold increase in the conversion of 17OHP4 to A4. Yet, the reaction rate does not compete with that of 17OHP5 to DHEA. An additional activity has been attributed to the human CYP17A1 in which P4, and not P5, is hydroxylated at C16 yielding 16OHP4. This activity was attributed to CYP17A1 after inhibitory studies supported a mutual active site for the conversion of P4 to both 17OHP4 and 16OHP4 (Swart *et al.*, 1993).

2.2.5.2 Hydroxysteroid dehydrogenase

Unlike the P450 proteins, HSD enzymes have no heme group and function as an oxidative and/or reductive enzymes which receive electrons via a hydride transfer mechanism from NAD^+/NADH or their phosphates (Miller and Auchus, 2011). Generally, these enzymes catalyse the bidirectional conversion (mainly unidirectional *in vivo*) of an alcohol to a ketone or the isomerization of the carbon-carbon double bond from position 5 to position 4. The directional preference is dependent on the cofactors type and availability (Miller and Auchus, 2011). These enzymes may be classified in terms of structure as either short chain dehydrogenases/reductases (SRD) or aldo-keto reductases (AKR) and include 3α-hydroxysteroid dehydrogenases (3αHSDs), 3βHSDs, 11β-hydroxysteroid dehydrogenases (11βHSDs) and multiple 17βHSDs (Miller and Auchus, 2011). SRD enzymes are structurally characterized by a Rossman fold (Agarwal and Auchus, 2005). AKR enzymes can be identified by a triosephosphate isomerase-barrel motif. Another enzyme grouped as HSD is 5α-reductase type 1 and type 2 which function by saturating the olefinic carbon-carbon double bond (Miller and Auchus, 2011).

2.2.5.2.1 SRD5A

SRD5A1 and SRD5A2 enzymes are 5α-reductase isozymes which, together with the cofactor NADPH and a hydride anion (H^-), catalyse the irreversible reduction of the double bond between C4 and C5 of C_{19}- and C_{21} steroids containing a C3 keto moiety. These enzymes are hydrophobic, membrane-bound cytoplasmic or nuclear proteins catalysing the metabolism of a wide range of substrates ranging from androgens to corticosteroids (Tomkins and Michael, 1957). Although SRD5A1 and SRD5A2 share the 5α-reductase function and some homology, the genes are located on different chromosomes and they differ with respect to biochemical properties such as pH and kinetic parameters (Azzouni *et al.*, 2012). A third isozyme called SRD5A3 has also been detected in CRPC tissue and is classified as a polyprenol reductase which differs in function and substrate preference to SRD5A1 and SRD5A2 (Uemura *et al.*, 2008). SRD5A1 and SRD5A2 enzymes are crucial in normal physiological steroid metabolism and produce vital steroids such as DHT required in the regulation of sexual development in males. The 5α-reduction of steroids often leads to an increased potency as in the case of the conversion of T to DHT, and as such the enzyme is also a target in PCa treatments (Aggarwal *et al.*, 2010). In contrast,

reducing C_{21} steroids such as P4, renders the steroid less potent in terms of PR agonist activity (Jewgenow and Meyer, 1998; Van Rooyen et al., 2017).

SRD5A1 expression has been detected in the liver, brain, non-genital skin, and to a lesser degree the scalp, kidney and adrenal gland (Bayne *et al.*, 1999; Thigpen *et al.*, 1993) whereas SRD5A2 is expressed in the liver, prostate and prostate adenocarcinoma, epididymis, seminal vesicle and genital skin, adrenals, kidney, ovaries and hair (Thigpen *et al.*, 1993). Elevated expression of 5α-reductases have been associated with diseases such as PCa, in which the ratio of SRD5A1:SRD5A2 increased, (Lehlé *et al.*, 1999; Stanbrough *et al.*, 2006; Thomas *et al.*, 2005; Titus *et al.*, 2005), in breast cancer, in which both isozymes are elevated (Lewis *et al.*, 2004), ovarian cancer, presenting elevated SRD5A1, and PCOS (Fassnacht *et al.*, 2003; Torchen *et al.*, 2016; Vassiliadi *et al.*, 2009) resulting in the progression of the diseases. Increased 5α-reductase activity has also been detected in obese men and women exhibiting elevated cortisol levels (Andrew *et al.*, 1998).

2.2.5.2.2 AKR1C2

AKR1C2, or 3α-hydroxysteroid dehydrogenase type 3, forms part of the aldo-keto reductase family which are a group of oxidases and/or reductases metabolising the hydroxyl- or keto groups at C3, 17 or 20 (Penning *et al.*, 2000). These enzymes share a high homology, especially AKR1C1 and AKR1C2 which differs by only 7 amino acids (Dufort *et al.*, 1996). As mentioned, these enzymes may be bidirectional in function and although AKR1C2 mainly acts as a reductase, it has been shown to catalyses both the reduction and oxidation of the C3 keto- and hydroxyl groups of the C_{19} steroids, DHT and 5α-androstane-3α, 17β-diol (3α-Adiol) (Penning *et al.*, 2000). These reactions are dependent on cofactors such as NAD(P)$^+$ and NAD(P)H. The C3 reduction of androgens such as DHT and T makes these steroids more susceptible for conjugation by UGT2B17. AKR1C2 expression has been detected in PCa tissue (Rižner *et al.*, 2003), the brain (Griffin and Mellon, 1999) and uterus.

2.2.5.2.3 17β-hydroxysteroid dehydrogenase type 6 (17βHSD6)

17βHSD6, also known as retinal dehydrogenase or 3(α→β))-hydroxysteroid epimerase, catalyses the NAD$^+$ dependent oxidation reaction in the prostate in which 3α-Adiol is converted to DHT. In ovaries of PCOS patients, 17βHSD mRNA, together with CYP17A1 and CYP11A1, are elevated (Wood *et al.*, 2003).

2.2.6 Steroid inactivation and conjugation

Cholesterol is metabolised through a series of reduction and hydroxylation reactions with each step influencing the bio-activity of the steroid. The polycyclic ring metabolites remain active as it is not degraded by metabolism. Hydroxysteroid hormones are inactivated by conjugation with either a glucuronic acid or sulfonic acid thus increasing the hydrophilicity of the steroid allowing it to be excreted in the urine.

2.2.6.1   Uridine diphosphate glucuronosyltransferase

In the liver phase I metabolism of hydrophilic compounds and steroids, amongst others, the inducible P450s mediate the oxygenation of the molecule. The compound is subsequently further metabolised in phase II of metabolism by the microsomal enzymes which include sulfatases, acetylases, and the uridine diphosphate glucuronosyltransferase (UGT) enzymes. These enzymes are primarily situated in the liver and kidney.

UGT transfers a glucuronic acid obtained from uridine diphosphoglucuronic acid to molecules with oxygen, nitrogen or sulfur as functional groups. The addition of the acid group (containing multiple polar groups and a carboxylic acid,) makes the steroids more polar and thus more water soluble and accessible for excretion. Glucuronic acid is predominately conjugated with steroid hormones containing a hydroxyl group (alpha configuration) at C3, C16, C17, C18 and C21 (Baulieu and Kelly, 1990). In humans, the UGT enzymes are divided into the two UGT1 and UGT2 families. Predominantly catalysing steroid hormone glucuronidation are the UGT2 enzymes which are further classified into subfamilies with UGT2B15 conjugating C_{19} steroids such as DHT and 3α-Adiol but not androsterone (AST), whereas UGT2B17 glucuronidate T, DHT, 3α-Adiol and AST – steroids containing a hydroxyl group at C17 or C3, both of which are exclusively expressed in the prostate (Hum *et al.*, 1999).

2.2.6.2   Sulfotransferase and sulfatase

Sulfotransferases (SULT) are phase 2 detoxification cytosolic enzymes which catalyse the sulfonation of compounds including steroids, xenobiotics and pharmaceutical drugs and are expressed in numerous tissue including the liver, kidney and skin. In addition, membrane bound SULT catalyse the sulfonation of proteins and polysaccharides (Nowell and Falany, 2006).

A sulfonyl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is transferred to Δ^5-steroids rendering them temporarily inactive and preventing further metabolism of the Δ^5-steroids to Δ^4-steroids by 3βHSD. Main hydroxysteroid SULTs include SULT2A1, SULT2B1 and SULT1E1, which mediate the sulfonation of steroids (Azziz *et al.*, 2000; Nowell and Falany, 2006), with the latter two catalysing the sulfonation of sterols and aromatic estrogens, respectively (Miller and Auchus, 2011). SULT2A1 catalyses the sulfonation of DHEA, an androgen precursor, in the adrenal and liver, impacting the DHEA:DHEA-S ratio and thereby regulating the steroid hormone concentrations in target tissue (Luu-The *et al.*, 1995; Nowell and Falany, 2006).

2.3   Gonadal steroid biosynthesis

Steroid hormone biosynthesis in gonadal tissue differs vastly from that in the adrenal due to the absence of CYP21A2 and CYP11B1 and CYP11B2 expression. Apart from the adrenal steroidogenesis, male and female sex hormones are produced in gonadal pathways in the testes and ovaries governed by the collection of enzymes specific to these tissues.

2.3.1   Steroidogenesis in the Leydig cells

T biosynthesis, in the male, predominantly occurs in the testes, in a similar manner as in the ZR of the adrenal, via the Δ^5-pathway to DHEA. The difference between the pathways exist in the regulation system. In the testes, T biosynthesis is regulated by luteinizing hormone (LH) and GnRH in a negative feedback manner. The hypothalamus releases GnRH to stimulate the secretion of LH and follicle-stimulation hormone (FSH) by the pituitary. LH binds to the LH-receptor and stimulates the secretion of T which subsequently signals the hypothalamus to decrease GnRH and LH secretion. Leydig cells express all the enzymes necessary to transport and convert cholesterol to P5. CYP17A1 and cyt b_5 expression allows the rapid conversion of P5 to 17OHP5 and DHEA, and due to the lack of SULT2A1, DHEA is further metabolised to A4 and T by 3βHSD2 and 17βHSD3. As the conversion of 17OHP4 to A4 is not characteristic of the human CYP17A1 lyase activity, the Δ^4-pathway to T production is negligible (Fig. 2.8).
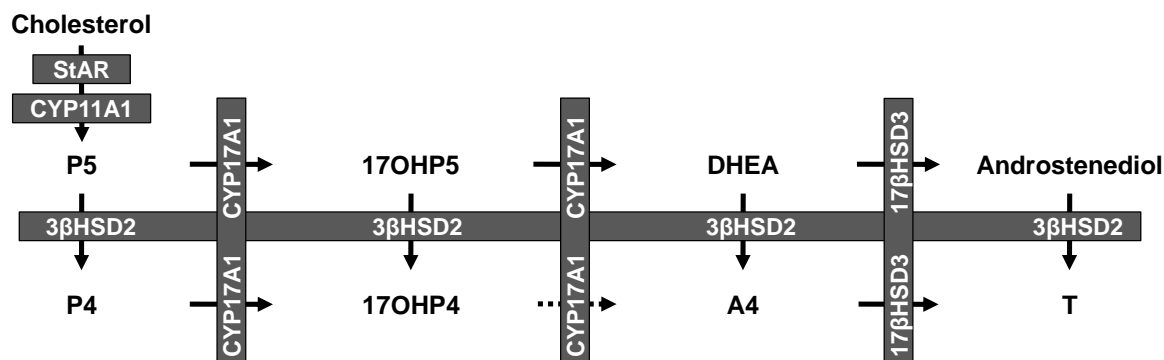
Figure 2.8   Testicular T biosynthesis in Leydig cells. The lack of SULT2A1 and high expression of 17βHSD3 promotes the biosynthesis of T in the testes.

2.3.2   Ovarian steroidogenesis

The ovarian steroidogenesis pathway is a complex two cell model due to factors such as the expression of specific enzymes being compartmentalized, and the signalling pathways regulating the change in hormone production during the menstrual cycle (Fig. 2.9) (Jamnongjit and Hammes, 2006; Miller and Auchus, 2011). The ovarian follicle contains four cell types in which steroidogenesis mainly occurs in the granulosa and theca cells regulated by FSH and LH, respectively. Estrogens are produced in the granulosa cells expressing high levels of CYP11A1, CYP19A1 and 17β-hydroxysteroid dehydrogenase type 1. Interestingly, CYP11A1 expression is low thus granulosa cells are dependent on theca cells for estrogen precursor steroids. Theca cells lack CYP19A1 expression but due to high expression levels of the LH-receptor (which stimulates StAR), CYP11A1 and CYP17A1, cholesterol is utilized in the biosynthesis of androgens including DHEA and A4. Thus, for estradiol biosynthesis, the steroid metabolites are required to diffuse between the granulosa and theca cells. StAR activity is upregulated when LH binds to the LH-receptor or FSH binds to the FSH receptor ($G\alpha_s$ protein-coupled receptors) and induce a signalling pathway in which adenylyl cyclase stimulates the release of second messenger (cAMP) to activate protein kinase A (PKA). Alternatively, activation of Gq/Gβγ indirectly activates PKA

through intracellular Ca^{2+}. These G-coupled proteins activate phospholipase C triggering inositol-1,4,5-triphosphate and diacylglycerol production (Wood and Strauss, 2002). Other regulatory factors include extracellular-regulated kinases, insulin-like growth factors and epidermal growth factor receptor but will not be discussed in this study (Jamnongjit and Hammes, 2006).

In addition, both CYP17A1 and CYP19A1 activities are regulated by cAMP. In the ovary, P4, estrogen and androgen steroid concentrations vary throughout the menstrual cycle with androgens upregulating cAMP levels by binding the AR inducing FSH signalling (Hillier and de Zwart, 1982). 16OHP4 has also been detected in the ovary and corpus luteum (Huang, 1967; Warren and Salhanick, 1961; Zander *et al.*, 1962) with urinary concentrations also changing in a cyclic manner in menstrual cycle - reaching a maximum concentration during the luteal phase (Stiefel and Ruse, 1969). Interestingly, concentrations declined with the use of contraceptive agents (Stiefel and Ruse, 1969). Similar to the adrenal production of 16OHP4 and 17OHP4, the production of 17OHP4 and 16OHP4 remain proportional to each other in the human ovary during menstrual cycle changes (Yumiko *et al.*, 1981).
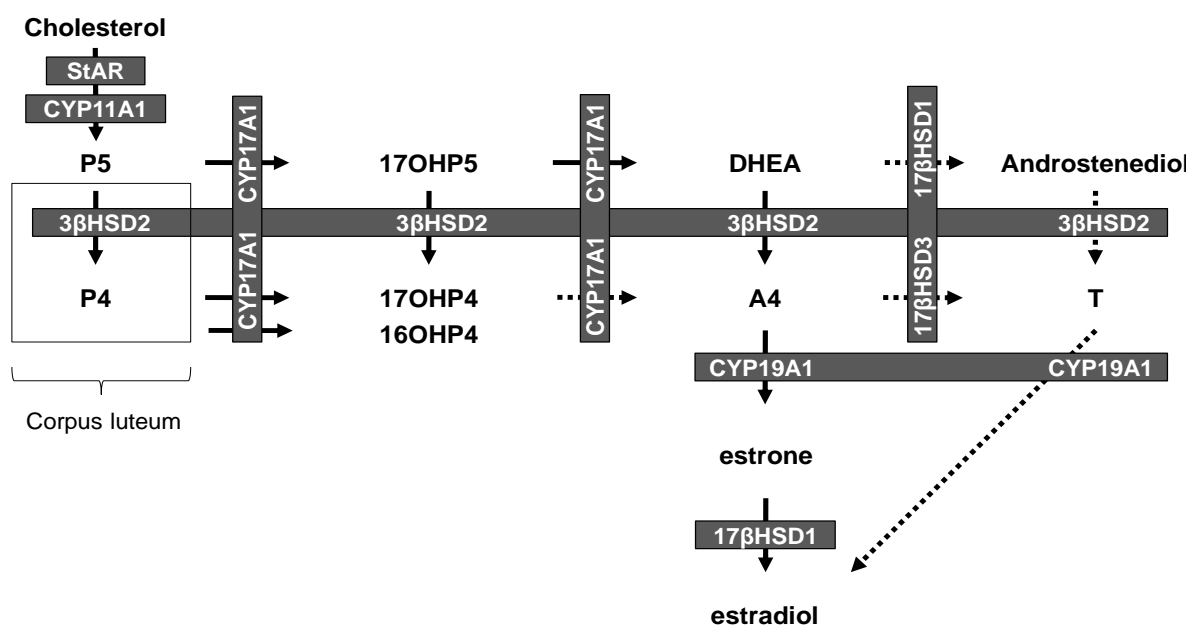
Figure 2.9   Major ovarian steroidogenic pathways in the granulosa, theca cells and corpus luteum.

2.4   Androgen biosynthesis pathways

2.4.1   *The classical or conventional androgen biosynthesis pathway*

Although the adrenal cortex produces small amounts of T, the bulk of male T is biosynthesized in the testes. Even though T exhibits androgenic activity, it generally serves as a precursor to the more potent androgen, DHT (Storbeck *et al.*, 2013). For decades DHT was considered the most potent androgen produced in the conventional pathway. DHEA is biosynthesized as in the adrenal and subsequently converted to A4 by 3βHSD2 followed by the C17 reduction of A4 by 17βHSD3 and/or 17βHSD5 to T. DHT is finally biosynthesized from T catalysed by 5α-reductases (SRD5A1 and SRD5A2) (Fig. 2.10). The testes lack SRD5A thus conversion of T to DHT is localized in tissue expressing 5α-reductases

such as in the prostate in which both isozymes are expressed (Thigpen *et al.*, 1993). In addition, the expression of 17βHSD type 2, 3 and 5 (Payne and Hales, 2004) have been detected in the prostate, allowing the interconversion of A4 and T, and 5α-dione and DHT (Luu-The *et al.*, 2008; Payne and Hales, 2004; Suzuki *et al.*, 2000a).
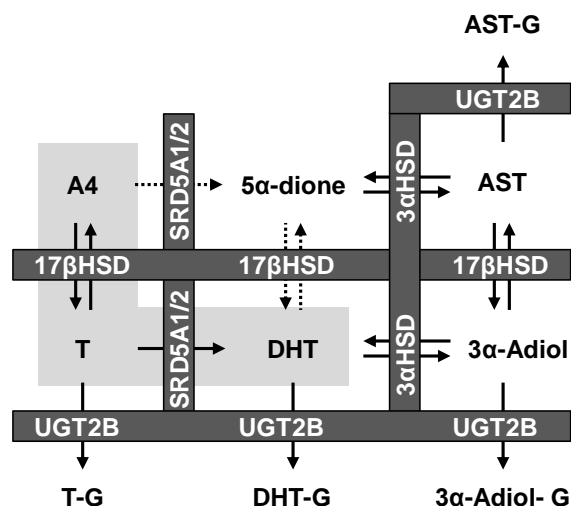
Figure 2.10   Biosynthesis of DHT in the conventional androgen pathway. A4 is converted to T and subsequently DHT by 17βHSD and SRD5A, respectively (shaded reactions). 3αHSD catalyses the reversible reaction in which DHT is inactivated to 3α-Adiol. In addition, T, DHT, 3α-Adiol and AST can be irreversibly glucuronidated. Reproduced and modified from Du Toit (2015).

2.4.2   The alternative 5α-dione pathway

In CRPC, DHT is chiefly biosynthesised via the 5α-dione alternative pathway, in which A4 instead serves as the substrate for SRD5A type 1 or type 2 to 5α-dione (Fig. 2.11). Chang *et al.* (2011) demonstrated the superiority of A4 over T as a substrate for SRD5A1 (Chang *et al.*, 2011). The flux through the 5α-dione pathway in CRPC is promoted by the increased expression of SRD5A1 (Sharifi, 2013). 17βHSD subsequently catalyses the reduction of the C17 keto group of 5α-dione leading to the production of DHT (Auchus, 2004; Chang *et al.*, 2011; Luu-The *et al.*, 2008). Temporary inactivation of DHT and 5α-dione is achieved by the 3αHSD catalysed reduction of the C3 ketone yielding 3α-Adiol and AST, respectively. Due to the reversibility of the 3αHSD reaction, 3α-Adiol and AST may be oxidised back to DHT and 5α-dione. T, DHT, AST and 3α-Adiol are substrates for UGT2B and are thus conjugated irreversibly while AST may also be sulfonated and secreted from the prostate.
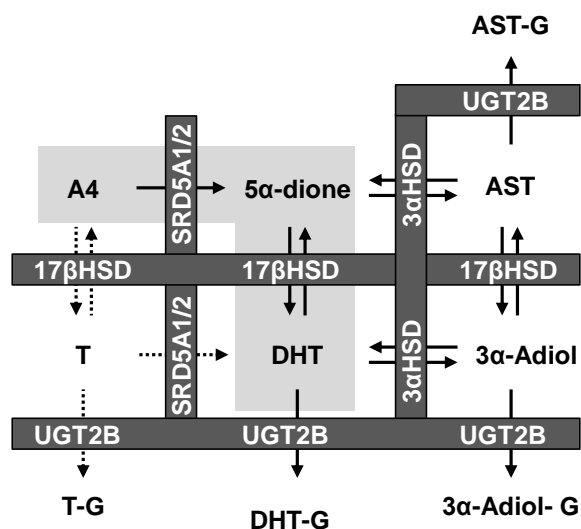
Figure 2.11 Alternative pathway to DHT production. A4 is converted to 5α-dione by SRD5A and subsequently by 17βHSD to DHT, with 5α-dione as an intermediate as an alternative of T. Reproduced and modified from Du Toit (2015).

2.4.3 11OHA4 pathway

11OHA4 was detected in the mid-1900's, as an adrenal metabolite but considered unimportant due to its low androgenic activity (Bélanger *et al.*, 1993; Goldzieher *et al.*, 1978; Touchstone *et al.*, 1955). The reaction was considered an inactivation step preventing the biosynthesis of T from A4 in the conventional androgen pathway (Bélanger *et al.*, 1993). It was subsequently excluded in the search for treatment regimens, even though it is one of the most abundant adrenal C_{19} steroids (Hudson and Killinger, 1972). In recent years, investigations into 11OHA4 identified this adrenal metabolite to be the precursor to novel potent C11-oxy androgens as it is metabolised in the 11OHA4 pathway to 11KT and 11KDHT (Storbeck *et al.*, 2013).

Biosynthesis of 11OHA4 has been investigated in numerous studies of which results eliminated P4 and cortisol as a precursor to 11OHA4 (Cohn and Mulrow, 1963; Lantos *et al.*, 1968) with the steroid rather being metabolised from DHEA by 3βHSD and CYP11B activity. Since then, the production of 11OHA4 from A4 has been accredited to both CYP11B1 and CYP11B2 activity (Swart *et al.*, 2013). The production of 11OHA4 has also been shown to be upregulated by ACTH in castrated guinea pigs (Bélanger *et al.*, 1993), human adrenal slices (Bloch *et al.*, 1957; Cohn and Mulrow, 1963; Xing *et al.*, 2011), with forskolin stimulating adrenal H295R cells (Schloms *et al.*, 2012; Swart *et al.*, 2013; Xing *et al.*, 2011) and increasing 11OHA4 production. Increased levels were also detected in adrenal vein samples after ACTH stimulation (Rege *et al.*, 2013). Thus, the involvement of CYP11B is further supported as ACTH upregulates the expression of CYP11B1 (Kowal, 1969; Kowal *et al.*, 1970). Although the adrenal accounts for the bulk production of 11OHA4, it is not limited to the adrenal. C11-oxy C_{19} steroids have been detected in testicular tumours (Savard *et al.*, 1956), PCa tissue and plasma (Du Toit *et al.*, 2017), testicular adrenal rest tumours (TART) (Bercovici *et al.*, 2005) as well as

in serum samples obtained from 21-hydroxylase deficiency patients (21OHD) (Turcu *et al.*, 2016). Furthermore, CYP11B1 and CYP11B2 mRNA expression has been shown in human primary- and metastatic primary PCa (Mitsiades *et al.*, 2012) while CYP11B2 has also been detected by RT-qPCR in the LNCaP cell line (Biancolella *et al.*, 2007).

In an earlier study, analysis of human adrenal homogenates incubated with labelled T detected mainly 11OHT, together with lower levels of A4 and 11OHA4 (Chang *et al.*, 1963). LC-MS/MS analysis detected low levels of T and 11OHT in adrenal vein samples pre- and post ACTH administration, with a ±6 fold increase with ACTH (Rege *et al.*, 2013). The metabolism of 11OHA4 and 11OHT was subsequently investigated by the research group of A.C. Swart (Biochemistry, Stellenbosch University) with respect to 11βHSD, 17βHSD, SRD5A and 3αHSD generating the bicubic 11OHA4 pathway (Bloem *et al.*, 2015).

In the top tier of the 11OHA4 pathway (Fig. 2.12), 11OHT is metabolised by 17βHSD2 to 11OHA4 but the reverse reaction does not occur. Both 11OHA4 and 11OHT are substrates for 11βHSD2 yielding 11K4 and 11KT, respectively. The SRD5A isozymes convert the C11-oxy A4 and C11-oxy T metabolites to their respective 5α-reduced metabolites yielding the middle tier metabolites − 11β-hydroxy-5α-androstanedione (11OH-5α-dione), 11-keto-5α-androstanedione (11K-5α-dione), 11β-hydroxydihydrotestosterone (11OHDHT) and 11KDHT. Catalysed by 11βHSD2, 11OH-5α-dione is metabolised to 11K-5α-dione and 11OHDHT to 11KDHT, whereas 17βHSD catalyses the inter-conversion of 11K-5α-dione and 11KDHT. Metabolism of 11OHDHT to 11OH-5α-dione is unidirectional. The steroids of the middle tier, excluding 11OHDHT, are subsequently metabolised by 3αHSD yielding 11β-hydroxyandrosterone (11OHAST), 11keto-androsterone (11KAST) and 11K-3α-Adiol. These downstream metabolites form the bottom tier of the bicubic 11OHA4 pathway where 11βHSD2 and 17βHSD catalyse the conversion of 11OHAST to 11KAST and the interconversion between 11KAST and 11K-3α-Adiol (Swart and Storbeck, 2015).

Studies into the biological relevance showed A4 and 11OHA4 to be weak androgens. For this reason, the 11β-hydroxylation was considered an inactivation, preventing the peripheral metabolism of A4 to DHT through the conventional- and 5α-dione pathway. In contrast to the weak androgenicity of A4, 11OHA4 and to most of the 11OHA4 pathway metabolites, an increase in AR agonist activity is observed at 1 nM for 11OHT < 11OHDHT < 11KT < 11KDHT with the androgenicity of the latter two compounds being comparable to that of T and DHT (Storbeck *et al.*, 2013).

It was recently shown by Du Toit *et al.* (2017) that the C11-oxy group hampers the inactivation of the androgens. 11KT and 11KDHT are less readily metabolised by 3αHSD and UGT compared to T and DHT (Du Toit *et al.*, 2017). Hence these steroids are active in circulation for longer periods making them biologically more relevant.
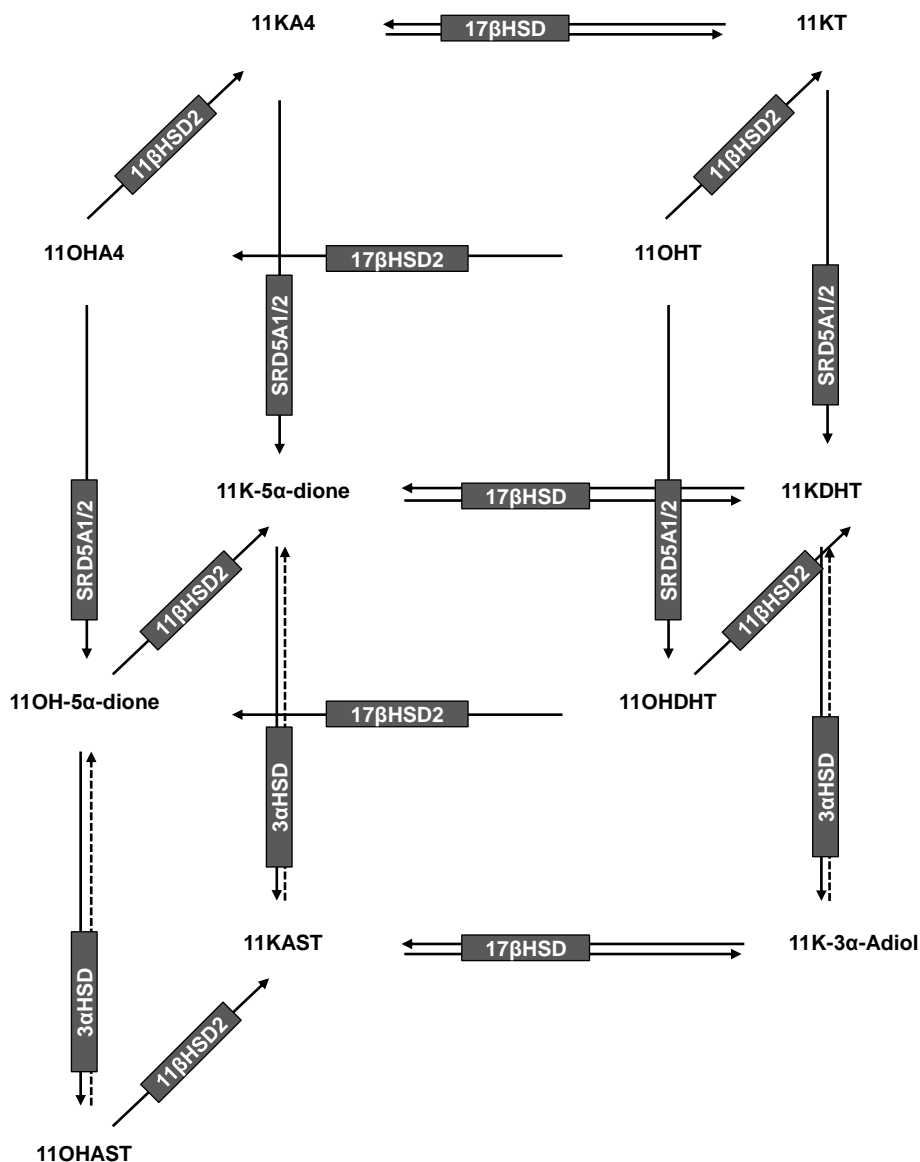
Figure 2.12   11OHA4 pathway to 11KDHT production. 11βHSD2, 17βHSD, SRD5A and 3αHSD catalyse the metabolism of 11OHA4 and its C11-oxy metabolites. Solid arrows correspond with established reactions and the dashed arrows to be established.

2.4.4   *Backdoor pathway*

Investigations into the downstream metabolism of 17OHP4 led to the discovery of the backdoor pathway, another pathway yielding DHT. First studied in tammar wallabies' testes, DHT is produced bypassing the classical pathway, biosynthesizing AST from 17OHP4 and ultimately DHT (Fig. 2.13) (Auchus, 2004). An earlier clinical study investigating the urinary excreted steroid metabolites in CAH patients, associated with elevated P4 and 17OHP4 levels, showed AST to be a product of 17OHP4 (Masuda, 1957). In the initial reaction C_{21} Δ^4-steroids, such as P4 and 17OHP4, are metabolised by the 5α-reductase isozymes (SRD5A1 and SRD5A2), followed by the reduction of the C3 keto group by

AKR1C2. The 3α-5α-reduced C_{21} steroids are subsequently cleaved by CYP17A1 yielding AST (Fig. 2.13).

P4 is reduced by SRD5A yielding 5α-pregnane-3, 20-dione (DHP4) (Gupta *et al.*, 2003; Sinreih *et al.*, 2015) which is subsequently reduced by AKR1C2 to 5α-pregnane-3α-ol-20-one (allopregnanolone) (Fig. 2.13 dashed red arrows). In addition, both DHP4 and allopregnanolone are good substrates for the 17-hydroxylase activity of CYP17A1. Similarly, 17OHP4 is 5α-reduced by SRD5A to 5α-pregnan-17α-ol-3,20-dione (Pdione) and subsequently 3α-reduced to 3α-hydroxy-5β,17α-pregnan-20-one (Pdiol) by AKR1C2 prior to being further metabolised by the lyase activity of CYP17A1 to produce AST (Turcu and Auchus, 2015). Pdione is a weak substrate for the lyase reaction whereas Pdiol is a potent substrate for the 17, 20-lyase activity of CYP17A1, independent of cyt b_5 (Gupta *et al.*, 2003). Thus, Pdiol is transformed to AST and subsequently DHT by 17βHSD5 and 17βHSD6, respectively. The 5α-reductase isozymes have a preference for 17OHP4 and P4 compared to A4 and T which leads to the formation of Pdione and DHP4 (Frederiksen and Wilson, 1971; Gupta *et al.*, 2003). The metabolism DHP4 by CYP17A1 (with or without cyt b_5) is limited to the hydroxylase activity producing Pdione though in certain systems small amounts of A4 is biosynthesized in the presence of cyt b_5 (Gupta *et al.*, 2003). Human CYP17A1 catalyses the lyase reaction of 17OHP5, 17OHP4 or Pdiol but differs in terms of the conversion rates and the dependence on cyt b_5. In a study by Gupta *et al.* (2003), Pdiol holds superiority over the 17OH-C_{21} steroids of the classical pathway for the lyase reaction catalysed by CYP17A1 in the presence and absence of cyt b_5, yielding a 3-fold increase in the presence of the cofactor. Both 17OHP5 and Pdiol contain a hydroxyl group at C3 and C17 which seems to be mandatory for the 17,20-lyase activity due to geometrical configuration, however, the mechanism is still poorly understood and results are limited (Gupta *et al.*, 2003). These studies clearly showed the biosynthesis of DHT via a pathway other than the classical pathway, which results mainly from the peripheral 5α-reductase and CYP17A1 activities. Kamrath *et al.* (2012) studied the steroid flux in the pathway, more specifically studied the competition between the 5α-reductase and the lyase activity. Investigating the urinary levels of the different metabolites associated with the classical- and backdoor pathway and analyses of steroid ratios contributed to the elucidation of the backdoor metabolic pathway, shown to dominate steroid biosynthesis in 21OHD patients. Etiocholanolone, a metabolite specific to the classical pathway, is not elevated in 21OHD patients compared to the controls. Available data clearly suggest that the 3α,5α-reduced C_{21} steroids are a major source of 5α-reduced C_{19} steroids contributing to the androgen excess observed in PCOS and 21OHD patients (Kamrath *et al.*, 2012a). Investigating these steroids C11-oxy C_{21} steroids in the current study may thus elucidate the contribution of these steroids to active androgens in androgen sensitive conditions or diseases such as PCa, CAH and PCOS.

DHT may therefore be formed not only by the 5α-reduction of T and A4 but also via the 5α-reduction of C_{21} steroids in the backdoor pathway (Auchus, 2004). Understanding the contribution of this pathway to C_{19} steroids may contribute to the management of androgen dependent disorders (Auchus, 2004).
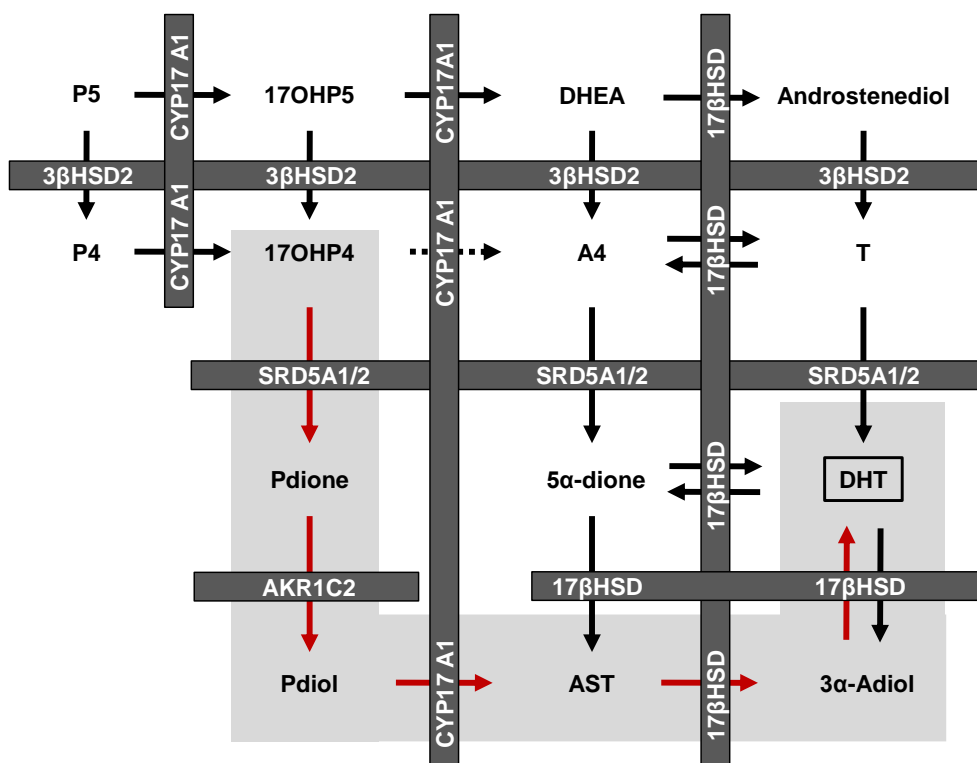
Figure 2.13 Backdoor pathway to DHT. 17OHP4 is metabolised by SRD5A1 and SRD5A2 and subsequently AKR1C2 to Pdione and Pdiol. CYP17A1 cleaves Pdiol yielding AST. Metabolism of AST by 17βHSD enzymes yields DHT.

2.5 Selected clinical conditions associated with 16OHP4 and C11-oxy steroids.

2.5.1 Congenital adrenal hyperplasia

CAH is a recessive autosomal defect of enzymes catalysing the adrenal steroidogenesis resulting in hormonal abnormalities (Speiser and White, 2003). These enzyme defects, in decreasing frequency, include CYP21A2, CYP11B1, 3βHSD2 and rarely CYP17A1, CYP11A1 and POR (Turcu and Auchus, 2015). Classic CAH occurrences differ between ethnic populations and geographic locations (Merke and Bornstein, 2005) with new-born screening showing a carrier frequency of 1:55 in the general population whereas 1:15,000 Caucasians phenotypically display CAH (Baumgartner-Parzer *et al.*, 2005).

The most common form of CAH, accounting for about 90 – 95% of all reported CAH cases, is the deficiency of CYP21A2 (Nurhaen and Duarsa, 2012; Speiser and White, 2003). Impairment of the 21-hydroxylase activity in adrenal steroidogenesis results in decreased biosynthesis of mineralocorticoids and glucocorticoids. The regulatory systems such as the HPA axis and RAAS are subsequently stimulated to compensate for the steroid hormone deficiency and as a consequence result in the accumulation of 17OHP4 (Turcu *et al.*, 2016). The excessive secretion of ACTH leads to hyperplasia of the adrenal cortex (New and Levine, 1984). In addition, the lack of ALDO results in a

25

decrease in plasma sodium and blood pressure which leads to further stimulation of the adrenal cortex. Steroids which accumulate can serve as substrates for functional adrenal enzymes such as CYP17A1 in the androgen pathway subsequently resulting in hyperandrogenism as well as elevated 17OHP4 levels. Other C_{19} and C_{21} steroid hormones associated with 21OHD include 16OHP4, 21-desoxycortisol (DOF) and 11OHP4 and C11-oxygenated C_{19} steroids (Fiet *et al.*, 1989, 1988; Turcu *et al.*, 2016).

Phenotypes include ambiguous genitalia with female patients, increased phallus in males, hirsutism, hypokalaemia, hypotension, subfertility or infertility, early onset of puberty, metabolic syndrome and PCOS-like symptoms can be ascribed to hyperandrogenism (Speiser and New, 1985; Speiser and White, 2003; Turcu *et al.*, 2016; Witchel and Azziz, 2011). Current 21OHD diagnosis are based on elevated plasma 17OHP4 levels, detected after stimulation with ACTH, in consideration with factors such as hormone treatments, age and weight which can frequently lead to false positive and false negative clinical diagnostic results.

21OHD is clinically described as classic or non-classical with the latter being a mild, late-onset form. In the non-classic form of 21OHD, cortisol and ALDO biosynthesis is normal and fertility is rarely influenced (Speiser and White, 2003). On the other hand, androgen levels are slightly increased thus accounting for the symptoms such as hirsutism, irregular menstrual cycles and acne. Non-classical 21OHD is more frequent (1:8000) than the classic form (Merke and Bornstein, 2005; Speiser *et al.*, 1985) with the symptoms of the non-classic form comparable to that of PCOS (Sahin and Kelestimur, 1997). Classic 21OHD, being the severe form of the disease, can be sub-categorized as simple virilising or salt-wasting.

A third of all classic 21OHD cases are categorized as simple virilising which is the most common cause of genital ambiguity in females (Merke and Bornstein, 2005). One in every 16,000 births is diagnosed with this severe form which is presented with hyperandrogenism pre- and post-parturition (Nurhaen and Duarsa, 2012). Excess androgens during early development result in the virilising of the external genitalia. For this reason, females are diagnosed earlier and males later in childhood or even early adulthood due to early onset of male secondary sexual characteristics (Nurhaen and Duarsa, 2012; Speiser and White, 2003). These patients are presented with an inefficient production of cortisol whereas ALDO levels may or may not be normal –depending on the severity if the enzyme defect (Speiser and White, 2003).

The classic salt wasting 21OHD comprise about a 66% of 21OHD cases, and is characterized by low ALDO levels and the inability to retain sodium or regulate electrolyte homeostasis (Horner *et al.*, 1979). Plasma renin activity (PRA) is increased because of the role of sodium and ALDO in the RAAS, and may lead to hyperplasia of the juxtaglomerular apparatus (Cara and Gardner, 1963). Investigations into the correlation between the renin activity and ALDO secretion presented hyperreninemia and negligible ALDO secretion in patients with severe salt wasting 21OHD. In addition, restriction of sodium intake in non-salt and salt wasting patients results in elevated renin levels but not in increased ALDO levels, suggesting a defect in the production of ALDO in 21OHD patients (Godard *et al.*, 1968). This was further

supported in a study by Imai *et al.* (1968) wherein administration of DOC and NaCl to salt wasting 21OHD phenotypes was shown to decrease PRA (Imai *et al.*, 1968). As previously mentioned, renin can indirectly result in vasoconstriction (Bongiovanni, 1968) thus increasing the blood pressure. Nevertheless, renin levels are normal in hypertensive CAH (Imai *et al.*, 1968).

Interestingly, in some cases the decrease in sodium in 21OHD patients appears to be age dependent as conditions improve with age (Godard *et al.*, 1968) whereas in other cases, mineralocorticoid and glucocorticoid therapies are crucial for survival (Horner *et al.*, 1979). The lack of ALDO is further exacerbated by P4 and 17OHP4 which both act as mineralocorticoid antagonist (Speiser and White, 2003). Investigations into the elevated biosynthesis of 16OHP4 detected in CAH patients led to results associating 16OHP4 with natriuresis and ACTH secretion (George *et al.*, 1965; Janoski *et al.*, 1969; Uete and Venning, 1963). Janoski *et al.* (1969) investigated 16OHP4 and 16α-hydroxypregnenolone (16OHP5) levels in the urine of salt wasting 21OHD patients and demonstrated markedly elevated 16OHP4 levels in salt wasting 21OHD before (24-28 mg/day) and after (0.6-7.9 mg/day) glucocorticoid therapy compared to 1 mg/day detected in control subjects (Janoski *et al.*, 1969). With the levels of 16OHP4 exceeding 16OHP5 together with the presence of 5α-pregnane-3α,17α,20α-triol (pregnanetriol) and pregnanediol as urinary metabolites, Δ^4-3-ketosteroids were suggested to be precursors. Characteristic of 21OHD in females is the pseudohermaphrodism of the external genitalia which is presented more severely in the salt wasting 21OHD than in simple virilising 21OHD (Godard *et al.*, 1968).

Male 21OHD patients, especially salt wasting (Combes-Moukhovsky *et al.*, 1994), frequently show tumours in the testis which have an adrenal origin termed TART (Clark *et al.*, 1990). In early development, ±5 weeks into gestation, the development of the gonads and adrenal glands takes place in close vicinity of each other. In some 21OHD cases, the adrenal cells adhere to the gonadal tissue prior to the formation of the adrenal groove and the subsequent separation of the organs. This causes adrenal cells to migrate with the testes and subsequently results in the TART found in 21OHD males. These tumours are histologically distinct from Leydig cell tumours. The adrenal origin is further supported by the tumour's response to ACTH and glucocorticoid therapy (Blumberg-Tick *et al.*, 1991; Rutgers *et al.*, 1988; Stikkelbroeck *et al.*, 2004). In addition, *in vitro* and *in vivo* studies have shown cortisol biosynthesis localized to the testes of classical 21OHD patients (Fore *et al.*, 1972; Franco-Saenz *et al.*, 1981). Adrenal-like properties in TART are further emphasized by the detection and elevated circulating levels of 11β-hydroxylated steroids such as 11OHP4, DOF and 11OHA4 (Bercovici *et al.*, 2005; Combes-Moukhovsky *et al.*, 1994; Turcu *et al.*, 2015). These patients experience impairment of T biosynthesis, spermatogenesis and testicular function often leading to infertility (Stikkelbroeck *et al.*, 2001).

2.5.2 *Polycystic ovarian syndrome*

PCOS, also referred to as Stein-Leventhal syndrome, is an endocrine disorder differentiated from known adrenal and pituitary disorders, which globally affects about 5-10% of premenopausal women of

reproductive age (Azziz *et al.*, 2009; Jamnongjit and Hammes, 2006). The etiology of the disorder is still unclear but the condition is associated with hyperandrogenism leading to irregular menses and chronic anovulation resulting in infertility (Hull, 1987), metabolic syndrome (Wild, 2002), polycystic ovaries, as well as acne and hirsutism. In addition, women with PCOS often display insulin resistance which can progress to type 2 diabetes mellitus (Franks, 1995; Legro *et al.*, 1999; Ovalle and Azziz, 2002). Insulin also promotes steroidogenesis in granulosa cells of normal and PCOS ovaries and enhances LH activity (Franks *et al.*, 1999), while synergism with LH may account for androgen upregulation.

PCOS is often difficult to distinguish from the non-classic 21OHD due to the similarities in the symptoms. In addition, CYP11B1, CYP21A2, and 3βHSD2 deficiency have been detected in some PCOS patients (Sahin and Kelestimur, 1997). The polycystic appearance of the PCOS ovary is due to the overstimulation of follicular development due to excess androgens (Weil *et al.*, 1999) with basal androgen, DHEA, T, A4, 11OHA4 and Adiol levels being elevated in PCOS together with increased LH levels (Carmina *et al.*, 1992, 1986; Franks, 1995; Lachelin *et al.*, 1982; Yen *et al.*, 1970). In addition, an increase in CYP17A1 activity was detected in theca cells which, as previously mentioned, express all the enzymes required for androgen production) (Nelson *et al.*, 1999; Wickenheisser *et al.*, 2000). An increase in follicle cell proliferation is associated with excess androgens. Vendola *et al.* (1998) showed that androgens induce follicular growth and Weil *et al.* (1999a) subsequently demonstrated the mRNA co-localization and positive correlation between the AR and FSH-receptor expression in the follicle (Vendola *et al.*, 1998; Weil *et al.*, 1999).

Over the years, numerous studies have questioned the site of androgen biosynthesis in PCOS patients and ascribed it to adrenal-, ovarian- and peripheral origin (Azziz *et al.*, 1998; Fassnacht *et al.*, 2003; Sahin and Kelestimur, 1997; Stahl *et al.*, 1973). Stahl *et al.* (1973) detected elevated T levels in both the adrenal and ovary vein of clinically diagnosed PCOS patients, while peripheral conversion to T was also identified (Stahl *et al.*, 1973). Ovarian androgen production is further supported by the sustained elevated androgen levels detected upon adrenal suppression with dexamethasone (Lachelin *et al.*, 1982). The adrenals also contribute to elevated androgens in PCOS as was shown in patients in whom ovarian steroidogenesis was suppressed using a GnRH agonist, consequently presenting with abnormal androgen levels while ACTH reportedly exacerbated androgen production (Anapliotou *et al.*, 1990; Azziz *et al.*, 1998).

2.5.3 *Prostate cancer and castration-resistant prostate cancer*

PCa is the second most common cancer and second leading cause of cancer morbidity among men of which approximately thirteen percent will be diagnosed in their lifetime (National Cancer Institute, 2015). This androgen-dependent disease is augment by androgenic steroids such as T, DHT (Chang *et al.*, 2011; Geller *et al.*, 1978; Titus *et al.*, 2005) as well as adrenal A4 (Luu-The *et al.*, 2008; Montgomery *et al.*, 2008; Stanbrough *et al.*, 2006) and the C11-oxy derivatives (Storbeck *et al.*, 2013).

The healthy prostate is dependent on T and DHT to mediate virilisation, sexual development, maturation and maintenance of the prostate tissue. T is secreted by the testes and subsequently 5α-reduced in the prostate yielding DHT which has a higher androgenic activity and binding affinity for the AR compared to T. In disease states, such as benign prostate hyperplasia (BPH) and PCa, these androgens bind the AR leading to transcriptional activation and cell proliferation (Auchus, 2004; Luu-The *et al.*, 2008).

Treatments for metastatic PCa target the reduction of these androgens via androgen deprivation therapies (ADT) in which the men are either surgically- or chemically castrated (Luu-The *et al.*, 2008; Mostaghel, 2014). Even with treatment, numerous investigations have shown the inability of these therapies to suppress androgen biosynthesis evident in recurring cancer as in the case of CRPC. This form of PCa is characterised by high androgen levels which are able to activate the AR with an even higher morbidity rate (Mostaghel, 2014). In CRPC, DHT is produced independent of testicular T biosynthesis using different precursors. In the 5α-dione pathway, DHT is biosynthesized from A4 with 5α-dione as intermediate whereas in the backdoor pathway 17OHP4 is metabolised by SRD5A, AKR1C2 and CYP17A1 to AST and subsequently converted to DHT. In addition, the 11OHA4 pathway has provided another alternative to the conventional pathway to yield potent androgens including 11KT and 11KDHT. Thus, in CRPC weak circulating androgens are converted to active androgens able to activate the androgen receptor.

2.6   Summary

Steroidogenesis is complex in terms of regulation, the biosynthetic pathways, tissue distribution of enzymes, as well as the disease's ability to adapt to its environment. Although the conventional adrenal steroidogenic pathway described in this chapter provides a background for understanding the biosynthesis of mineralocorticoids, glucocorticoids and adrenal androgen precursors, there are certain reactions and products such as 11OHP4, 11KP4 and 16OHP4 which is not accounted for. As is evident from the numerous androgen producing pathways, that human steroidogenesis is complex with unexplored avenues, impacting specifically in steroid hormone related diseases. 11OHA4 was initially regarded as unimportant in PCa. Subsequent studies, nevertheless, showed 11β-hydroxylated androgens to be more potent than T and DHT -due to exhibiting androgenic activity with their inactivation and conjugation being impeded, rendering these steroids active for longer. It is therefore critical to not only investigate the metabolic fate and the biological relevance of the adrenal steroids such as 11OHP4, 11KP4 and 16OHP4 but also to establish their position in steroidogenic pathways. The following chapters discuss the metabolism of 11OHP4, 11KP4 and 16OHP4, and the physiological importance which they may hold.

CHAPTER 3

An investigation into the biosynthesis, metabolism and biological relevance of adrenal 11β-hydroxyprogesterone and 11keto-progesterone

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3.1 Introduction

Contrary to the general adrenal steroidogenic pathway depicting the clearly designated pathways producing mineralocorticoids, glucocorticoids and adrenal androgens, it has been shown that the substrate preference of CYP11B1 and CYP11B2 are not limited to DOC and deoxycortisol, but also extends to other C_{19} and C_{21} steroids such as 16OHP4, A4, T and P4 (Fig. 3.1) (Schloms et al., 2012; Swart et al., 2013; Van Rooyen et al., 2017). The significance of C11-oxy steroids has recently come to the fore with the initial focus on 11OHA4.
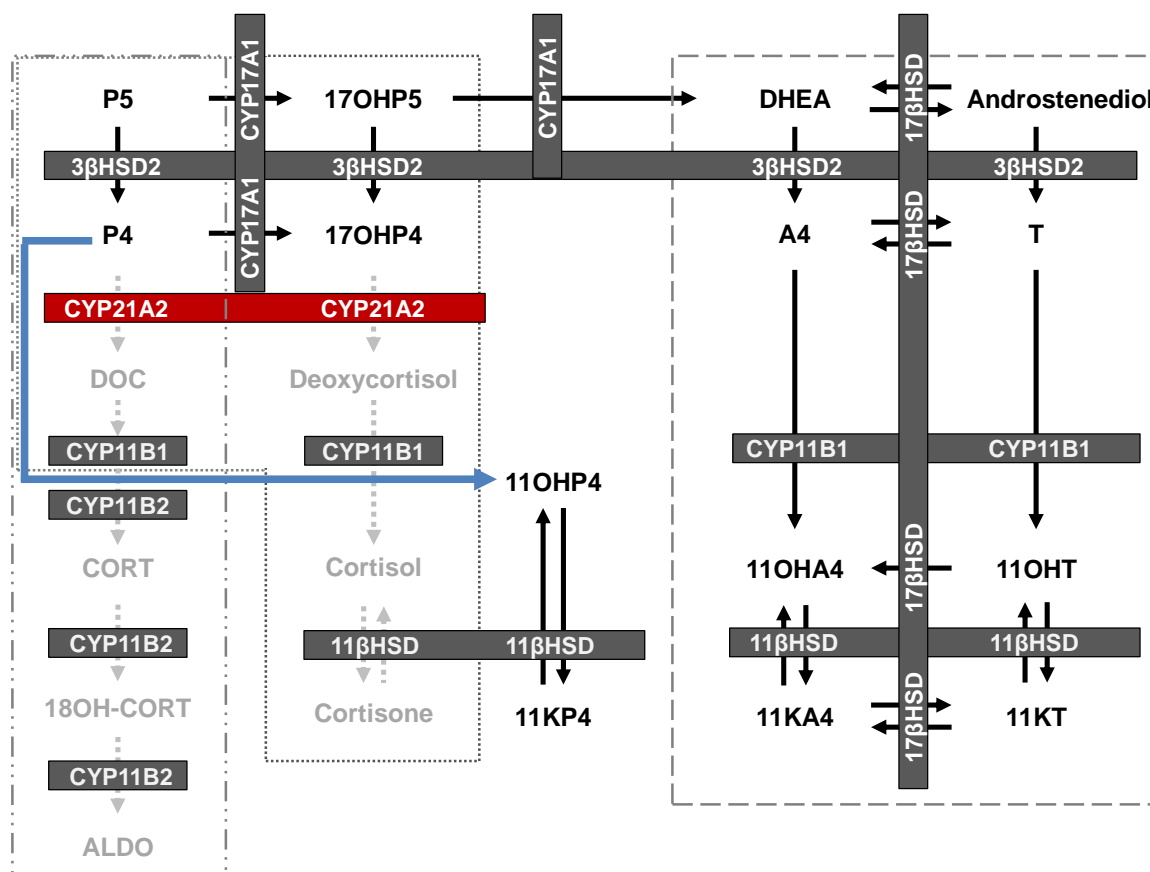
Figure 3.1   Schematic representation of adrenal steroidogenesis in 21OHD. The mineralocorticoid pathway (−··−··−), glucocorticoid pathway (········) and the adrenal androgen precursors (−−−−). Defective biosynthesis of mineralocorticoids and glucocorticoids due to a deficiency of CYP21A2 (red block) in 21OHD is indicated in grey.

This C11-oxy C_{19} steroid is uniquely synthesized in the adrenal and its downstream metabolism to potent androgens such as 11KT and 11KDHT (Fig. 3.1 and Fig. 3.2) has been associated with clinical conditions characterized by hyperandrogenism such as CAH due to 21-hydroxylase deficiency (21OHD), and PCOS (Azziz *et al.*, 2004; Turcu *et al.*, 2016). With ever-growing advances in analytical technologies, the spotlight has recently fallen on another class of steroids, the C11-oxy C_{21} steroids, however, aspects regarding their biosynthesis, metabolism and biological relevance remain limited. 11OHP4 and 11KP4, both C11-oxy C_{21} steroids, are currently being associated with a number of clinical conditions.
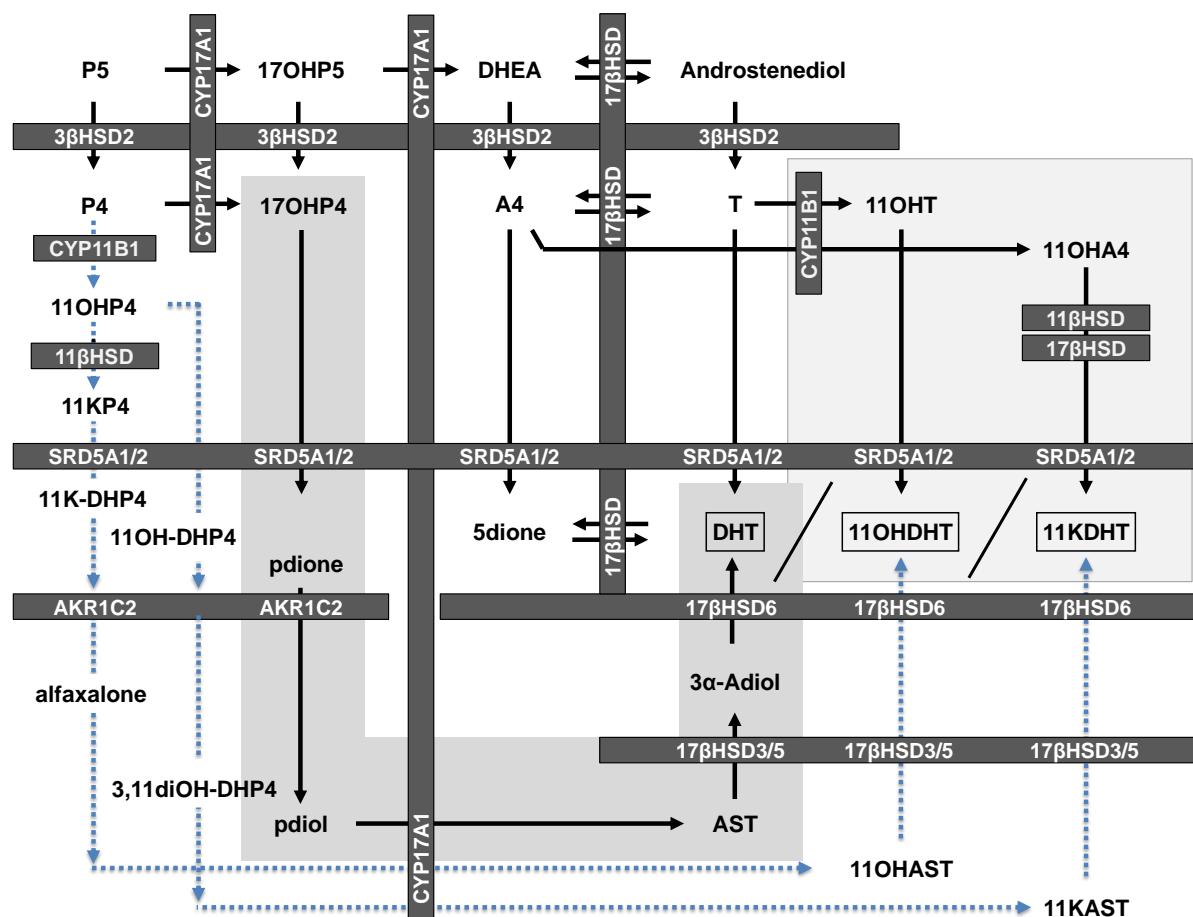
Figure 3.2   Peripheral steroid metabolism. The proposed metabolism of 11OHP4 and 11KP4, and their metabolites in the backdoor pathway to 11OHDHT and 11KDHT, dotted blue lines; the backdoor pathway converting 17OHP4 to DHT, reactions shaded dark grey; a simplified 11OHA4 pathway converting 11OHA4 and 11OHT to 11OHDHT and 11KDHT, reactions shaded light grey.

11OHP4 was first detected in 1951 in P4 perfused ox-adrenals, albeit at very low concentrations (<1%). The steroid was later shown to be metabolised to CORT and DOC in rat and frog quartered adrenal tissue (Kraulis and Birmingham, 1964). Gueux *et al.* (1987) subsequently deduced that the production of 11OHP4 originated in the adrenal and not in the gonads due to a lack of variation in different menstrual cycle phases in basal 11OHP4 and DOF levels, in contrast to 17OHP4, as assessed in males and in females. Furthermore, the basal levels of 11OHP4 (18-19 pM) increased 4- to 6-fold (±73 pM)

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after the injection of ACTH in healthy patients (Fiet *et al.*, 1989; Gueux *et al.*, 1987). These findings, together with the aforementioned studies, suggest that the biosynthesis of 11OHP4 in the adrenal is dependent on CYP11B1, the expression of which is upregulated by ACTH.

Investigations into the metabolism of 11OHP4 and 11KP4 by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and 11βHSD2, which catalyse the interconversion of cortisol and cortisone as well as 11OHA4 and its ketone, 11keto-androstenedione (11KA4) (Schloms *et al.*, 2012; Swart *et al.*, 2013) are limited (Fig. 3.1). In 1995, Souness *et al.* reported the potent inhibitory activity of 11OHP4 towards 11βHSD2 expressed in JEG-3 cells. However, only product formation was assayed and as such endogenous conversion of the steroid substrate was not considered. Unpublished data by the research group of A.C. Swart (Biochemistry, Stellenbosch University) showed the conversion of 11OHP4 to 11KP4 by 11βHSD2 (50% after 11 hrs), as well as 11KP4 being metabolised to 11OHP4 by 11βHSD1 (40% after 24 hrs), both isozymes transiently expressed in human embryonic kidney (HEK-293) cells – clearly showing these conversions. *In vivo* conversions of these C11-oxy steroids will, however, also depend on the expression levels of 11βHSD1 and 11βHSD2 in the specific tissue as well as cofactor availability.

More recently, Turcu *et al.* (2015) reported on peripheral sera levels of C_{21} steroids in 21OHD patients and in cultured TART cells. Low circulating levels of 11OHP4 were detected, together with increased 17OHP4, 16OHP4, and DOF levels. However, in TART samples basal DOF levels were significantly higher than in ACTH stimulated cells (Turcu *et al.*, 2015). Earlier studies reported basal 11OHP4 levels observed in 21OHD and TART's of 21OHD patients to be comparable to ACTH-stimulated levels in normal subjects with the ACTH-stimulated levels in 21OHD subjects being significantly higher than those of ACTH-stimulated healthy subjects (Bercovici *et al.*, 2005; Blumberg-Tick *et al.*, 1991; Claahsen-van der Grinten *et al.*, 2007; Combes-Moukhovsky *et al.*, 1994; Fiet *et al.*, 1989).

Further investigations into steroid levels in 21OHD patients receiving hydrocortisone treatment detected significantly increased 11OHP4 levels together with variations observed between the patients with salt-wasting and non-salt-wasting. Levels were found to be dependent on angiotensin stimulation which resulted in elevated PRA and on the degree of severity of the 21OHD. The salt-wasting phenotype receiving a sub-dosage of hydrocortisone treatment exhibited higher levels of 11OHP4 and PRA compared to levels in the non-salt wasting 21OHD phenotype, which also stabilized after administrating the correct dosage to resemble the levels of non-salt wasting patients in terms of 11OHP4 and PRA (Gueux *et al.*, 1987). This indicates that ACTH plays a role in the biosynthesis of 11OHP4 as low cortisol levels signal the HPA negative feedback mechanism, upregulating the production of glucocorticoids, accounting for the increase in 11OHP4 levels in the salt-wasting 21OHD patients.

Claashen-van der Grinten *et al.* (2007) subsequently reported significantly higher levels of 11β-hydroxysteroids (11OHA4, DOF and 11OHP4) in the spermatic vein compared to the adrenal- or peripheral vein samples of 21OHD patients with TART. They also showed the presence of mRNA transcripts of the adrenal-specific enzymes, CYP11B1 and CYP11B2 and ACTH and AngII receptors in the TART tissue, supporting the adrenal-like characteristics of the testicular tumour. The elevated

C11 steroids in the spermatic vein were attributed to the expression of these adrenal specific enzymes and, together with receptor expression, suggested that ACTH and AngII were contributing factors to tumour growth (Claahsen-van der Grinten *et al.*, 2007).

Increased 11OHP4 and 11KP4 levels in 21OHD may also contribute to increased androgens and in turn, contribute towards virilisation observed in 21OHD female patients. It was already shown in 2004 that 17OHP4 is converted to DHT (Auchus, 2004) with the metabolism of 17OHP4 in peripheral tissue described years later as the backdoor pathway depicted in Fig. 3.2.

Metabolites of the backdoor pathway have been detected in 21OHD patients between 1-25 years of age with high levels of Pdiol and androsterone (AST) being detected in urinary samples (Kamrath *et al.*, 2012b). Confirming the role of the C11-oxy steroids in disease conditions, the analysis of urine obtained from children receiving treatment for 21OHD showed 11OHAST to be the main androgen metabolite formed (Kamrath *et al.*, 2017). In addition, AST levels were also detected to be 7-fold higher compared to controls. Turcu *et al.* (2016) also showed that A4, T and four C11-oxy C_{19} steroids (11OHA4, 11KA4, 11OHT and 11KT) were elevated (±3-4-fold) in serum samples of 21OHD patients (male and females). These elevated C11-oxy C_{19} steroid levels were detected in the adrenal vein but not in the inferior vena cava, thus localizing the biosynthesis of the C11-oxy C_{19} steroids from A4 to the adrenal. It was hypothesised that the C11-oxy C_{19} steroids may therefore act as novel biomarkers to identify excessive androgen biosynthesis in the adrenal (Turcu *et al.*, 2016).

The induction of AR and PR responsive genes by 11OHP4 and 11KP4 and their downstream metabolites have not been investigated. Limited studies regarding the C11-oxy C_{21} steroids have been reported to date in terms of steroid receptor interaction. 11OHP4 has been shown to bind to the glucocorticoid receptor (GR) whereas 11KP4 did not (Galigniana *et al.*, 1997). This may have implications in PCa in which GR-driven resistance developed after continued treatment with enzalutamide, a PCa treatment drug. The GR has been shown to induce target genes similar to those activated by the AR (Arora *et al.*, 2013). The relative binding affinities of 11OHP4 and 11KP4 for the MR, have been assayed and 11KP4 shown to have a significantly greater affinity for the receptor, although the metabolism of the steroids were not considered (Galigniana *et al.*, 1997). Futhermore, the C11-oxy C_{21} steroid metabolites have not been considered as contributing to the active androgen pool in 21OHD, PCOS, CAH or PCa. These steroids may bind reversibly to nuclear steroid receptors –a group of transcriptional regulators which include the GR, AR, PR, MR and estrogen receptor (ER) (Olefsky, 2001). These receptors share a common structure and contain a promotor region, ligand-binding domain (LBD), DNA-binding domain and hinge region. In its inactive form, nuclear receptors are directly associated with a phosphorylated heat shock protein (HSP) bound to the LBD – stabilizing the conformation of the receptor to allow the ligand to bind. Once the ligand binds to the LBD, the HSP dissociates and a conformational change allows homodimerization or heterodimerization to occur. In the activated state, the dimer translocates to the nucleus where the DNA-binding domain binds to the hormone response element of the target gene manipulating the cellular genetic machinery, thus eliciting transactivation or transrepression of the target gene (Baulieu and Kelly, 1990; Chawla *et al.*, 2001). Although the receptor types expressed in different tissues may be similar, activation depends on the

ligand specificity of the receptor. Moreover, genes regulated by the receptors differ, resulting in different physiological consequences induced via a single receptor type (Hall and Guyton, 2011).

The aim of this study was to determine the biosynthesis and metabolism of the C11-oxy C_{21} steroids and to include adrenal- and peripheral enzymes (Fig. 3.2; blue dotted). Enzymatic assays catalysed by enzymes transiently expressed in cell model systems, together with steroid (substrate and product) identification and quantification using quantitative time-of-flight mass spectrometry (Q-TOF MS), ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS), were carried out. The biosynthesis of 11OHP4 from P4, catalysed by CYP11B1 and CYP11B2 in HEK-293 cells, was firstly investigated. Enzymatic conversion of 11OHP4 and 11KP4 by 5α-reductase (SRD5A1/2) and AKR1C2 was subsequently investigated in order to determine their metabolic fate and the potential contribution of the C11-oxy C_{21} steroids to the C11-oxy C_{19} androgen pool. The possibility exists that the product of the latter reaction may also serve as substrates for CYP17A1, similar to the metabolism of 17OHP4 in the backdoor pathway which would lead to the production of 11OHAST and 11KAST. The metabolism of 11OHP4 and 11KP4 was therefore subsequently investigated in the LNCaP cell model. Since the physiological relevance of these C11 oxy-C_{21} steroids is unknown, the interaction of 11OHP4 and 11KP4 with the AR and PR isoforms (PR-A and PR-B) was therefore assayed together with their 5α-reduced metabolites.

3.2 Methods and materials

3.2.1 Materials

Methyl tert-butyl ether (MTBE), Dulbecco's modified Eagle's media (DMEM), Roswell Park Memorial Institute 1640 media (RPMI-1640), β-glucuronidase (Type VII-A from *E. coli*; 5 292 units/mg), D-(+)-Glucose, and steroids (A4; T; 5α-androstanedione, 5α-dione; AST; DHT; P4; 17OHP4; Pdione; DOC; CORT; ALDO; deoxycortisol, cortisol and cortisone) were purchased from Sigma-Aldrich (St. Louis, USA). DHP4; 11OHP4; 11KP4; 11keto-dihydroprogesterone, 11K-DHP4; 5α-pregnan-3α-ol-11,20-dione, alfaxalone; Pdiol; 11OHAST; 11KAST; 11OHA4; 11OHT; 11KA4; 11KT; 11KDHT; and DHT-glucuronide, DHT-G were obtained from Steraloids (Wilton, USA). The deuterated steroids purchased from Cambridge isotopes (Andover, USA) included deuterated testosterone (D2-T/Testosterone 1,2-D2, 98%), deuterated cortisol (D4-cortisol/9,11,12,12-D4-cortisol) deuterated progesterone (D9-P4/Progesterone 2,2,4,6,6,17A,21,21,21-D9, 98%), deuterated 17α-hydroxyprogesterone (D8-17OHP4/17-hydroxyprogesterone 2,2,4,6,6,21,21-D8, 98%), deuterated androstenedione (D7-A4/ androstenedione 2,2,4,6,6,16,16-D7; 97%) and deuterated 11β-hydroxyandrostenedione (D7-11OHA4/11β-hydroxyandrostenedione 2,2,4,6,6,16,16-D7; 98%). Promegestone (R5020) and mibolerone (Mib) were acquired from Perkin Elmer (Massachusetts, USA). HEK-293 cells were purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). LNCaP cells were purchased from Sigma's European Collection of Cell Cultures (St. Louis, USA). Corning® CellBIND® surface tissue culture ware (T75 flasks, 100 mm dishes, 12-, 24-, and 96 well

plates) were purchased from Corning® Life Science (NY, USA). Trypan blue stain and cell count plates were purchased from Invitrogen (Eugene, USA) and the MColorpHast™ non-bleeding pH-indicator strips from (Merck Millipore, Germany). XtremeGENE HP® and 9 DNA transfecting reagents were obtained from Roche Diagnostics (Mannheim, Germany). Phosphate buffered saline (PBS), trypsin-EDTA, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Oxoid limited (Hampshire, England). Biochrom (Berlin, Germany) supplied the sodium pyruvate, 100 mM, and HEPES, 1 M. Nucleobond® Maxiprep DNA isolation kits were purchased from Macherey-Nagel (Duren, Germany). The Kinetex PFP column was purchased from Phenomenex Incorporated (Torrence CA). The Promega Luciferase Assay System and Promega Wizard® Plus Midipreps DNA purification system (Madison, USA) was purchased from Anatech and the Bradford protein assay kit and all other chemicals used were purchased from reliable scientific supply houses.

3.2.2   Plasmid constructs and preparation

The ovine pcDNA3.1-CYP17A1 was a gift from Prof. P. Swart (Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa). The CYP11B plasmid constructs (human pCMV-CYP11B1 and pCMV-CYP11B2) were obtained from Prof. W.L. Miller (School of Medicine, University of California, San Francisco, USA). The human pTrc99A-ADX plasmid was obtained from Prof. R.C. Tuckey (School of Chemistry and Biochemistry, University of Western Australia, Crawley, Australia). The human pCMV7-SRD5A1 and pCMV7-SRD5A2 plasmid constructs were obtained from Prof. D.W. Russell (Southwestern Medical School, University of Texas, Dallas, USA) and Prof. T.M. Penning (Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA) supplied the human pcDNA3-AKR1C2 plasmid construct. For the luciferase reporter assays, the response element plasmid (pTAT-GRE-E1b-luc) was obtained from Prof. G. Jenster (Erasmus University of Rotterdam, Netherlands). The plasmids expressing the human PR isoforms (PR-A; pSG5hPR-A and PR-B; pSG5hPR-B) and human wild-type androgen receptor (pSG5/hAR) was obtained from Dr. E. Kalkhoven (University Medical Center Utrecht, Utrecht, Netherlands) and Dr. H Klocker (Innsbruck Medical University, Innsbruck, Austria, EU), respectively. The pCI-neo plasmid was available in the laboratory and was transiently transfected in HEK-293 cells in parallel with the enzymatic conversion assays as a negative control.

Briefly, Luria-Bertani (LB) medium containing ampicillin (100 µg/mL) was inoculated with plasmid cDNA transformed in *E. coli* cells (0.001%; $^v/_v$) and incubated at 37°C for 12-16 hrs, shaking at 220 rpm (Innova shaking incubator, New Brunswick). The plasmid DNA was isolated and purified from either a 100 mL culture using the Wizard Plus Midipreps DNA purification system (Promega) or from a 300 mL culture using the Nucleobond® Xtra Maxiprep DNA isolation kit (Machery-Nagal) as per instructions stipulated by the manufacturer.

3.2.3   Accurate mass determination and steroid analysis

Although most steroids were commercially available, some steroids used in this project were unavailable and thus needed to be prepared. In order to identify and characterise these compounds, the steroids to be extracted were required to be at relatively high concentrations in the medium. For preparative purposes therefore, 10 µM substrate was incubated with HEK-293 cells transfected with the relevant enzymes to ensure sufficient product yield. The accurate molecular mass and product ions were determined as previously described (Storbeck *et al.*, 2013) on a Waters Synapt G2 quadrupole time-of-flight (Q-TOF) mass spectrometer (Milford, USA) using positive ionisation (ESI+). The molecular and product ion values were subsequently used to analyse and quantify the steroids using internal standards by UHPLC-MS/MS on a ACQUITY UHPLC (Waters, Milford, USA) using a Phenomenex UHPLC Kinetex PFP column (2.1 mm x 100 mm 2.6 µm) (Torrance, CA, USA) and a Xevo triple quadrupole mass spectrometer (Waters, Milford, USA); analysed in multiple reaction monitoring (MRM) mode using an electrospray probe in the positive ionization (ESI+) mode. The mobile phases consisted of analytical grade deionised water containing (1%) formic acid (A) and 49%: 49%: 2% methanol: acetonitrile: isopropanol (B) and the gradient was set up according to the steroids of interest. An injection volume of 5 µL was used per run time.

Steroid metabolism in LNCaP cells was analysed using ACQUITY UPC^2-MS/MS (Waters, Milford, USA) and the steroids separated using a ACQUITY UPC^2 BEH column (3 mm x 100 mm, 1.7 µm). An injection volume of 2 µL was used per run time with a flow rate of 2 mL/min. CO_2 and methanol was used as the mobile phase. The settings and gradients used are set out in chapter 5. Masslynx Software 4.1 assisted in data collection.

3.2.4   Steroid conversion assays

3.2.4.1   The conversion assay of P4 by CYP11B1 and CYP11B2

HEK-293 cells were cultured to confluency (80%) at 37°C, 90% relative humidity and 5% CO_2 in DMEM, supplemented with 0.0015% NaHCO$_3$ (^{m}/$_v$) (pH 7.0), FBS (10%; ^{v}/$_v$) and antibiotics (1%) consisting of penicillin-streptomycin (10 000 U/mL and 10 mg/mL, respectively). The cells were cultured and replated to a minimum of 3, maximum of 6 passages prior to experimental use. Time course assays were carried out to investigate the metabolism of P4 by CYP11B1 and CYP11B2. DOC, deoxycortisol, A4, and T were included as control reactions. HEK-293 cells were replated in Corning® CellBIND® 100 mm dishes at a cell concentration of 2×10^5 live cell/mL and incubated for 24 hrs or until confluency of 50% was reached. Cells were transiently co-transfected with human adrenodoxin (ADX) and CYP11B1 or CYP11B2 using the protocol set out for XtremeGene HP® DNA transfection reagent. Following the 48 hrs transfection period, the media was replaced with 13.5 mL fresh DMEM media supplemented with 1 µM steroid under investigation. For the duration of 20 hrs, 500 µL aliquots were taken at 30 min, 1, 2, 4, 6, 8, 12, 16 and 20 hrs. A liquid-liquid extraction method using a 3:1, MTBE:media, ratio was used to extract the steroids. Prior to extraction, 15 ng of each deuterated internal standard (D2-T, D4-cortisol, D7-A4, D7-11OHA4, D9-P4 and D8-17OHP4) was added to each sample. The samples were vortexed

for 15 mins, frozen at -80°C for 15-20 min and the organic phase removed and dried under nitrogen gas (N_2). The steroids were resuspended in 150 µL HPLC-grade methanol (50%) and analysed using UHPLC-MS/MS as described under section 3.2.3.

3.2.4.2   The conversion assay of 11OHP4 and 11KP4 by SRD5A1 and SRD5A2

The 5α-reduction of 11OHP4 by SRD5A1 and SRD5A2 was subsequently investigated in an end-point conversion assay. HEK-293 cells were cultured as previously described and re-plated (0.5 mL/well) in Corning® CellBIND® 24 well plates (1.9 cm^2) at a cell density of 2 x 10^5 live cell/mL and incubated for 24 hrs. The cells were then transfected with the human SRD5A1 and SRD5A2 plasmids, respectively, and incubated for 48 hrs. For the initial experiment, the transfected cells were incubated with 10 µM 11OHP4 in order to obtain sufficient product (5α-pregnan-11β-ol,3,20-dione (11OH-DHP4)) yield for steroid identification using Q-TOF MS analysis due to the steroid being commercially unavailable. After the identification of 11OH-DHP4, a similar study was conducted investigating the conversion of 1 µM 11OHP4 after 1.5 and 3 hrs. The metabolism of T to DHT by SRD5A1 and SRD5A2 was assayed as positive controls. Having established full conversion, a time course assay was carried out as described above. HEK-293 cells replated in 100 mm dishes were incubated for 24 hrs, prior to transfection with the respective SRD5A isozymes. After 48 hrs, cells were incubated with fresh medium supplemented with 11OHP4, 5 µM, after which a 500 µL aliquot was sampled at 15, 30 min, 1, 2, and 3 hrs. D9-P4 and D8-17OHP4 was added as internal standards prior to steroid extraction as previously described and steroids analysed using UHPLC-MS/MS.

The metabolism of 11KP4 to 11K-DHP4 by SRD5A1 and SRD5A2 was subsequently studied. As commercial 11K-DHP4 was not yet available at this stage of the project, the steroid product was determined as in the case of 11OH-DHP4, starting with the metabolism of 10 µM substrate for 12 hrs in a 24 well plate. The steroids were extracted and analysed. Q-TOF MS analysis identified 11K-DHP4 together with an unknown additional steroid compound equal to the *m/z* of 11K-DHP4 plus 2H$^+$. The molecular and product ions were used in UHPLC-MS/MS analysis and the substrate was quantified. In addition, the ion signal (the area below the peak) detected by UHPLC-MS/MS was used to compare the signals prior to substrate conversion at the time point zero (T_0) (substrate only) and at 12 hrs (substrate, 11KP4, and the two products, 11K-DHP4 and unknown [11K-DHP4 + 2H$^+$]). Once analysed, a time course assay for the metabolism of 11KP4, 5 µM, to 11K-DHP4 was carried out as described for 11OHP4. It should be noted that once 11K-DHP4 became available commercially the quantification of both 11KP4 and 11K-DHP4, without the aforementioned laborious analyses, could be carried out. Subsequently aliquots were taken, internal standards added and the steroids extracted and analysed as described previously (3.2.3).

3.2.4.3   The conversion assay of 11OH-DHP4 and 11K-DHP4 by AKR1C2

Following the investigation into the 5α-reduction of 11OHP4 and 11KP4, the metabolism of 11OH-DHP4 to 3α,11β-dihydroxy-5α-pregnan-20-one (3,11diOH-DHP4) by AKR1C2 was studied. Due to the

unavailability of both 11OH-DHP4 and the novel 3,11diOH-DHP4, a carry-over experiment was conducted to investigate the catalytic activity of AKR1C2 towards 11OH-DHP4. HEK-293 cells were replated in Corning® CellBIND® 12 well- (1 mL/well; 3.8 cm^2) and 24 well plates (0.5 mL/well) at a density of 1 x 10^5 live cells/mL and 2 x 10^5 live cells/mL, respectively, and allowed to grow for 24 hrs. Cells replated in the 12 well plates were transfected as previously described with the human SRD5A1 and SRD5A2 plasmids for 48 hrs, the 24 well plate with the human AKR1C2 plasmid for 60 hrs. After the 48 hrs, the medium of the SRD5A transfected cells was replaced with fresh medium containing 11OHP4, 10 µM. A T$_0$ sample was collected in order to evaluate full conversion of 11OHP4 to 11OH-DHP4. After 12 hrs incubation, a 500 µL aliquot was taken from each well as a T$_0$ control sample (in order to evaluate conversion of 11OH-DHP4 to 3,11diOH-DHP4) and the remaining 500 µL media containing the synthesized 11OH-DHP4 was carried-over to the AKR1C2 transfected cells in the 24 well plate. AKR1C2 expressing cells were incubated for 24 hrs with the steroid containing media after which the final aliquot, 500 µL, was taken and a single sample used for Q-TOF MS analysis. Upon identifying 3,11diOH-DHP4, internal standards (D9-P4 and D8-17OHP4) were added and the steroids extracted, separated and analysed using UHPLC-MS/MS. Data was presented as the ionisation signal of the respective substrate (11OHP4) and products (11OH-DHP4 and 3,11diOH-DHP4) and the decrease in 11OHP4 levels quantified.

Investigation into the reduction of 11K-DHP4 to alfaxalone was carried out using a single time point conversion assay. HEK-293 cells were replated in a 24 well plate seeded at 1 x 10^5 live cells/mL and incubated for 24 hrs. The cells were then transiently transfected with AKR1C2 for 72 hrs, followed by incubating the cells in medium supplemented with 11K-DHP4, 10 µM, for 24 hrs. Aliquots were extracted as previously described. Alfaxalone was initially unavailable and the data was thus expressed as the decrease in substrate and the ionisation signal of the substrate and product (see chapter 5.2.1). Upon obtaining alfaxalone, the experiment was reanalysed and the biosynthesis of alfaxalone quantified.

3.2.4.4   The conversion assay of alfaxalone by CYP17A1

The conversion of alfaxalone by CYP17A1 was determined in transiently transfected cells. P4 and Pdiol were included as controls as these steroids are readily metabolised by CYP17A1. HEK-293 cells were replated as previously described in a Corning® CellBIND® 24 well plate and transfected with ovine CYP17A1 plasmid for 48 hrs. Steroid substrate, 1 µM, was added in fresh medium and the cells incubated for 24 hrs, followed by the extraction and analysis of the steroids using UHPLC-MS/MS. The biosynthesis of 17OHP4 and A4 from P4, AST from Pdiol and 11KAST from alfaxalone by CYP17A1 was determined. All steroid addition volumes were increased by 10% well volume to compensate for evaporation. pCI-neo transfected HEK-293 cells were used in negative control experiments in parallel with all the assays.

3.2.5   Steroid metabolism assay in LNCaP cells

The LNCaP cells were grown at 37°C, 90% relative humidity and 5% CO_2. The culture media used was RPMI-1640 (pH 7) supplemented with $NaHCO_3$ (0.0015%; $^m/_v$), D-(+)-Glucose (0.0025%), penicillin-streptomycin (1%), HEPES (1%), sodium pyruvate (1%) and FBS (10%). The cells were cultured and replated in Corning® CellBIND® T75 flasks until the third passage prior to experimental use, after which the cells were replated in Corning® CellBIND® 12 well plates (2×10^5 live cells/mL; 1 mL/well) and incubated for 48 hrs. The media was replaced with fresh RPMI-1640 media (1000 µL) containing the steroid substrates, 1 µM, (11OHA4, 11OHP4 and 11KP4. After 48 hrs, duplicate aliquots were taken of which one set was treated with β-glucuronidase (as described in chapter 3.2.5.1) prior to extraction. Internal standards, 15 ng each, were added prior to extraction and included D2-T, D7-A4, D7-11OHA4, D9-P4 and D8-17OHP4. Liquid-liquid extraction with MTBE at a volume ratio of 3:1 was carried out. The samples were vortexed for 15 min, frozen at -80°C for 15-20 mins and the organic phase removed and dried at 40°C under N_2. Steroid residues were resuspended in 150 µL UPLC-grade methanol (50% analytical grade deionised water) and analysed using UPC^2-MS/MS as described in section 3.2.3.

3.2.5.1   The deconjugation of glucuronidated steroid metabolites

Due to the presence of UGT enzymes in LNCaP cells, steroids may be conjugated and thus not extracted in the organic phase during the preparation of samples for analyses by UPC^2-MS/MS. Thus, to determine total steroid production, the steroids were deglucuronidated in order to assay all the steroids produced (total steroid = conjugated and unconjugated metabolites). Therefore experimental aliquots (500 µL) were collected in duplicate of which one set was extracted with MTBE without prior treatment, representing free, unconjugated steroids, while the other samples were subjected to β-glucuronidase digestion prior to extraction, representing total steroid production. The pH of collected aliquots for deglucuronidation was decreased to 6.5 using ±20 µL acetic acid (1%) per sample. A control was added in which the deconjugation of DHT-G was assayed (section 5.5). The samples were treated with 400 units of β-glucuronidase (76 mL; *E. coli* Type VII-A) and incubated at 37°C for 24 hrs, prior to the addition of internal standard and liquid-liquid extraction as previously described (3.2.5).

3.2.6   Luciferase reporter assay

HEK-293 cells were cultured in phenol red DMEM supplemented with 10% FBS, 1% antibiotics and 1.5 g $NaHCO_3$/L (pH 7) as described previously in chapter 3.2.4. The cells were plated in 100 mm culture dishes at 2×10^6 live cells/mL and incubated for 48 hrs. The cells were transiently transfected with 9 µg luciferase reporter plasmid construct (pTAT-GRE-E1b-luc) and 0.9 µg of the respective receptor plasmid construct (pSG5hPR-A; pSG5hPR-B; pSVARo-hAR) using XtremeGene 9® DNA transfection reagent according to the manufactural protocol. Following the 24 hrs transfection period, cells were replated in 96 well culture plates (0.2 mL/well) at a cell density of 1×10^4 live cells/mL in phenol red-free DMEM supplemented with 10% FBS and 1% antibiotics and incubated for 72 hrs. The cells were subsequently treated for 24 hrs with a vehicle control (ethanol, 0.1%), positive control (R5020 and P4

for PR or Mib and DHT for AR) and steroids (DHP4, 11OHP4, 11KP4, 11K-DHP4 or 11OH-DHP4) at 1 nM. 11OH-DHP4 was prepared as described in chapter 3.2.6.1. The medium used for steroid treatment was phenol red free growth medium supplemented with 4 mM L-glutamine and 25 mM glucose, stripped of FBS without antibiotics. After the 24 hrs, the cells were washed with PBS (10 mM, pH 7.4), lysed using 25 µL passive lysis buffer and stored at -20°C until conducting the luciferase assay. The luciferase activity in the lysate was measured using a Luciferase Assay System and Veritas microplate luminometer. The data was normalised to protein which was determined using the Bradford protein assay method. The luciferase activity (relative light units) was determined and set relative to ethanol set as 1. The data was then expressed as a percentage of the luciferase activity fold normalised to protein levels with the fold induction values relative to the synthetic agonist controls R5020 or Mib set as 100%.

3.2.6.1   Preparation of 11OH-DHP4

Investigation into the metabolism of commercially unobtainable steroids required the *in vitro* production of the steroid metabolites. The steroids were prepared as adapted from the method described in (Van Rooyen et al., 2017). Briefly, HEK-293 cells were cultured and transiently transfected with the human SRD5A1 encoding plasmid in Corning® CellBIND® 100 mm dishes as described (chapter 3.2.4.2). Following the transfection period, 11OHP4, 10 µM, was made in unsupplemented phenol red-free DMEM. Prior to substrate addition, the media was removed from the transfected cells, and cells washed with 2 x 500 µL phenol red-free and steroid-free DMEM media. Following the wash, the 11OHP4 containing media was added to the cells and incubated for 12 hrs (maximum conversion). The media containing the newly synthesised 11OH-DHP4 was sampled and subsequently used in luciferase reporter assays.

3.2.7   Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc, CA, USA) was used to determine the statistical relevance of the data by either a one-way ANOVA followed by Dunnett's multiple test or an unpaired t-test and expressed as means ±SEM. Statistical significance was measured as $P \leq 0.05$ (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). One-way ANOVA followed by a Newman-Keuls post-test was used in the analyses of the luciferase reporter assay data. The letters a, b and c were used to indicate statistical significance. Values which differed significantly were assigned a different letter.

3.3   Results

Currently interest in the C11-oxygenated steroids is increasing due to their association with clinical conditions linked to adrenal disorders and disease progression. While limited studies into the biosynthesis and metabolism of C11-C_{19} steroids have been conducted, the metabolism of C11-C_{21} steroids and their physiological significance remain unclear.

3.3.1   Identification and characterisation of 11OHP4 and 11KP4 metabolites

The metabolism of 11OHP4 and 11KP4 by peripheral enzymes, SRD5A1/2, AKR1C2 and CYP17A1 yielded steroid products of which identities were confirmed by accurate mass determination. For each metabolite, a theoretical protonated mass [M+H]$^+$ was calculated which aided in identifying the metabolites on the Q-TOF mass spectrometer in ESI+ mode. The accurate molecular mass of the compound and its fragmentations are shown in table 3.1 (Fig. 3.3) and were subsequently used to analyse steroid metabolites in conversion assays after UHPLC-MS/MS.

Table 3.1

Accurate mass determination of 11OHP4 and 11KP4 metabolites.

Steroid	Theoretical mass [M+H]$^+$	Observed accurate mass [M+H]$^+$	Chemical formula	Product ion [M+H]$^+$
Alfaxalone	333.2430	333.2421	$C_{21}H_{32}O_4$	159; 147; 85
11OH-DHP4	333.2430	333.2433	$C_{21}H_{32}O_3$	173; 84.8
11K-DHP4	331.2273	331.2278	$C_{21}H_{30}O_3$	147; 105
3,11diOH-DHP4	335.2686	335.2589	$C_{21}H_{34}O_3$	241.2; 159; 119.2
Unknown [≈ 11K-DHP4 +2H$^+$]	-	333.2439	-	297.1; 84.8
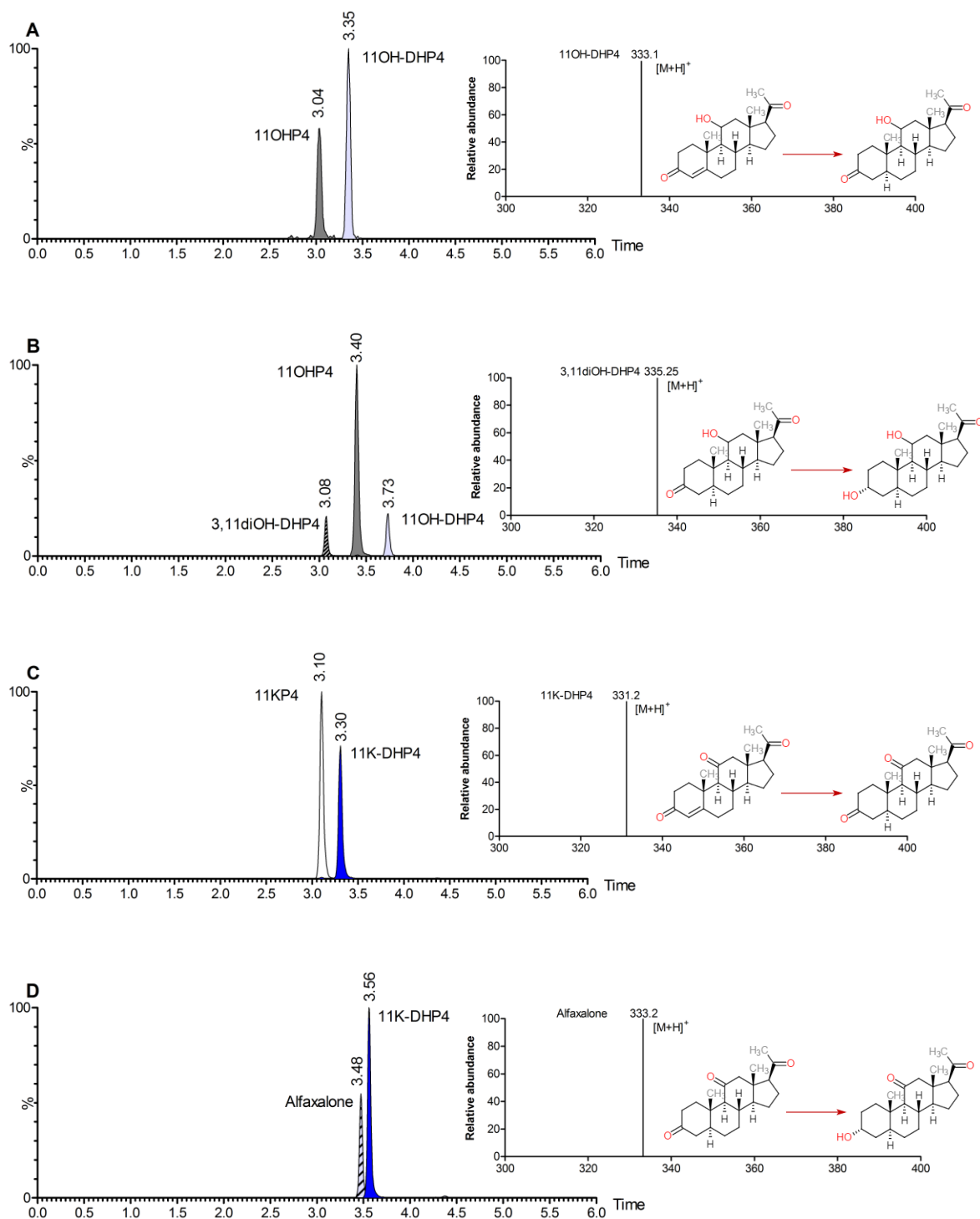
Figure 3.3 UHPLC-MS/MS chromatographic separation of 11OHP4 and 11KP4 metabolites. Insert: MS spectra of novel steroids [M+H]+ and chemical structures. (A) 11OHP4 and 11OH-DHP4 with 11OH-DHP4, *m/z* 333.1; (B) 11OHP4, 11OH-DHP4 with 3,11diOH-DHP4, *m/z* 335.25; (C) 11KP4 with 11K-DHP4, *m/z* 331.2; and (D) 11K4, 11K-DHP4 with alfaxalone, *m/z* 333.2. Retention times are shown representing steroid compounds of a single injection shown in MRM mode. The steroids are indicated by relative abundance (%).

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3.3.2 The 11β-hydroxylation of P4 by CYP11B1 and CYP11B2

11OHP4 and 11KP4 have been identified in patients diagnosed with PCOS and PCa serum (T du Toit, personal communication), therefore it was determined whether CYP11B1 and CYP11B2 would catalyse the conversion of P4 to 11OHP4. Steroid conversion assays were carried out to determine the metabolism of five steroid hormones (P4, DOC, deoxycortisol, A4 and T; 1 µM) over 20 hrs by the human CYP11B1 (Fig. 3.4) and CYP11B2 (Fig. 3.5), transiently co-transfected with ADX, in HEK-293 cells.

The conversion of P4 to 11OHP4 by both CYP11B1 (Fig. 3.4 A) and CYP11B2 (Fig. 3.5 A) was less efficient than conversions of the natural substrates with 50% conversion being obtained after 20 hrs. The metabolism of DOC and deoxycortisol were assayed as positive controls. Analysis of DOC showed a 50% conversion after 4 and 8 hrs by CYP11B1 (Fig. 3.4 B) and CYP11B2 (Fig. 3.5 B), respectively. After 20 hrs, the substrate was fully converted by both isozymes, with negligible ALDO produced by CYP11B1. Significant production of ALDO was, however, detected in the conversion by CYP11B2 only. Deoxycortisol was rapidly converted to cortisol by CYP11B1 (Fig. 3.4 C) reaching 50% after 5 hrs with relatively little substrate remaining after 20 hrs. Only 73% deoxycortisol was metabolised by CYP11B2 (Fig. 3.5 C) of which 50% was metabolised after 14 hrs. By 7 hrs 50% conversion of A4 to 11OHA4, by CYP11B1, was achieved (Fig. 3.4 D) whereas the conversion catalysed by CYP11B2 was less efficient with only 20% 11OHA4 converted after 20 hrs (Fig. 3.5 D). CYP11B1 converted 50% of T to 11OHT within the first 9 hrs (Fig. 3.4 E) but only 25% T was converted CYP11B2 to 11OHT (Fig. 3.5 E).

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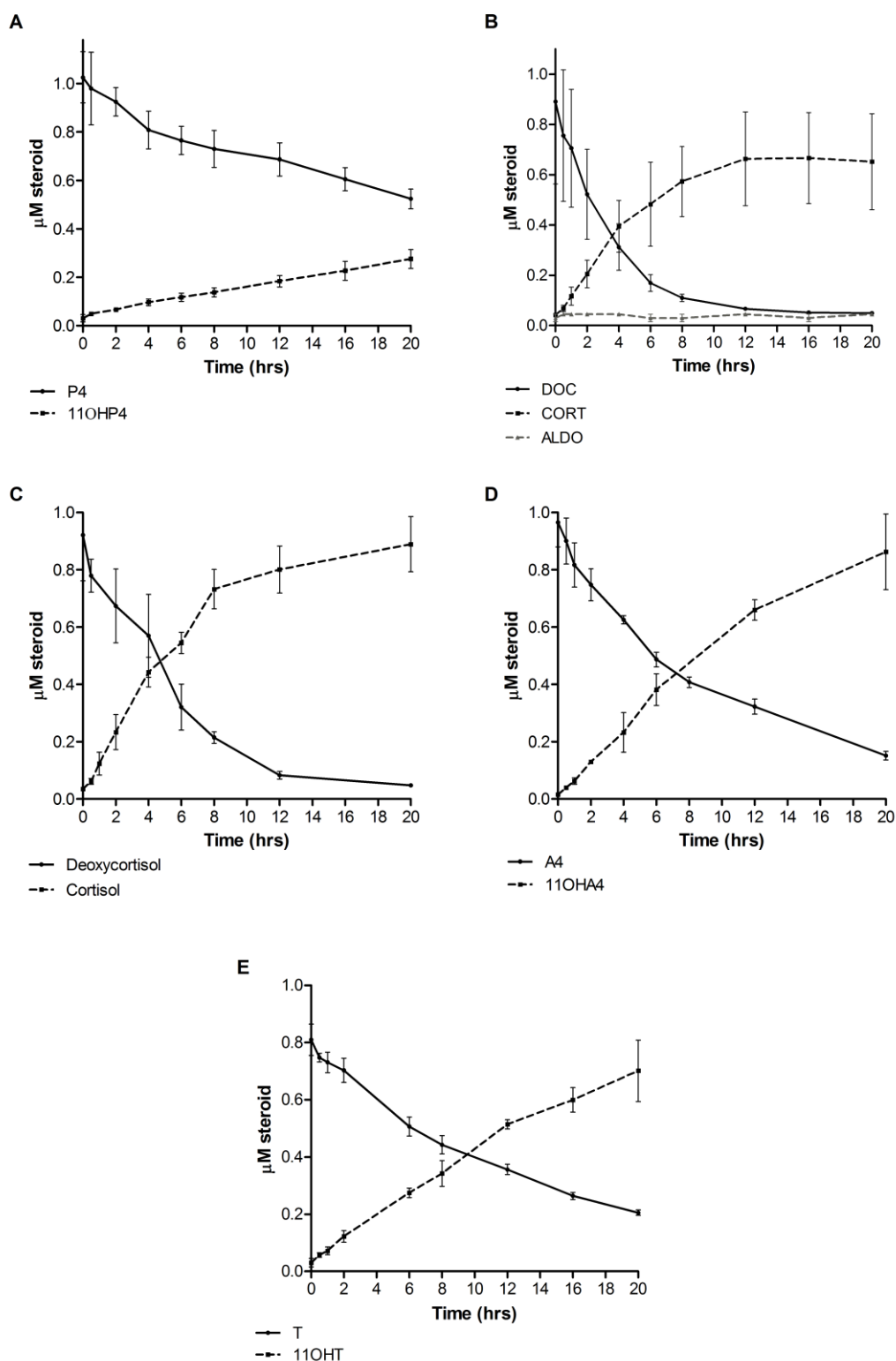
Figure 3.4 Conversion of 1 µM steroid substrate by human CYP11B1. HEK-293 cells were co-transfected with CYP11B1 and ADX and substrate conversion assayed: (A) P4, (B) DOC, (C) deoxycortisol, (D) A4, and (E) T. Results are expressed as the mean ±SEM of an experiment performed in triplicate.

44
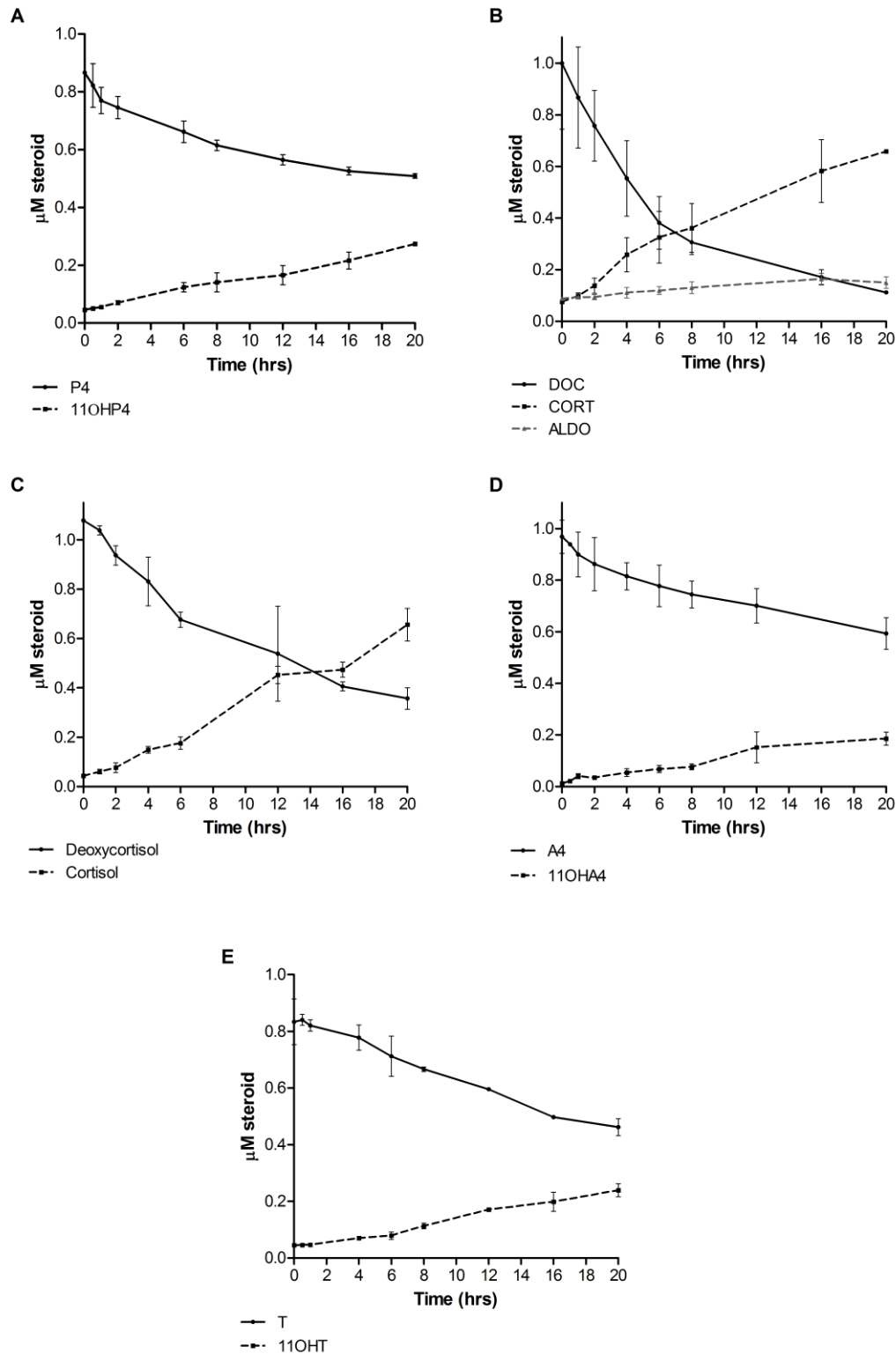
Figure 3.5   Conversion of 1 µM steroid substrate by human CYP11B2. HEK-293 cells were co-transfected with CYP11B2 and ADX and the substrate conversion assayed: (A) P4, (B) DOC, (C) deoxycortisol, (D) A4, and (E) T. Results are expressed as the mean ±SEM of an experiment performed in triplicate.

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3.3.3 The 5α-reduction of 11OHP4 by SRD5A1 and SRD5A2

The 5α-reduction of 11OHP4 by SRD5A1 and SRD5A2 was investigated in transiently transfected HEK-293 cells. Enzymatic assays were conducted to determine the catalytic activity of these isozymes towards 11OHP4. Initial studies showed full conversion of 11OHP4 (10 µM) after 12 hrs by both subtypes (Fig. 3.6 insert). The conversion of 11OHP4, 1 µM, to 11OH-DHP4 was subsequently assayed for a shorter period. At 1.5 hrs 77% was converted by SRD5A1 and 80% by SRD5A2 with 0.232 µM and 0.202 µM substrate remaining. After 3 hrs 0.030 µM and 0.020 µM substrate remained in the conversion by SRD5A1 and SRD5A2 respectively (Fig. 3.6).
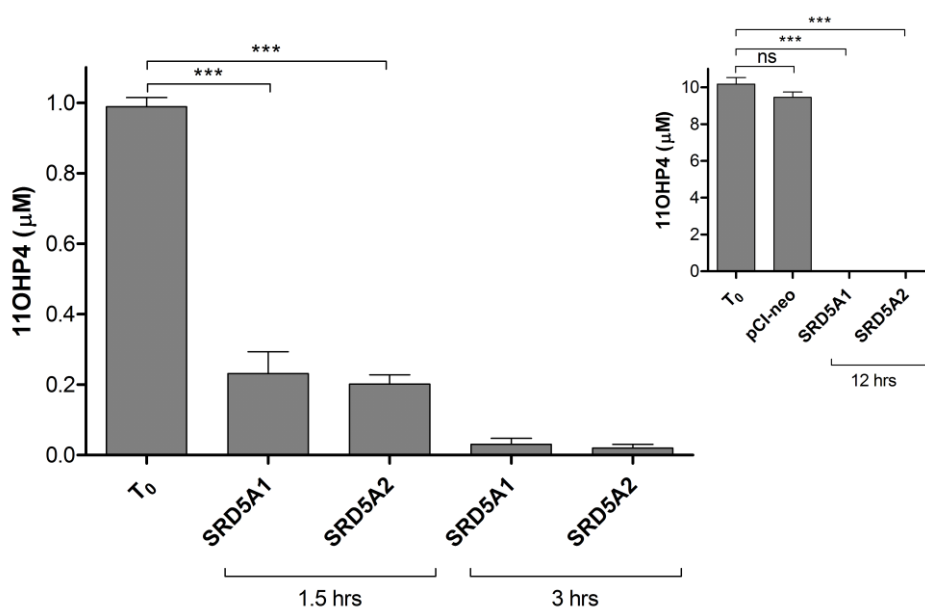
Figure 3.6   Conversion of 1 µM 11OHP4 by SRD5A1 and SRD5A2 expressed in HEK-293 cells. Insert: negative control and complete conversion of 10 µM 11OHP4 after 12 hrs. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM of an experiment performed in triplicate (***$P < 0.001$).

Although full conversion of 11OHP4 by both isozymes was observed at 3 hrs, additional assays showed a 3-fold higher initial conversion rate of 11OHP4 to 11OH-DHP4 by SRD5A1 when compared to SRD5A2. The reduction of 5 µM 11OHP4 reached 50% conversion after 15 min in the case of SRD5A1 and after 45 min in the case of SRD5A2 (Fig. 3.7). No conversion of 11OHP4 in pCI-neo transfected HEK-293 cells was detected in the control experiments carried out in parallel investigating the 5α-reduction of 11OHP4.
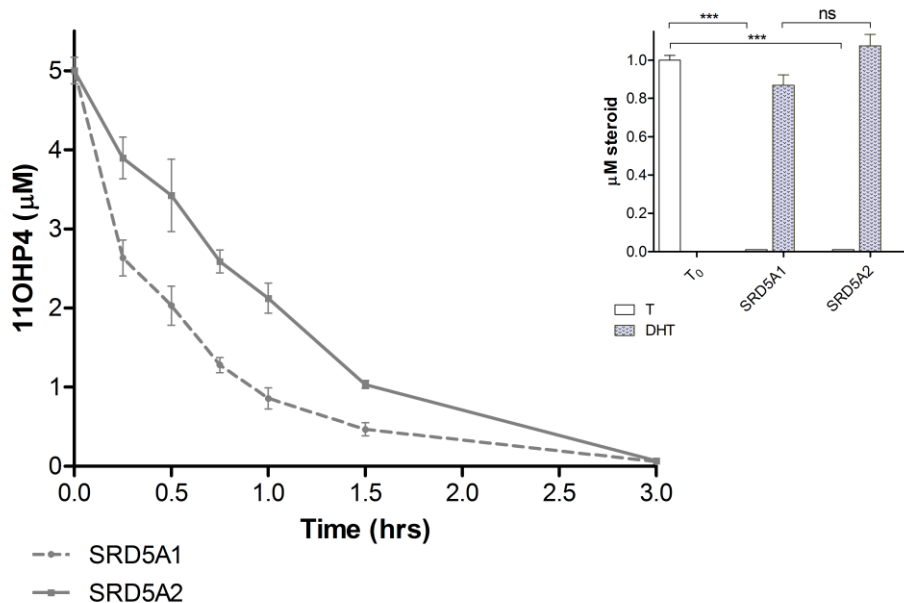
Figure 3.7   Time course of 11OHP4, 5 µM, conversion to 11OH-DHP4 by human SRD5A1 (dashed line) and SRD5A2 (solid line) expressed in transiently transfected HEK-293 cells. Insert: conversion of T to DHT by SRD5A1 and 2 (positive control). Results are expressed as the mean ±SEM of three independent experiments performed in triplicate (n = 3).

3.3.4   The 5α-reduction of 11KP4 by SRD5A1 and SRD5A2

The metabolism of 11KP4 by SRD5A1 and SRD5A2 was also investigated. As is the case of 11OHP4 in chapter 3.3.3, initial experiments showed full conversion of 11KP4 (10 µM) after 12 hrs (Fig. 3.8) as indicated by the ionisation signal of 11KP4 and 11K-DHP4 detected in the aliquots taken from the assays catalysed by SRD5A1 and SRD5A2. In addition, a second metabolite was identified with a *m/z* of 333.24. Together with the determined product ions, the unknown metabolite was detected and depicted as the ion signal/area below the peak. This compound was not quantifiable due to its identity being unknown and the lack of standards with which to reference the compound.

11K-DHP4, which was initially unavailable was subsequently used to quantify this compound in experimental assays. Time course analysis of the conversion of 11KP4 to 11K-DHP4 (Fig. 3.9) by SRD5A1 and SRD5A2 showed full conversion of 11KP4 after 3 hrs by both isozymes. The initial conversion rate of SRD5A1 was 1.6-fold faster, having metabolised ±50% of the substrate after 30 min whereas SRD5A2 reached 50% after 50 min. Upon completion of the assay (after 3 hrs) the levels of 11K-DHP4 had not reached the initial substrate concentration of 11KP4, supporting the formation of another metabolite, possibly catalysed by an endogenous enzyme. Analysis of the ionisation with respect to 11KP4 and 11K-DHP4 showed the presence of 11K-DHP4 as well as the unknown steroid metabolite with the *m/z* possibly that of 11K-DHP4 + 2H⁺.
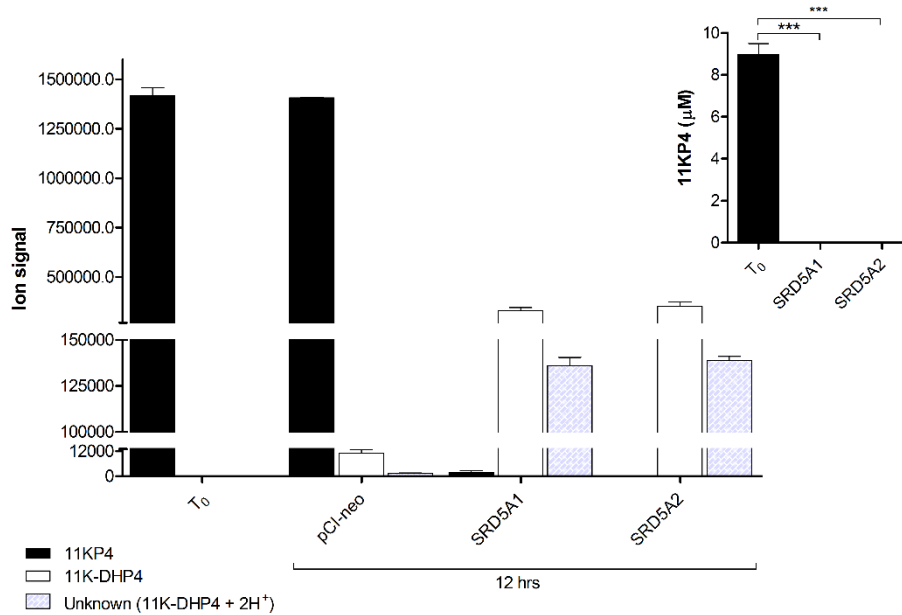
Figure 3.8   Ion signals detected for 11KP4 and its products following conversion by SRD5A1 and SRD5A2 expressed in HEK-293 cells. Insert: 11KP4, 10 µM, complete conversion after 12 hrs. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. The experiment was performed in triplicate and expressed as the mean ±SEM (***$P < 0.0001$).
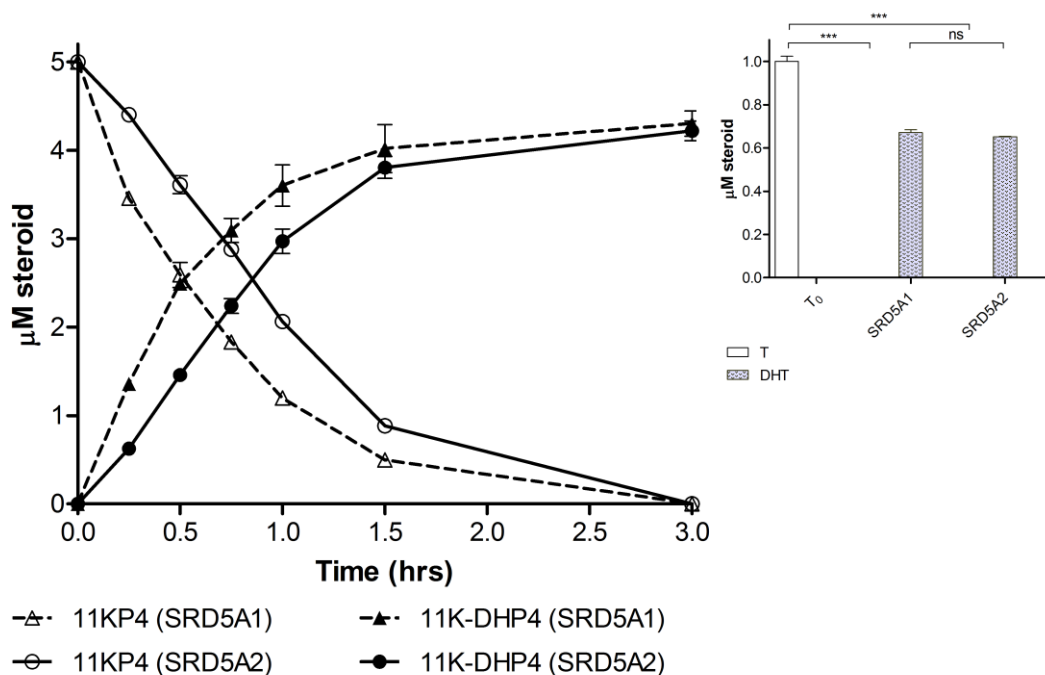
Figure 3.9   Time course of 11KP4, 5 µM, metabolism by human SRD5A1 (dashed lines) and SRD5A2 (solid lines) in HEK-293 cells. Insert: conversion of T to DHT by SRD5A1 and 2 (positive control). Results are expressed as the mean ±SEM of three independent experiments performed in triplicate (n = 3).

An endogenous conversion assay in HEK-293 cells was subsequently carried out investigating the conversion of 11K-DHP4, Pdione and Pdiol in an attempt to identify the reductase activity. 11OHP4 was not included since the unknown metabolite (11K-DHP4 + 2H$^+$) was not detected in the conversion assay (Fig. 3.6 and 3.7) which suggests that 11K-DHP4 is the substrate of choice, with the C11-OH moiety of 11OH-DHP4 possibly hampering conversion by the unknown enzyme. Possible reduction of the keto group are at either C3, C11 or C20 of the 11K-DHP4 molecule. In an attempt to determine the position at which the reduction took place, untransfected HEK-293 cells were incubated for 24 hrs with three steroids (1 µM) which were selected based on the keto moieties – 11K-DHP4 (keto group at C3, C11 and C20) > Pdione (keto group at C3 and C20) > Pdiol (keto group at C20). The samples were extracted and quantified as previously described in section 3.2.3. Analyses showed the conversion of 11K-DHP4 to alfaxalone and of Pdione to Pdiol. These steroids both contain keto groups at C3 and C20 which suggest the presence of an enzyme able to reduce one of these keto groups (Fig. 3.10 A and B). Incubation of Pdiol, which has a ketone at C20 only, yielded no product, indicating that the C3 keto moiety is reduced (Fig. 3.10 C). Although this was a preliminary investigation the data suggest that an enzyme, with catalytic activity similar to that of AKR1C2 or reductive 17βHSD, is endogenously expressed in HEK-293 cells.
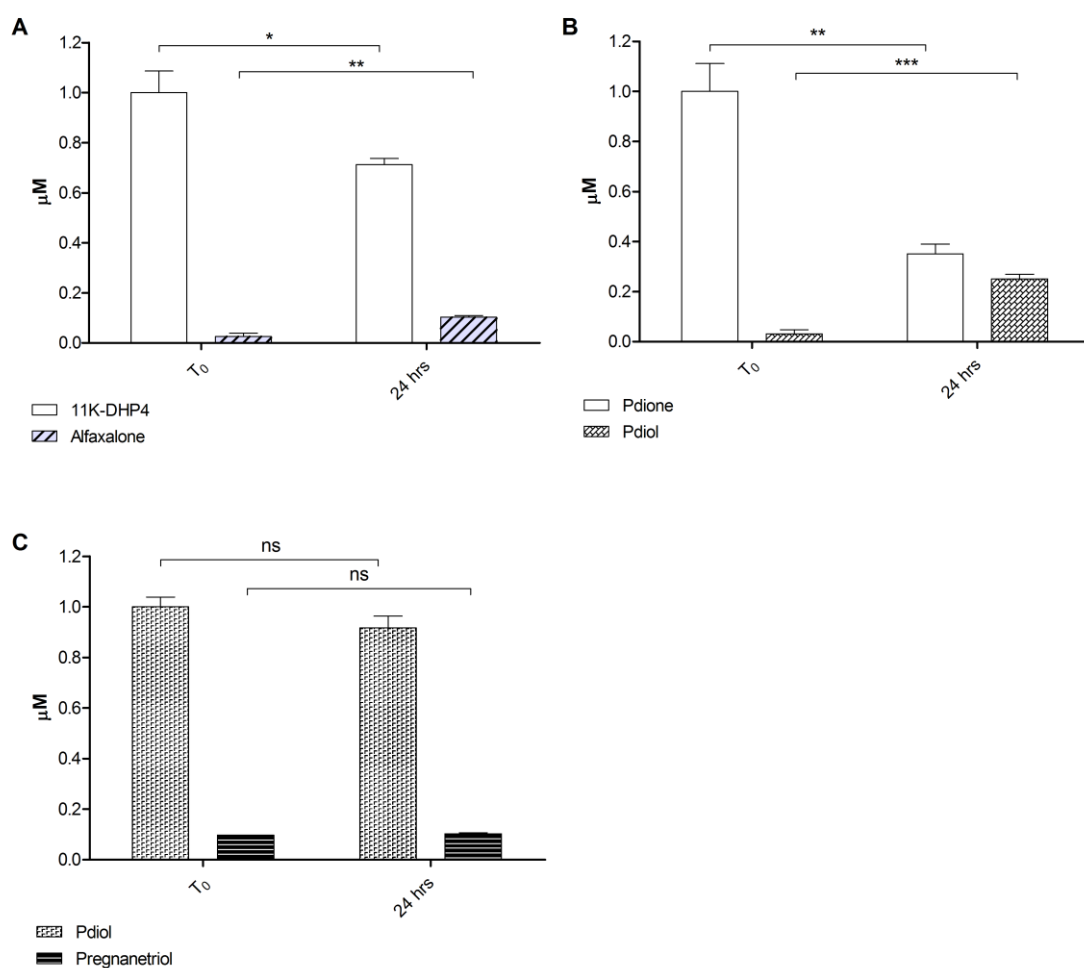
Figure 3.10   Endogenous conversion of 1 µM 11K-DHP4, Pdione and Pdiol in HEK-293 cells.

3.3.5   *The C3-reduction 11OH-DHP4 by AKR1C2.*

Having established the 5α-reduction of 11OHP4 by SRD5A, it was considered that 11OH-DHP4 may present as substrate for AKR1C2 in the formation of 3,11diOH-DHP4. This HSD enzyme catalyses the reduction of the keto group at C3 to a hydroxyl group and plays a crucial role in, for example, the inactivation of DHT. The conversion by AKR1C2 was investigated in order to further assess the potential contribution of 11OHP4 to the production of androgens via the backdoor pathway.

11OH-DHP4 was prepared as previously described (chapter 3.2.4) and the media incubated with transiently transfected AKR1C2 HEK-293 cells for 24 hrs. The media was analysed for the 3α,5α-reduced 11OHP4 product and the conversion yielded a steroid with the molecular weight equal to alfaxalone. Quantitative analysis of the conversion showed 85-98% conversion of 11OHP4 by the SRD5A isozymes after 12 hrs with no significant reduction in the remaining 11OHP4 levels when the media was incubated with AKR1C2 (Fig. 3.11 A and B), indicating that AKR1C2 does not reduce the steroid unless the C4/5 double bond is reduced. Analysis of the ion signal showed the presence of both 11OHP4 and 11OH-DHP4 (Fig. 3.11 C) after the 12 hr incubation period – showing incomplete conversion of 11OHP4 by the SRD5A isozymes. Upon incubation of the media with AKR1C2, 3,11diOH-DHP4 is evidently biosynthesized as indicated by the ion signal analysis - with 11OHP4 and 11OH-DHP4 still present after the 24 hr incubation period with AKR1C2 (Fig. 3.11 D).
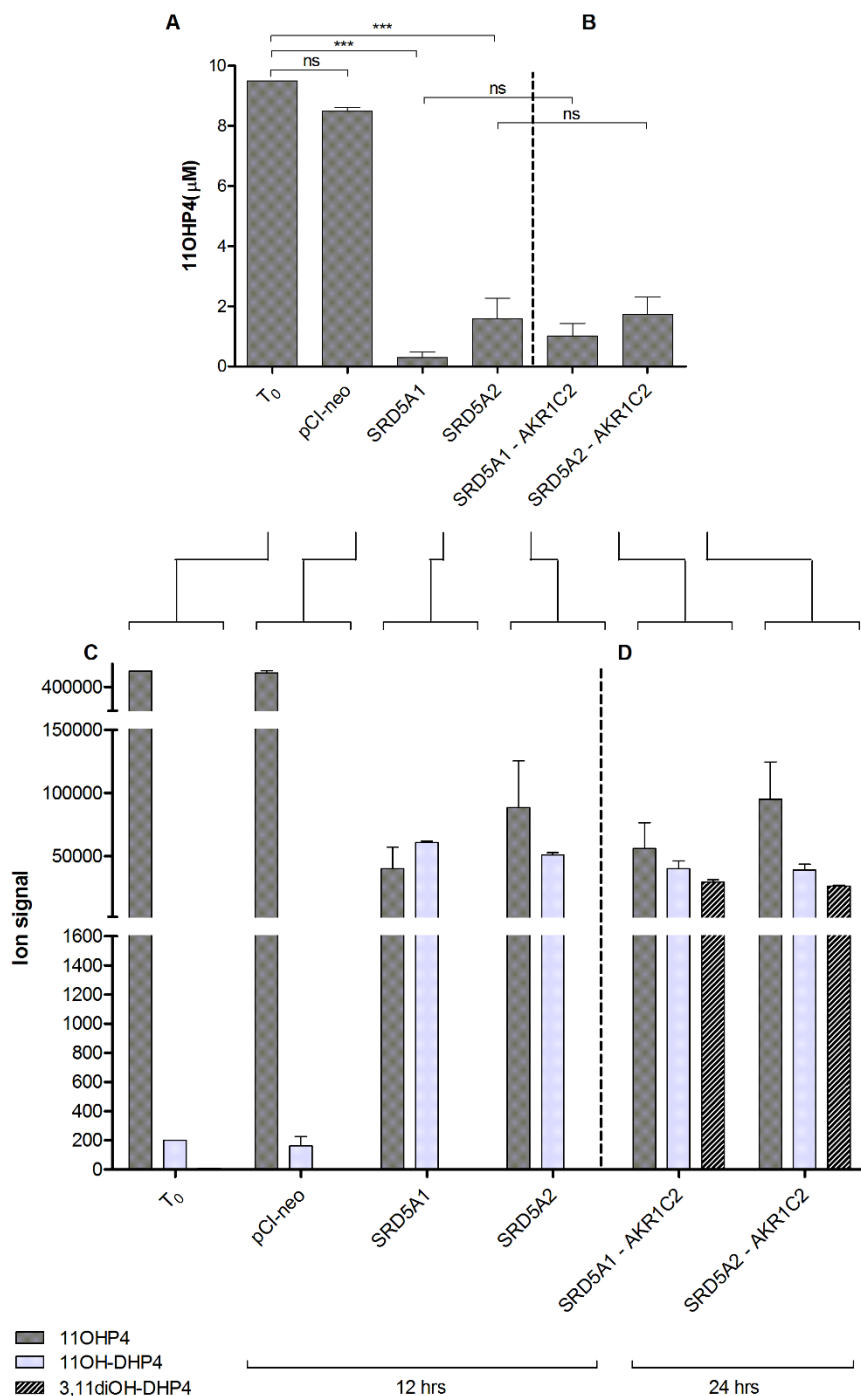
Figure 3.11   Metabolism of 11OHP4 by SRD5A and 11OH-DHP4 by AKR1C2 in transiently transfected HEK-293 cells. (A) 11OHP4, 10 µM, conversion by SRD5A1 and SRD5A2 after 12 hrs; (B) 11OHP4 conversion by AKR1C2 after 24 hrs; (C) Ion signal of 11OHP4 and 11OH-DHP4 in the SRD5A assay; and (D) ion signal representing 11OHP4, 11OH-DHP4 and 3,11diOH-DHP4 in the AKR1C2 assay. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM of an experiment performed in triplicate (***P ≤ 0.001).

3.3.6   The C3-reduction of 11K-DHP4 by AKR1C2.

The metabolism of 11K-DHP4, 10 µM, to alfaxalone by AKR1C2 was assayed in transiently transfected HEK-293 cells. Initial studies showed a significant decrease in 11K-DHP4 levels (Fig. 3.12) after 24 hrs with 4.28 µM substrate remaining. Alfaxalone, 2.95 µM, was detected after 24 hrs incubation of 11K-DHP4 with HEK-293 cells expressing AKR1C2 with negligible levels in the negative pCI-neo control (Fig. 3.12).
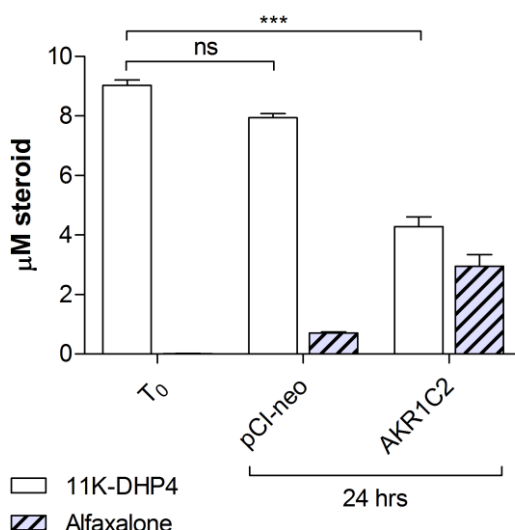
Figure 3.12   11K-DHP4, 10 µM, conversion in transiently transfected HEK-293 cells expressing AKR1C2. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM of an experiment performed in triplicate ($***P < 0.001$).

3.3.7   The metabolism of alfaxalone by CYP17A1

The metabolism of alfaxalone was assayed in HEK-293 cells expressing ovine CYP17A1. Steroid substrate, 1 µM, was incubated with the transfected cells for 12 hrs. The conversion of alfaxalone to 11KAST (Fig. 3.13) was investigated. This dual activity of CYP17A1 entails the hydroxylation at C17 of alfaxalone, followed by the 17,20-lyase reaction, which would yield 11KAST. In the analyses, only alfaxalone and 11KAST were monitored and results indicated the formation of 11KAST, 0.62 µM, with no alfaxalone remaining after 12 hrs. It is possible that 17OH-alfaxalone may have formed accounting for ±0.4 µM.

P4 and Pdiol were included as positive controls to monitor the conversion of P4 to 17OHP4 and A4, and Pdiol to AST. Results showed P4 to be fully converted after 12 hrs, with both 17OHP4 and A4 being produced at 0.51 and 0.11 µM, respectively (Fig. 3.14 A). Analysis of Pdiol samples showed CYP17A1 catalysing the 17,20-lyase reaction forming AST, 0.67 µM (Fig. 3.14 B).
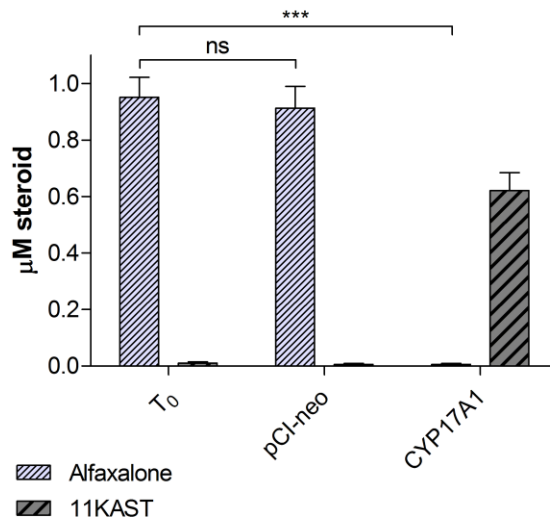
Figure 3.13 Metabolism of alfaxalone by ovine CYP17A1. Alfaxalone conversion was assayed for 12 hrs in HEK-293 cells transiently transfected with CYP17A1. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM of two independent experiment performed in triplicate (***$P < 0.001$, $n = 2$).
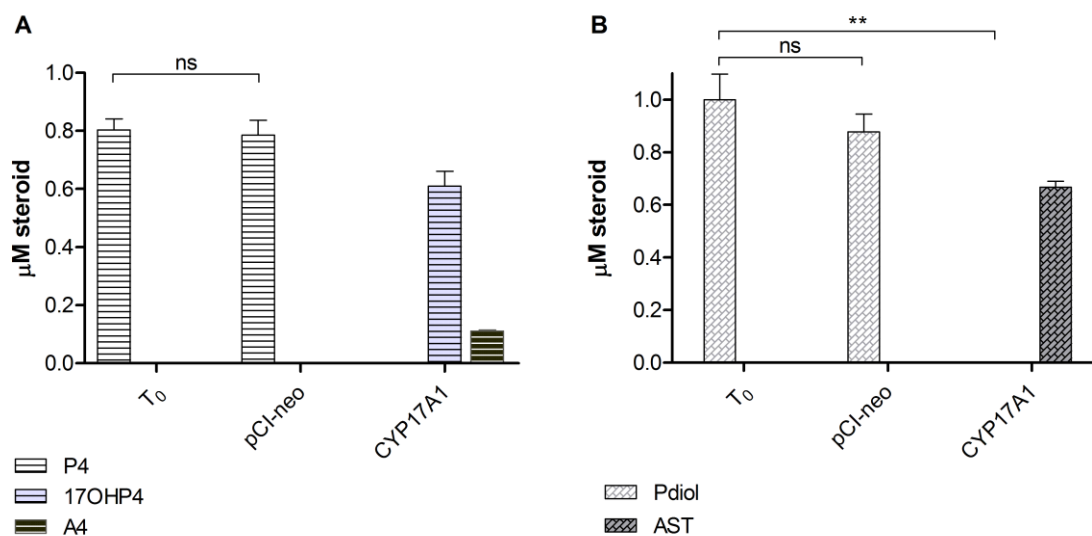
Figure 3.14 Conversion of 1 μM P4 and Pdiol by ovine CYP17A1 expressed in HEK-293 cells after 12 hrs. (A) P4 conversion to 17OHP4 and A4; (B) Pdiol conversion to AST. Statistical analysis was carried our using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ±SEM of two independent experiment performed in triplicate (***$P < 0.001$, $n = 2$).

3.3.8   11OHP4 and 11KP4 metabolism in the LNCaP cell model

The metabolism of 11OHP4 and 11KP4, together with 11OHA4 as a control reaction, was investigated in a preliminary study in LNCaP cells. This cell model endogenously expresses 11βHSD2, SRD5A1 and 17βHSD3 (Ji *et al.*, 2003), but the expression of CYP17A1 is uncertain (Robitaille *et al.*, 2015; Vasaitis *et al.*, 2008). In addition, UGT enzymes are expressed, therefore steroids were deglucuronidated to allow the determination of both the free (unconjugated) and total steroid (conjugated and unconjugated) production.

The metabolism of 11OHP4 and 11KP4 was assayed in LNCaP cells after 48 hrs and analysed using UPC^2-MS/MS. Analyses of steroid profiles are presented in Fig. 3.15 A and B, respectively. The metabolism of both C_{21} steroids was less efficient than that of 11OHA4 shown below. Only 33% of 11OHP4 was metabolised (Fig. 3.15 A) of which alfaxalone was detected as the main steroid produced with levels of 0.048 and 0.045 μM for free and total, respectively, followed by 11KP4 quantified at 0.032 μM (free) and 0.029 μM (total). 11K-DHP4 and 11KDHT were detected at low levels (0.0072 μM), both below the LOQ. The metabolism of 11KP4 was marginally higher, 38%. 11KP4 was mainly converted to 11K-DHP4 and alfaxalone with 11KDHT below the LOQ (Fig. 3.15 B). Free and total concentration of 11K-DHP4, 0.054 and 0.052 μM, were comparable to that of free alfaxalone levels, 0.05 μM, whereas the total alfaxalone levels were higher at 0.073 μM. 11KDHT was detected, but only in the glucuronidated form, below the LOQ. In the control reaction, in which the conversion of 11OHA4 (1 μM) was assayed (Fig. 3.16), negligible levels (±0.01 μM) remained after 48 hrs. The steroid was converted to 11KA4 (±90%) and the glucuronidate form could not be detected. In addition, 11KT was detected, albeit at low levels, both as free and in the conjugated form with 0.048 and 0.106 μM free and total steroid, respectively.
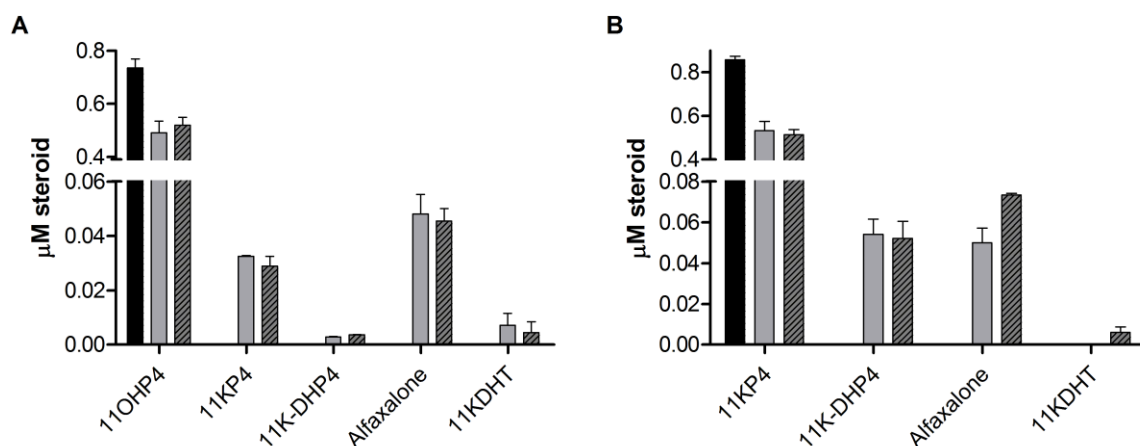
Figure 3.15   Metabolism of 11OHP4 (A) and 11KP4 (B) in LNCaP cells: Steroid substrate, 1 μM, was assayed after 48 hrs and the metabolites profiled using UPC^2-MS/MS analyses. Black bars, time point zero (initial concentration); grey bars, free steroids; and grey patterned bars, total steroids (free + conjugated). The results are expressed as the mean ±SEM of an experiment performed in triplicate.
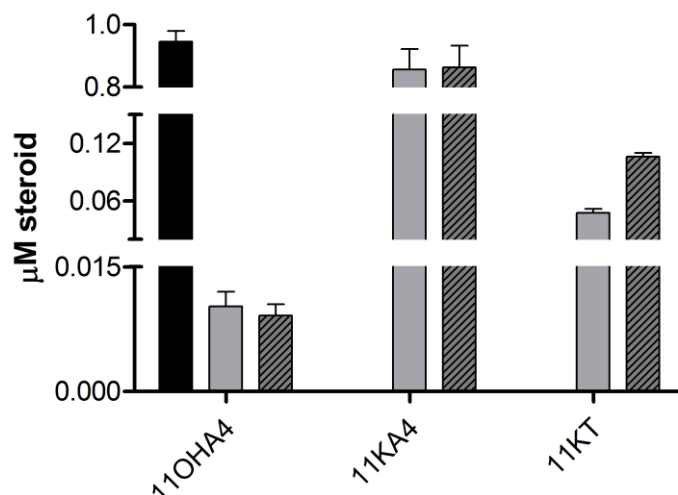
Figure 3.16   Metabolism of 1 µM 11OHA4 in LNCaP cells. Metabolites were analysed after 48 hrs and profiled using UPC^2-MS/MS. Black bars, time point zero (initial concentration); grey bars, free steroids; and grey patterned bars, total steroids (free + conjugated). The results are expressed as the mean ±SEM of an experiment performed in triplicate.

3.3.9   Receptor interaction

The physiological function of steroid metabolites depends on the interaction with steroid receptors and whether the expression of the target genes is affected (induced or inhibited). Earlier studies showed that the MR exhibits affinity for both 11OHP4 and 11KP4 with the affinity for the latter being 17-fold higher (Galigniana *et al.*, 2004). Nevertheless, since 11OHP4 has been detected in tissue associated with clinical conditions in which the AR and/or PR is expressed, such as in TART in 21OHD patients and in PCOS characterized by hyperandrogenism, the interaction with the wild-type AR and the PR-A and PR-B was investigated. A luciferase reporter assay was thus conducted to determine whether DHP4, 11OHP4, 11KP4 and their respective 5α-reduced metabolites possess agonistic activity towards PR-A, PR-B and the AR. Plasmid vectors containing the cDNA encoding human PR-A, PR-B or AR together with the P4 response element (PRE)-containing promoter-reporter construct was transiently transfected in HEK-293 cells. The P4 response element was used in both PR and AR studies due to the high homology shared between the DNA binding domain of steroid receptors allowing numerous receptor types to bind to a single response element. The luciferase activity of the natural agonist (Mib for the AR and R5020 for the PR isoforms) was set to 100% while the activity of the steroids were set to their respective controls.

Results showed that, of all the steroids assayed, 11OHP4 exhibited significant agonist activity towards the PR-A and the AR, while exhibiting only partial agonist activity towards the PR-B isoform. Regarding both the PR-A and PR-B (Fig. 3.17 A and B), the other C11-oxy C$_{21}$ derivatives assayed 11K-DHP4 exhibited partial agonist activity and 11OH-DHP4 and 11KP4 weak partial agonist activity towards both isoforms. Partial agonist activity was exhibited by DHP4 towards both PR-A and PR-B while the steroid

exhibited agonist activity towards the AR, comparable to Mib. Regarding the AR, 11KP4 and 11K-DHP4 exhibit full agonist- and partial agonist transactivation activity whereas 11OH-DHP4 is a weak partial agonist.
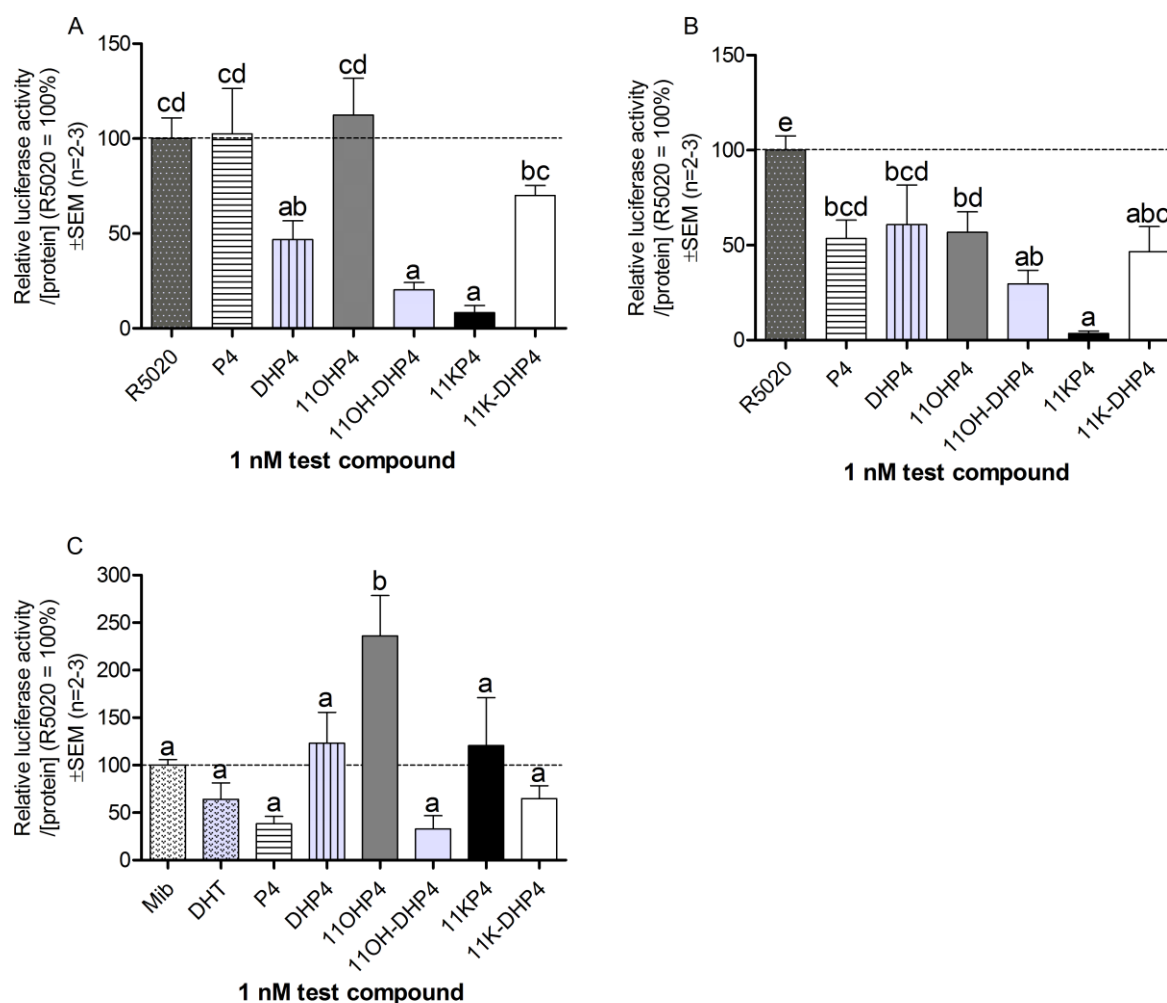
Figure 3.17   Receptor interaction of P4, DHP4, 11OHP4, 11OH-DHP4, 11KP4 and 11K-DHP4 with the hPR isoforms and the hAR. HEK-293 cells expressing a luciferase promoter-reporter construct (pTAT-PRE-E1b-luciferase) and human receptors (A) hPR-A (pSG5-hPR-A), (B) hPR-B (pSG5-hPR-B) and (C) hAR (pSG5-hAR), were treated with synthetic ligand (hAr, Mib; hPR,R5020), natural ligand (hAR, DHT; hPR, P4), and C_{21} steroids (1 nM) for 24 hrs. Induction is shown as % luciferase activity, with the synthetic receptor-selective agonists R5020 (A and B) and Mib (C) set as 100%. Results shown are the averages (±SEM) of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by a Newman-Keuls post-test (comparing all pairs of columns). Statistical significance is designated by different letters (a, b, c or d).

It is interesting to note that in terms of the PR the 5α-reduction of 11KP4 to 11KDHP4 increased the agonist activity while, in terms of the AR, its activity was reduced with the 5α-reduction of P4 increasing the agonist activity. The effect of the 5α-reduction is not as prominent considering the PR-B with the agonistic activity of 11OHP4, 11K-DHP4 and DHP4 for PR-B remaining comparable to that of P4, the

natural PR ligand, at 1 nM. In terms of the AR, while 11OHP4 exhibited strong agonistic activity the 5α-reduction of the steroid reduced the agonist activity significantly, however, the potential metabolism by 11βHSD of 11OH-DHP4 would increase the activity from a weak- to a partial agonist (Fig. 3.17 C). 11KP4 exhibited full agonist activity for the AR, comparable to that of Mib, which was also reduced upon the 5α-reduction of the ketone to a partial agonist comparable to DHT.

3.4   Discussion

Recent reports on the detection of C11-oxy C_{21} steroids in clinical conditions and recent publications regarding the C11-oxy C_{19} steroids and their role in PCa (Bloem *et al.*, 2015; Turcu *et al.*, 2016), has brought to the fore renewed interest in both C11-oxy C_{19} and -C_{21} steroids –their metabolic fate and biological relevance. 11OHA4 has been the topic of several studies as a precursor to potent C11-oxy androgens such as 11KT and 11KDHT (Du Toit *et al.*, 2017; Pretorius *et al.*, 2017; Storbeck *et al.*, 2013; Swart *et al.*, 2013). Both 11OHA4 and 11OHT are metabolised by the SRD5A, 11βHSD and 17βHSD to downstream steroids which can interact with the AR. In contrast to the conventional pathway in the biosynthesis of DHT, 11OHA4 is the precursor to active androgens in the 11OHA4 pathway in which it gives rise to C11 hydroxylated T, -DHT, -5α-dione, AST and their respective ketone metabolites (Swart and Storbeck, 2015). Steroid metabolism by 11βHSD2 was initially regarded only in the context of glucocorticoid inactivation, in cortisol's conversion to cortisone, a less potent glucocorticoid. In contrast, 11β-hydroxy androgens increase in potency after oxidation of the C11 functional group. Interestingly, the binding affinity of 11KP4 for the MR is higher than that of its reduced metabolite, 11OHP4 (Galigniana *et al.*, 1997). Furthermore, the 5α-reduction of androgens has been demonstrated to increase the androgenicity of the compound. C11-oxy C_{19} steroids are detected as the dominant androgens in 21OHD patients, BPH, PCa tissue and plasma samples, as well as in PCOS patients (Du Toit *et al.*, 2017; Turcu *et al.*, 2016). Upon stimulation with ACTH, the C11-oxy C_{19} steroid levels were shown to be increased in 21OHD patients (Turcu *et al.*, 2016). Furthermore, Du Toit *et al.* (2017) demonstrated that 11KT and 11KDHT are less prone to glucuronidation and would thus remain active for longer resulting in prolonged androgenic induced effects. This led to the current study investigating the metabolism of C11-oxy C_{21} steroids 11OHP4 and 11KP4.

CYP11B1 and CYP11B2 are generally accepted to be adrenal specific enzymes. However, Q-PRC, Western blot and immunohistochemical analysis localized CYP11B1 expression in Leydig cells and ovarian theca cells (Imamichi *et al.*, 2016) and CYP11B2 in an androgen dependent PCa cell model using a microarray (Biancolella *et al.*, 2007). Both CYP11B1 and CYP11B2 mRNA were shown in human vascular endothelium (Takeda *et al.*, 1996), in rat myocardium, with reports of elevated ALDO levels detected in the myocardium compared to plasma levels (Silvestre *et al.*, 1998); in the human heart with increased expression in myocardial fibrosis (Satoh *et al.*, 2002). Moreover, mRNA expression of 11βHSD2 and both CYP11B isozymes has been detected in the human brain/central nervous system (Yu *et al.*, 2002) while both CYP11B isoforms are expressed in TART and human primary PCa (Claahsen-van der Grinten *et al.*, 2007; Mitsiades *et al.*, 2012). The investigation into the catalytic

activity of CYP11B1 and CYP11B2 towards P4 was conducted since elevated 11OHP4 levels are associated with the adrenal in 21OHD patients, and have also been detected in the spermatic vein (Blumberg-Tick *et al.*, 1991; Combes-Moukhovsky *et al.*, 1994) as these reactions have, to date, not been shown to be catalysed by these two enzymes.

Corresponding to results previously obtained by our group (Swart *et al.*, 2013), the data shows the full conversion of DOC, catalysed by CYP11B1 and CYP11B2, to CORT, with low levels of ALDO being detected in metabolism by CYP11B2. Deoxycortisol is readily converted to cortisol by CYP11B1 whereas the reaction catalysed by CYP11B2 was not as efficient. Compared to the metabolism of A4 and T to their respective C11-hydroxyl metabolites, deoxycortisol and DOC remain superior substrates for CYP11B1 and CYP11B2. CYP11B1 convert T and A4 more readily than CYP11B2, corroborating data previously been shown by Swart *et al.* (2013). With regards to the C_{21} steroids, the metabolism of P4 by either of the CYP11B isoforms are significantly slower than that of the natural ligands and the C_{19} steroids. Thus, in the presence of the natural ligands, DOC and deoxycortisol would be the preferred substrate over P4. Nevertheless, in abnormal conditions such as 21OHD, the biosynthesis of DOC and deoxycortisol is impaired which will result in the accumulation of P4 and 17OHP4. P4 will subsequently be hydroxylated to form 11OHP4, accounting for the elevated levels observed in this condition. Moreover, numerous studies demonstrated the upregulation of the expression of these enzymes by ACTH, AngII and potassium (Bassett *et al.*, 2004). In salt-wasting 21OHD, low sodium and elevated potassium levels result in high plasma renal activity (renin) which in turn upregulates AngII (Horner *et al.*, 1979). Overstimulation of the adrenal cortex by ACTH results in hyperplasia and excess adrenal C_{21} and C_{19} steroids of which the latter lead to virilisation (Horner *et al.*, 1979). Furthermore, AngII receptors are expressed in testicular tumours with testicular levels in 21OHD comparable to that of healthy adrenals. (Claahsen-van der Grinten *et al.*, 2007). The CYP11B time course data shows low levels of steroid product forming over 20 hrs in the conversion of T and A4 by CYP11B2 and P4 by both isozymes. Low expression of endogenous SRD5A in HEK-293 would convert the substrates to their respective 5α-reduced metabolites (Panter *et al.*, 2005) as P4, T, A4, 11OHT and 11OHA4 are all excellent substrates for the 5α-reductase catalysed reaction (Frederiksen and Wilson, 1971; Quanson, 2015). Unfortunately the reduced metabolites were not analysed in these assays as the focus was the conversion of the steroids by CYP11B1 and CYP11B2.

Once in circulation 11OHP4 is available for further downstream metabolism. The data presented in this chapter clearly shows the rapid metabolism of 11OHP4 by SRD5A1 and SRD5A2 to the novel 11OH-DHP4 product. To the best of our knowledge, this is the first report of the 5α-reduction of 11OHP4. The conversion rate of 11OHP4 to 11OH-DHP4 is more efficient than that of the reduction of the androgens such as T, A4, 11OHA4 and 11OHT to their respective 5α-reduced metabolites (Quanson, 2015; Swart *et al.*, 2013). 11OHP4, like P4 and 17OHP4, are excellent substrates for SRD5A (Compagnone and Mellon, 2000; Frederiksen and Wilson, 1971; Gupta *et al.*, 2003; Sinreih *et al.*, 2015). These data suggest that 11OHP4 would be reduced once in the presence of the SRD5A enzymes and that the C11 hydroxyl group does not hinder the reduction rate in the manner as observed for the C11-oxy C_{19} steroids (Quanson, 2015). These enzymes are expressed at various levels in tissue

including the reproductive systems of males and females, breast tissue, the skin, the liver, kidneys, and the prostate (Lewis *et al.*, 2004; Normington and Russell, 1992; Quinkler *et al.*, 2003; Thigpen *et al.*, 1993). Furthermore, the higher conversion rate by SRD5A1 is significant in PCa, breast cancer and endometrial cancer cell models in which the expression of SRD5A1 being upregulated in these cancers (Lewis *et al.*, 2004; Sinreih *et al.*, 2015; Zhu and Imperato-McGinley, 2009). Previous studies have indicated a marginally higher reduction rate for 11keto-C_{19} moieties by 5α-reductases compared to their 11-hydroxyl metabolites. A 50% conversion of 11OHT and 11KT by SRD5A was achieved after 8 and 5 hrs, respectively (Quanson, 2015). However, analysis of the 5α-reduction of 11KP4 showed equal or lower rates compared to the conversion of 11OHP4 to 11OH-DHP4. The data obtained from these steroid conversion assays thus show that the reduction of 11OHP4 and 11KP4 by SRD5A1 and SRD5A2, is more efficient than reported in previous investigations into P4, 17OHP4 and T (Frederiksen and Wilson, 1971). In addition, the unknown reduced metabolite detected in the metabolism of 11KP4 to 11K-DHP4 by SRD5A1 and SRD5A2 indicates conversion of 11K-DHP4 by an endogenous reductase expressed in HEK-293 cells.

The above findings led to further investigation into the metabolism by AKR1C2. The metabolism of 17OHP4 by SRD5A and AKR1C2 are the initial reactions of the backdoor pathway synthesizing Pdione and Pdiol (Gupta *et al.*, 2003). Furthermore, the 5α-reduction of androgens results in increased AR potency whereas the subsequent metabolism by AKR1C2 results in inactivation of the potent androgens. In contrast, the potency of P4 for the PR, and glucocorticoids for the GR decrease upon their 5α-reduction (Jewgenow and Meyer, 1998; Nixon *et al.*, 2012). However, as a neurosteroid, allopregnanolone, the 3α,5α-reduced P4 metabolite, elicits a higher $GABA_A$ response compared to DHP4. In this study, the reduction of the C3 ketone of 11OH-DHP4 and 11K-DHP4 was investigated to determine whether these C11-oxy C_{21} steroid metabolites are substrates for AKR1C2 which would result in the production of 3,11diOH-DHP4 and alfaxalone. The data clearly shows that even though the 3α-reduction of 11K-DHP4 does not appear to occur as rapidly as that of Pdione to Pdiol, the metabolism of the steroid by AKR1C2 was significant. In these assays steroid production could only be represented by the ionisation signal since the steroid was unavailable. Although this representation is by no means a measure of quantification, it confirmed the production of these novel steroid metabolites. Quantifying 11OH-DHP4 and 3,11diOH-DHP4 in the AKR1C2 conversion assay was problematic due to the lack of standards; however, the availability of 11K-DHP4 allowed the consumption of the steroid to be quantified.

The backdoor pathway is frequently activated in 21OHD and CRPC in order to bypass the conventional production of DHT (Kamrath *et al.*, 2012b; Locke *et al.*, 2009). Increasing evidence of backdoor metabolites (Pdione, Pdiol and AST) is being detected in studies reporting steroid profiles (Christakoudi *et al.*, 2013; Du Toit *et al.*, 2017). Furthermore, elevated levels of C11-oxy AST metabolites have also been detected in PCa cell models (Du Toit *et al.*, 2017) and 11OHAST was detected as the main metabolite in the urine of 21OHD children (Kamrath *et al.*, 2017). Earlier research by Dorfman *et al.* (1954) demonstrated the *in vivo* metabolism of DOF to 11OHAST (Dorfman, 1954) while Swart *et al.*

(2015) ascribed 11OH-5α-dione and 11K-5α-dione as the precursors in the production of 11OHAST and 11KAST.

CYP17A1 is the key enzyme in the conversion of C_{21} steroids to the C_{19} steroids in the Δ^5 and Δ^4 steroidogenic pathways. The conversion of alfaxalone to 11KAST by CYP17A1 was significant (±60%) when assayed with the heterologously expressed enzyme, without the presence of competitive enzymes. This reaction requires the dual activity of CYP17A1 - the 17α-hydroxylase and 17,20-lyase activity - in order to synthesize 11KAST from alfaxalone (Fig. 3.12). Although the 12 hr assay showed full conversion of alfaxalone, the detected 11KAST levels were lower than the initial substrate concentration, possibly indicating that the hydroxyl intermediate was also still present. As was reported in the conversion of 17OHP4 to AST in the backdoor pathway (Gupta *et al.*, 2003) it would appear that once the C3 keto group and the C4-5 double bond are reduced, alfaxalone and thus also 3,17diOH-DHP4 would serve as substrates for the CYP17A1 catalysed reactions (Fig. 3.18).
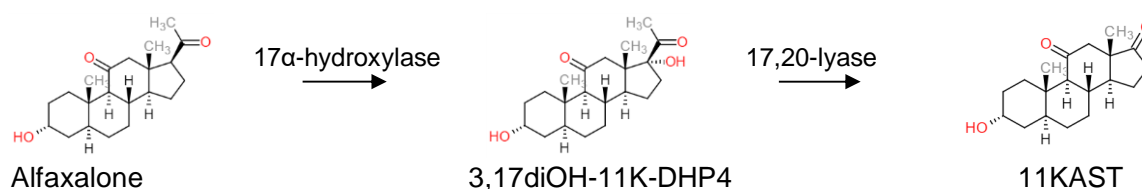
Figure 3.18   Proposed reaction for the metabolism of alfaxalone to 11KAST by CYP17A1. Alfaxalone is firstly hydroxylated at C17, prior to being lysed.

The novel conversions of 11OHP4 and 11KP4 presenting substrates for CYP17A1 in the backdoor pathway was furthermore shown in a physiological setting. For this purpose, the metabolism of 11OHP4 and 11KP4 was investigated in the LNCaP cell model endogenously expressing the relevant steroidogenic enzymes *viz.* 11βHSD2, SRD5A1, 17βHSD and AKR1C2. The data obtained in this model suggests that 11OHP4 was preferentially converted by 11βHSD2 to 11KP4 rather than to 11OH-DHP4 by SRD5A (Fig. 3.14 A). The formed 11KP4 was subsequently reduced by SRD5A to 11K-DHP4 of which the low levels indicate that it was readily converted to alfaxalone. This pathway was corroborated in the data obtained during the conversion of 11KP4 (Fig. 3.14 B) in which 11K-DHP4 and alfaxalone were the major products detected. The low levels of 11KDHT in both assays suggest conversion of either alfaxalone or 3,11diOH-DHP4 by CYP17A1 and subsequent catalysis by the endogenous 17βHSD3/5 and 17βHSD6 enzymes. It is possible that all the intermediates were not detected due to the lack of standards and steroids being present at very low levels as ±0.5 µM of the initial substrate remained after 48 hrs while the products identified only ranged between 100 – 150 nM steroid.

In the control reactions, the conversion of 11OHA4 to 11KA4 and 11KT in LNCaP cells corresponded to previous data published by our group in which 11KA4 was the main product formed, indicating the preferred conversion by 11βHSD2 over SRD5A. There are nevertheless, variations in steroid levels reported in these studies (Du Toit *et al.*, 2017; Swart *et al.*, 2013) which can be attributed to cell densities as well as experimental conditions. The general pathway remained comparable in the studies

nonetheless. In order to fully characterise the role of CYP17A1 in the conversion of the C11-oxy C_{21} steroids to C11-oxy C_{19} steroids in the backdoor pathway, the metabolism of 11OHP4 and 11KP4 will be assayed in a cell model system such as an ovary cell model which expresses the relevant steroidogenic enzymes as well as CYP17A1. The expression of CYP17A1 in LNCaP cells is controversial with conflicting reports of low levels of expression, possibly accounting for the low 11KDHT levels detected in this study, as well as the expression being absent in LNCaP cells.

The above findings led to an investigation into the biological relevance of 11OHP4, 11KP4 and their 5α-reduced metabolites with regards to their ability to induce transactivation via the PR-A, PR-B and the wild-type AR. Given the agonist activity of the C11-oxy C_{21} steroids towards these receptors shown in this study, together with the expression of these steroid receptors in normal steroidogenic tissue and in cancer tumour tissue, there can be no doubt as to the importance of these steroids. Previous studies by the research group of A.C. Swart (Biochemistry, Stellenbosch University) showed the C11-oxy C_{19} steroids such as 11KT and 11KDHT exhibit activation of the AR comparable to DHT (Storbeck *et al.*, 2013). Activation of the AR in the prostate results in cell proliferation and the progression of PCa with the AR having been detected in both benign and malignant prostate epithelial cells, LNCaP, DU-145 cell models as well as in BPH and prostate metastases with AR levels being elevated in the latter (Brolin *et al.*, 1992; Gregory *et al.*, 2001; Latil *et al.*, 2001). The mutated AR on the other hand, is detected in metastatic PCa and in patients unresponsive to PCa therapy while it has also been associated with increased resistance to therapy leading to CRPC (Culig *et al.*, 1993; Newmark *et al.*, 1992; Taplin *et al.*, 1995). Previous studies conducted by the research group of A.C. Swart (Biochemistry, Stellenbosch) showed that the 5α-reduction of the C11-oxy C_{19} steroids increased the agonist activity of these steroids in terms of the AR (Bloem *et al.*, 2015). In this study the 5α-reduction of 11OHP4 and 11KP4 reduced the agonist activity of these C_{21} steroids for the AR. Regarding the PR, the effect of the 5α-reduction differed in terms of the C11 moiety for both isoforms with the agonist activity of 11OHP4 decreasing significantly while that of 11KP4 increased significantly.

Evidence of AR activation by P4 has also been reported, primarily in association with PCa while induction of transactivation by P4 via the wild-type AR was shown to be lower than that of the mutated AR (Culig *et al.*, 1993). In addition, it has been suggested that P4 plays a role in PCa since elevated P4 levels are associated with PCa patients receiving AR antagonist therapy (Cho *et al.*, 2015). Compounding potential effects of C_{21} steroids is the expression of the PR in metastatic and androgen-insensitive adenocarcinomas which has also been demonstrated (Bonkhoff et al., 2001; Chen et al., 2017; Grindstad et al., 2015) with higher levels in benign hyperplastic- and recurring prostate compared to primary carcinoma (Brolin *et al.*, 1992).

The PR and AR are both expressed in the ovary together with variations in expression levels throughout the menstrual cycle (Horie *et al.*, 1992; Iwai *et al.*, 1990; Press and Greene, 1988; Sen and Hammes, 2010; Weil *et al.*, 1999). The PR has also been identified in numerous female reproductive tissue carcinomas (breast, endometrial and ovarian) (Lau *et al.*, 1999; Press and Greene, 1988) while the AR is also expressed in the human OVCAR-3, an ovarian carcinoma cell model (Hamilton *et al.*, 1983).

The effects of 11OHP4, 11KP4 and their 5α-reduced metabolites have to date not been considered in PCa progression, in female reproductive tissue carcinomas, in 21OHD or in PCOS. Although low

circulating 11OHP4 being detected in 21OHD at ±1.5 nM (Turcu *et al.*, 2015) 11KP4 was not included in the analyses (A. Turcu, personal communication) and as such these findings suggest that these C11-oxy C_{21} steroid at 1 nM, together with their downstream metabolites, would activate steroid receptors and affect cellular response even at these physiological concentrations.

It is evident from the results presented in this study that 11OHP4 plays a role in the transactivation of the PR-A with induction levels similar to that of P4, while towards the AR, the steroid exhibits super agonist activity. In addition, 11KP4 displayed activity comparable to Mib and DHT, the natural ligand. With the presence of both SRD5A and 11βHSD2 in the prostate and ovary (Albiston *et al.*, 1994) – enzymes responsible for the metabolism of 11OHP4 to these respective metabolites – it is prudent to assume that 11OHP4 and 11KP4 will impact on normal physiology and on that of disease states such as PCa, 21OHD and PCOS −even in the absence of CYP17A1 and the metabolism to their respective C_{19} metabolites in the backdoor pathway.

CHAPTER 4

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Manuscript: The metabolic fate and receptor interaction of 16α-hydroxyprogesterone and its 5α-reduced metabolite, 16α-hydroxy-dihydroprogesterone

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The biosynthesis of adrenal hormones has been well characterised and is firmly established together with their downstream metabolism and receptor interaction. However, 16OHP4 is less well studied and its metabolism in target tissue by steroidogenic enzymes had, up until this investigation, been unknown, while receptor interaction of the downstream metabolites has also not been investigated. The aim of the investigation was to determine the metabolism of 16OHP4 by steroidogenic enzymes as well as receptor interactions by its 5α-reduced metabolite.

The 16α-hydroxylase of P4 by CYP17A1 unique to humans and chimpanzees has been accredited to a single amino acid residue change, allowing a greater rotational freedom of the substrate in the active pocket of the enzyme (Swart *et al.*, 1993). P4 is converted by CYP17A1 to 17OHP4 and 16OHP4 at a ratio of 3:1, though the ratio varies marginally between cell type and tissues (Arlt *et al.*, 2002; Storbeck *et al.*, 2008; Swart *et al.*, 2010, 1993). 16OHP4 has been detected in conditions associated with elevated P4 levels *viz.* pregnancy, the luteal phase of the menstrual cycle and disorders such as PCOS, CAH as well as in PCa (Colla *et al.*, 1966; Den *et al.*, 1979; Griffiths *et al.*, 1963; Huang, 1967; Janoski *et al.*, 1969; Ruse and Solomon, 1966; Sharma *et al.*, 1965; Storbeck *et al.*, 2011; Takagi and Den, 1981; Villee *et al.*, 1961, 1962; Viscelli *et al.*, 1965; Ward and Grant, 1963). In a more recent study, Turcu *et al.* (2015) reported the C_{21} steroid profile of steroids in circulation in 21OHD patients and in TART cells in which increased levels of 17OHP4 as well as 16OHP4 were detected (Turcu *et al.*, 2015).

Urinary concentration of 16OHP4 in the menstrual cycle has been reported to change in a cyclic manner, reaching maximum levels in the luteal phase (Stiefel and Ruse, 1969). In addition, in the cyclic change in 17OHP4 and 16OHP4 levels, the production of these two hormones in the human ovary remains proportional to each other throughout the cycle (Yumiko *et al.*, 1981). Upon administration of a gonadotropin-hormone agonist, 17OHP4 increased and accumulated in the ovary −more so in functional ovarian hyperandrogenism and polycystic ovarian syndrome, thus acting as the source of C_{19} steroids in the backdoor pathway (Auchus, 2004).

Although previous studies conducted by the research group of A.C. Swart (Biochemistry, Stellenbosch University) detected adrenal 16OHP4 and found 16OHP4 to activate the PR, the downstream metabolism of these steroids has not been addressed. Since 16OHP4 is considered a dead end product of the adrenal and has not been considered in the context of 21OHD, this study investigated the potential metabolism of 16OHP4 by CYP11B1 and CYP11B2. Co-localization of 16OHP4 and SRD5A in the ovaries and the biosynthesis of DHP4 during the luteal phase (Auchus, 2004) suggest possible 5α-reduction of 16OHP4. The formation of the 11β-hydroxylated product thus prompted the further investigation into the metabolism of both 16OHP4 and 11,16diOHP4 by downstream steroidogenic

enzymes, SRD5A and AKR1C2, after which the receptor interaction with the 5α-reduced metabolite of 16OHP4 was assessed.

The metabolic fate and receptor interaction of 16α-hydroxyprogesterone and its 5α-reduced metabolite, 16α-hydroxy-dihydroprogesterone

Desmaré van Rooyen, Therina du Toit, Renate Louw-du Toit, Donita Africander, Pieter Swart, Amanda C. Swart[*]

Biochemistry Department, Stellenbosch University, Stellenbosch 7600, South Africa

ABSTRACT

16α-hydroxyprogesterone (16OHP4) is not well characterised in terms of metabolism and receptor interaction. We therefore investigated its metabolism by adrenal CYP11B and peripheral steroidogenic enzymes, SRD5A and AKR1C2. UHPLC-MS/MS analyses identified novel steroids: the biosynthesis of 4-pregnen-11β,16α-diol-3,20-dione catalysed by CYP11B2; the 5α-reduction of the latter and 16OHP4 catalysed by SRD5A yielding 5α-pregnan-11β,16α-diol-3,20-diovne and 5α-pregnan-16α-ol-3,20-dione (16OH-DHP4); and 16OH-DHP4 converted by AKR1C2 to 5α-pregnan-3α,16α-diol-20-one. Receptor studies showed 16OHP4, 16OH-DHP4, progesterone and dihydroprogesterone (DHP4) were weak partial AR agonists; 16OHP4, 16OH-DHP4 and DHP4 exhibited weak partial agonist activity towards PR-B with DHP4 also exhibiting partial agonist activity towards PR-A. Data showed that while the 5α-reduction of P4 decreased PR activation significantly, 16OHP4 and 16OH-DHP4 exhibited comparable receptor activation. Although the clinical relevance of 16OHP4 remains unclear the elevated 16OHP4 levels characteristic of 21OHD, CAH, PCOS, prostate cancer, testicular feminization syndrome and cryptorchidism likely contribute towards these clinical conditions, inducing receptor-activated target genes.

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1. Introduction

In adrenal steroidogenesis the hydroxylation of pregnenolone (P5) and progesterone (P4), by cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), follows on from the initial cleavage of cholesterol's side chain by cytochrome P450 side chain cleavage yielding P5, with 3β-hydroxysteroid dehydrogenase type 2 converting P5 to P4. The above mentioned conversions are common in all steroidogenic tissue where CYP17A1 catalyses the biosynthesis of 17α-hydroxypregnenolone (17OHP5) and 17α-hydroxyprogesterone (17OHP4), followed by the conversion of 17OHP5 to dehydroepiandrosterone (DHEA). In addition, human CYP17A1 also catalyses the hydroxylation of P4 to 16α-hydroxyprogesterone (16OHP4). CYP17A1 and cytochrome P450 21-hydroxylase (CYP21A2) compete for the P4 substrate with CYP21A2 catalysing the biosynthesis of deoxycorticosterone (DOC) and the former that

of 17OHP4 and 16OHP4. We previously reported that DOC, 17OHP4 and 16OHP4 are formed at a ratio of 2.95:3.4:1 in the human foetal adrenal, while recombinant CYP17A1 catalyses the biosynthesis of 17- and 16OHP4 at a similar ratio of 4:1. In addition, we reported that P5 is not hydroxylated at C16 and we showed conclusively that 17OHP4 is not converted to androstenedione (A4) by CYP17A1 in human testicular microsomal preparations. Even in the presence of cytochrome b_5, which enhances the CYP17A1 catalysed lyase reaction and aids the conversion of 17OHP5 to DHEA, which leads to the production of androgen precursors, 17OHP4 is not a substrate and does not contribute to androgen biosynthesis (Swart et al., 1993).

While P4 and its metabolites have been studied extensively, as they are involved in numerous physiological processes, the metabolism and biological effects of 16OHP4 remain largely unknown. 16OHP4 was first detected in 1961 upon incubation of tissue slices and human foetal adrenal homogenates with radiolabelled P4 followed by studies undertaken in feminising testicular tissue and human ovaries confirming the presence of this steroid *in vivo* (Griffiths et al., 1963; Sharma et al., 1965; Villee et al., 1961; Yumiko

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Steroid abbreviations

P5	pregnenolone	DOF	4-pregnen-11β,17α-diol-3,20-dione or 21-desoxycortisol
17OHP5	17α-hydroxypregnenolone	DOC	deoxycortisosterone
P4	progesterone	DHEA	dehydroepiandrosterone
DHP4	5α-pregnan-3,20-dione	5α-dione	5α-androstenedione;
allopregnanolone	5α-pregnan-3α-ol-20-one	A4	androstenedione
17OHP4	17α-hydroxyprogesterone	11OHA4	11β-hydroxyandrostenedione
16OHP4	16α-hydroxyprogesterone	T	testosterone
11OHP4	11β-hydroxyprogesterone	11OHT	11β-hydroxytestosterone
Pdione	5α-pregnan-17α-ol-3,20-dione	11KT	11keto-testosterone
Pdiol	3α-hydroxy-5β,17α-pregnan-20-one	DHT	dihydrotestosterone
16OH-DHP4	5α-pregnan-16α-ol,3,20-dione	11OHDHT	11β-hydroxydihydrotestosterone
3,16diOH-DHP4	3α,16α-dihydroxy-5α-pregnan-20-one	11KDHT	11keto-dihydrotestosterone
11,16diOHP4	4-pregnen-11β,16α-diol-3,20-dione	Mib	mibolerone
11,16diOH-DHP4	5α-pregnan-11β,16α-diol-3,20-dione	R5020	promegestone.

et al., 1981). These earlier studies also localised the production of 16OHP4 to the adrenal, as significant amounts of labelled P4 were recovered as 16OHP4 in the adrenal of the foetus, whereas none was detected in the adrenalectomized foetus (Bird et al., 1966; Wilson et al., 1966). More recently, the 16-hydroxylase activity of CYP17A1 has been uniquely accredited to humans and chimpanzees (Arlt et al., 2002; Swart et al., 2002, 1993), the ability of which is accredited to a single amino acid residue change, Ala105Leu, allowing for a greater rotational freedom of the substrate in the active site. In addition, the hydroxylation of P4 at C16 was also shown not to be augmented by cytochrome b_5 (Swart et al., 2010).

Lending itself to investigations into the P4 metabolites is the ovary, in which fluctuating P4 levels, associated with the female menstrual cycle/reproductive system, have shown the production of 17OHP4 and 16OHP4 to remain proportional during the menstrual cycle (Yumiko et al., 1981). 16OHP4 has also been detected in the P4-producing corpus luteum during pregnancy and while urinary 16OHP4 levels increase progressively throughout pregnancy even higher levels are observed in younger subjects. There is a significant decrease in 16OHP4 levels within hours after birth together with a rapid and significant decline in 16OHP4 in the umbilical artery (Ruse and Solomon, 1966a; Stiefel and Ruse, 1969). 16OHP4 levels have been shown to increase 2–4 fold from mid-pregnancy to term. This is due to foetal production, rather than by maternal activity, which was demonstrated following P4 administration via the umbilical cord, (Villee et al., 1961; Zander et al., 1962). In addition, umbilical artery 16OHP4 levels were shown to be higher during labour pains compared to suppressed labour pains during caesarean sections, suggesting a definite role for 16OHP4 in the birth process (Den et al., 1979). Interestingly, the absence of foetal CYP21A2 during the early stages of gestation results in increased levels of P4 and its C_{21} metabolites, similar to that which is observed in 21-hydroxylase deficiency (21OHD) (Villee et al., 1961). Elevated 16OHP4 levels have been detected in clinical conditions such as adrenal hyperplastic patients diagnosed with Cushing's disease, congenital adrenal hyperplasia (CAH), in polycystic ovarian syndrome (PCOS) and in 21OHD patients (28-fold) while also being detected in testicular adrenal rest tumour patients (TART) (7-fold) (Turcu et al., 2015; Villee, 1964; Warren and Salhanick, 1961; Zander et al., 1962).

The production of 16OHP4, in terms of the catalytic activity of CYP17, is well defined in the aforementioned studies, with these studies clearly showing that increased 16OHP4 levels are influenced by substrate availability. In disease conditions and adrenal disorders, 16OHP4 levels are also specifically associated with increased P4 levels. Earlier studies have, however, also shown that increased production of 16OHP4, not necessarily associated with increased P4 levels, is characteristic of abnormal male sexual development. Investigations into human foetal gonadal development showed that the *in vitro* hydroxylation of P4 was more active in male gonads with the ovary primarily producing 20α-hydroxy-4-pregnene-3-one (Bloch, 1964). It was subsequently shown that 3–5% of P4 was converted to 16OHP4 in the normal human testes (Viscelli et al., 1965) with our study showing the biosynthesis of 17OHP4 and 16OHP4 by CYP17A1 in adult testicular microsomal preparations in a ratio 3.25:1 (Swart et al., 1993). Deviations from this ratio are apparent in clinical conditions in which 17OHP4 and 16OHP4 were detected in percentile ratios of 2:1 and 1.3:1 in cryptorchid tumours and testicular tissue from testicular feminising patients, respectively (Griffiths et al., 1963). Subsequent studies corroborated these findings in testicular feminising testes tissue (Sharma et al., 1965), showing high levels of 16OHP4 (15%), linking 16OHP4 and abnormal sexual development (Colla et al., 1966). Interestingly, one of the strongest risk factors for testicular cancer and infertility is cryptorchidism, which is reportedly a common clinical diagnosis in newborn males (Hutson et al., 2013). 16OHP4 and 17OHP4 have also been identified as the major P4 metabolites in the testes of prostate cancer (PCa) patients (Oshima et al., 1967). In testicular microsomal preparations of PCa patients 16OHP4 inhibited the 17,20- lyase activity of CYP17A1 as well as the 20α-hydroxysteroid dehydrogenase activity, suggesting that 16OHP4 is not readily metabolised (Inano and Tamaoki, 1978). In contrast however, following the administration of labelled 16OHP4 in healthy males, urinary 3α,16α-dihydroxy-5α/β-pregnan-20-one, 5α/β-pregnane-3α,16α,20α-triol and 3α-hydroxy-5β,17α-pregnan-20-one were detected (Ruse and Solomon, 1966b) - some of which were also identified in porcine adrenals, in the urine of pregnant females and of salt-wasting CAH patients (Hirschmann et al., 1961; Villee et al., 1962; Warren and Salhanick, 1961). It would thus appear that the biosynthesis of 16OHP4 is relevant in males, and perhaps more so in clinical disease states since data suggest altered downstream metabolism of 16OHP4 compared to healthy males. While the 5α-reduction of C_{19} steroids produces more potent androgen receptor (AR) ligands, it remains to be determined whether the 5α-reduction of C_{21} steroids would yield active progesterone receptor (PR) or AR ligands. While we previously reported that 16OHP4 is able to interact with the human PR and bind both functional isoforms – PR-A and PR-B, exhibiting agonistic

activity (Storbeck et al., 2011) it was shown not to interact with the human mineralocorticoid receptor (MR), while 17OHP4 was shown to be a potent antagonist (Quinkler et al., 2002).

Although 16OHP4 features prominently as a product of P4, both in gonadal foetal development and in reproductive functions – in normal physiology, clinical disease states and in cancer, limited studies into its downstream metabolism and physiological role have been undertaken. The aim of this study was to firstly investigate the metabolism of 16OHP4 by adrenal CYP11B, since this enzyme has been implicated in the production of C11-oxy C_{21} steroids in 21OHD and CAH patients. In addition, the downstream metabolism of 16OHP4, by steroidogenic enzymes expressed in peripheral tissue, was investigated and analysed by UHPLC-MS/MS to determine the metabolic fate of the steroid. Receptor studies were subsequently undertaken to investigate the interaction of 16OHP4 and its 5α-reduced metabolite, 5α-pregnan-16α-ol,3,20-dione (16OH-DHP4), with the PR and the AR.

2. Materials and methods

2.1. Materials

HEK293 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). XtremeGENE HP® DNA transfecting reagent and X-tremeGENE 9 DNA transfection reagent were purchased from Roche Diagnostics (Mannheim, Germany). Penicillin-streptomycin, foetal bovine serum, trypsin-EDTA and phosphate buffered saline (PBS) were obtained from Oxoid limited (Hampshire, England). Dulbecco's modified Eagle's media (DMEM), P4, dihydrotestosterone (DHT) and methyl tert-butyl ether (MTBE) were purchased from Sigma-Aldrich (St. Louis, USA). 16OHP4 and 5α-pregnan-3,20-dione (DHP4) were purchased from Steraloids (Wilton, USA) and deuterated testosterone (Testosterone 1,2-D2, 98%), deuterated progesterone (Progesterone 2,2,4,6,6,17A, 21,21,21-D9, 98%) and deuterated 17OH-progesterone (17-hydroxyprogesterone 2,2,4,6,6,21,21-D8, 98%) were purchased from Cambridge isotopes (Andover, USA). Mibolerone (Mib) and promegestone (R5020) were purchased from Perkin Elmer (Massachusetts, USA). Nucleobond® Maxiprep DNA isolation kits were purchased from Macherey-Nagel (Duren, Germany). Corning® CellBIND® Surface T75 flasks, 100 mm dishes, 12-, 24- and 96-well plates were purchased from Corning® Life Science (NY, USA). Luciferase Assay System (Promega) was purchased from Anatech. The Kinetex PFP column was purchased from Phenomenex. The Bradford protein assay kit and all other chemicals used were purchased from reliable scientific supply houses.

2.2. Plasmid constructs

Human SRD5A1 and SRD5A2 pCMV7 plasmid constructs were obtained from Prof. D.W. Russell (Southwestern Medical School, University of Texas, Dallas, USA), human CYP11B1 pCMV and CYP11B2 pCMV plasmid constructs were obtained from Prof. W.L. Miller (School of Medicine, University of California, San Francisco, USA) and the human ADX plasmid construct was obtained from Prof. R.C. Tuckey (School of Chemistry and Biochemistry, University of Western Australia, Crawley, Australia). The human 3α-hydroxysteroid dehydrogenase (3αHSD) type 3 (AKR1C2) pcDNA3 plasmid construct was obtained from Prof. T.M. Penning (Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA). The plasmid pTAT-GRE-E1b-luc was obtained from Prof. G. Jenster (Erasmus University of Rotterdam, Netherlands). A plasmid expressing the human wild-type androgen receptor, pSG5/hAR was obtained from Dr. H Klocker (Innsbruck Medical University, Innsbruck, Austria, EU). The human PR-A and PR-B (pSG5hPR-A,

pSG5hPR-B) was obtained from Dr. E. Kalkhoven (University Medical Center Utrecht, Utrecht, Netherlands). The pCINeo plasmid was available for use in the laboratory, and was used in parallel with enzymatic conversion assays in transfected HEK293 cells as a negative control.

2.3. Enzymatic assays in transiently transfected HEK293 cells

Non-steroidogenic HEK293 cells were grown in DMEM supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin and 1.5 g $NaHCO_3$/L (pH 7) at 37 °C, 5.0% CO_2 and 90% relative humidity. Once confluency of 80% was reached, after the 3rd passage, the cells were plated into Corning® CellBIND® 24-well plates at a cell density of 2×10^5 live cells/mL (0.5 mL/well). After 24 hrs the cells were transiently transfected with the appropriate plasmid construct encoding the relevant enzymes using XtemeGene HP® DNA transfection reagent according to the manufacturer's instructions (0.25 μg CYP11B1 or CYP11B2 were co-transfected with 0.25 μg ADX; AKR1C2). The transfected cells were incubated for 48 hrs followed by the addition of 16OHP4 (1 μM) in media, which was incubated with the cells for 24 hrs. Thereafter aliquots (500 μL) of the media was taken and analysed for steroid content using UHPLC-MS/MS. Testosterone-D2 (15 ng), progesterone-D9 (15 ng) and 17OH-progesterone-D8 (15 ng) were added to each sample as internal standards prior to steroid extraction. Steroid extraction was achieved by liquid/liquid extraction with MTBE at a ratio of 1:3 (media:MTBE). The samples were vortexed and snap frozen at −80 °C to freeze the aqueous phase. The organic phase was subsequently removed and dried under nitrogen. Steroid residues were then redissolved in a 50% methanol/water mixture (150 μL) prior to analysis by UHPLC-MS/MS (Schloms et al., 2012; Swart et al., 2013).

Additional time course assays were conducted in HEK293 cells. HEK293 cells were cultured as described in section 2.3. The cells were then plated into 100 mm Corning® CellBIND® dishes prior to transfection according to the XtemeGene HP® DNA transfection reagent protocol (CYP11B1 or CYP11B2 were co-transfected with ADX; SRD5A1 and SRD5A2). After 48 hrs, 16OHP4 (1 μM) was added and 500 μL aliquots were collected at the appropriate time points. Steroids were extracted and analysed as described above.

2.4. Production of novel C_{21} steroids

2.4.1. Preparation of commercially unavailable steroids

As 16OH-DHP4 and 4-pregnen-11β,16α-diol-3,20-dione (11,16diOHP4) are commercially unavailable, it was necessary to produce these steroids *in vitro* for further investigatory purposes. The method of preparation was adapted from methods previously published (Storbeck et al., 2013). The respective enzymes (CYP11B2 and SRD5A1) were transiently expressed in HEK293 cells plated in a 12-well Corning® CellBIND® plate (1×10^5 live cells/mL; 1 mL/well) according to the manufacturer's supplied XtremeGene HP® protocol. After 48 hrs post transfection, 16OHP4 (10 μM) was added to the appropriate transfected cells and incubated for maximal conversion (24 hrs). HEK293 cells, expressing CYP11B2, were used to biosynthesise 11,16diOHP4 from 16OHP4 and HEK293 cells, expressing SRD5A1, were used to convert 16OHP4 to its 5α-reduced metabolite, 16OH-DHP4. These metabolites could then be utilised as steroid substrates in further conversion assays.

2.4.2. Enzymatic assays in transiently transfected HEK293 cells

The metabolism of the newly produced steroids, 11,16diOHP4 and 16OH-DHP4, by SRD5A1 and CYP11B2 respectively, was assayed in HEK293 cells, transiently transfected with AKR1C2, as described in Section 2.3. After 48 hrs transfection, the media was

aspirated and replaced with the media containing the appropriate steroid substrates (500 μL) as described in section 2.4.1 and incubated for 24 hrs. Additionally, a duplicate aliquot (500 μL) of the media containing the steroid substrates were analysed by UHPLC-MS/MS to confirm the amount of steroids present. After 24 hrs incubation of the cells with the steroid substrates, 500 μL aliquots were extracted as described in section 2.3 and analysed using UHPLC-MS/MS. All samples were stored at −20 °C prior to UHPLC-MS/MS analysis.

2.5. Accurate mass determination of novel steroid metabolites

The accurate mass of the novel steroids that were biosynthesised, as described in section 2.4.1 had to be determined to confirm their identities, as they were commercially unavailable. The steroids were biosynthesised at high concentrations (10 μM), and analysed on a Waters Synapt G2 quadrupole time-of-flight (Q-TOF) mass spectrometer (Milford, USA) using positive ionisation (ESI+) in order to determine the exact molecular mass and fragmentation as previously described (Storbeck et al., 2013).

2.6. Separation and quantification of steroid metabolites

A stock solution of 16OHP4 (2 mg/mL), in absolute ethanol (EtOH) was prepared and stored at −20 °C. Standards ranging from 0.02 to 5.0 ng/μL were extracted from media using the protocol described in section 2.3. The accurate mass and fragmentation of the novel steroids obtained after accurate mass determination was subsequently used to analyse and quantify the steroids by UHPLC-MS/MS on a ACQUITY UPLC (Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP column (2.1 mm × 100 mm 2.6 μm) coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, USA). Steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray probe in ESI + mode (Storbeck et al., 2013; Swart et al., 2013). The mobile phase consisted of 1% formic acid (in water) as solvent A and a methanol: acetonitrile: isopropanol (49%: 49%: 2%) mixture as solvent B (detail regarding the gradient is given in Supplemental Table 1). Masslynx Software 4.1 (Waters corporation) was used for data collection and Graph Pad Prism 5 (GraphPad Software, Inc, CA, USA) was used for statistical analysis.

2.7. Luciferase reporter assays

HEK293 cells were seeded into 100 mm dishes at 2×10^6 cells. After 48 hrs, the cells were transiently transfected with 9 μg of the pTAT-PRE-E1b-luciferase promoter-reporter construct and 0.9 μg of the appropriate expression vector for human (A) PR-A (pSG5-hPR-A), (B) PR-B (pSG5-hPR-B) or (C) AR (pSG5-hAR), using the XtremeGENE 9 DNA transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. After 24 hrs, the cells were replated into 96-well plates at a density of 1×10^4 cells per well (0.2 mL/well). After 72 hrs, the cells were treated with 0.1% EtOH (vehicle control) or 100 nM R5020, P4, DHP4, 16OHP4, 16OH-DHP4, Mib or DHT for 24 hrs. Following the 24 hrs, the cells were lysed and the luciferase activity in the lysate was measured using the Luciferase Assay System (Promega) and a Veritas microplate luminometer. Total protein concentration was determined using the Bradford protein assay method. Luciferase activity, expressed as relative light units (rlu), was normalised to protein concentration (mg/mL). Induction is shown as percentage luciferase activity, with (A and B) R5020 or (C) Mib set as 100%.

2.8. Statistical analysis

All experiments are presented as means ± SEM. Steroid quantifications using UHPLC-MS/MS were analysed with either an unpaired t-test or a one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 5 (GraphPad Software, Inc, CA, USA). A value of $P \leq 0.05$ was considered statistically significant ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). Luciferase reporter assay data was analysed by one-way ANOVA with Newman-Keuls (comparing all pairs of columns). The results shown are the averages (±SEM) of at least two independent experiments with each condition performed in triplicate. In some figures the letters a, b and c were used to indicate statistical significance. Values which differed significantly were assigned a different letter.

3. Results and discussion

Human CYP17 catalyses the biosynthesis of 17OHP4 and 16OHP4 with the role of 17OHP4 as precursor in glucocorticoid and sex steroids being well defined. The physiological significance of 16OHP4, however, remains uncertain. This study was therefore undertaken to determine the metabolism of 16OHP4 by CYP11B and peripheral enzymes, SRD5A and AKR1C2 and to investigate the receptor interaction of the C_{21} steroids with the AR and PR-isoforms.

3.1. Identification and characterisation of novel steroids

We firstly assayed the conversion of 16OHP4 to 11,16diOHP4 catalysed by CYP11B and its 5α-reduction to 5α-pregnan-11β,16α-diol-3,20-dione (11,16diOH-DHP4), together with the 5α-reduction of 16OHP4 to 16OH-DHP4 by SRD5A. The reduction of 16OH-DHP4 by AKR1C2 was also assayed after which the identities of these novel C_{21} steroids were confirmed by accurate mass determination (Table 1).

The theoretical protonated mass $[M+H]^+$ of the respective metabolites was calculated and the accurate mass of the four novel steroids identified (Table 1, Fig. 1) which were detected as follows: 11,16diOHP4 at 347.22 (Fig. 1 A) with daughter ions identified as 347.22 > 121.064 and 347.22 > 311.2; 16OH-DHP4 at 333.24 (Fig. 1 B) with daughter ions identified as 333.24 > 213.2, 333.24 > 159.1 and 333.24 > 145.1; 11,16diOH-DHP4 at 349.24 (Fig. 1 C) and daughter ions identified as 349.24 > 313.4 and 349.24 > 97.1; and 3α,16α-dihydroxy-5α-pregnan-20-one (3,16diOH-DHP4) at 335.26 (Fig. 1 D) with daughter ions identified as 335.26 > 145.2, 335.26 > 118.7 and 335.26 > 104.6.

Establishing the accurate mass and fragmentation of these steroid metabolites enabled the subsequent UHPLC−MS/MS analysis of these steroids by MRM using an electrospray probe in the positive ionisation mode (ESI+).

3.2. The 11β-hydroxylation of 16OHP4 by CYP11B2

The diversion of steroids catalysed by CYP11B in adrenal steroidogenesis is clearly demonstrated in the production of 21-desoxycortisol (DOF; 4-pregnen-11β,17α-diol-3,20-dione), characteristic of 21OHD in which 17OHP4 accumulates. In a recent quantification of circulating C_{21} steroids in 21OHD patients, 17OHP4 and DOF were detected at significantly elevated levels, 67- and 35-fold, respectively, as well as a 28-fold increase in 16OHP4 (Turcu et al., 2015). The increased level of 16OHP4 in vivo, presents the possibility that the latter would, as in the case of 17OHP4, present as a possible substrate for CYP11B. The 11β-hydroxylation of 16OHP4 by CYP11B1 and CYP11B2 was investigated to determine the production of 11,16diOHP4 (Fig. 1 A).

Table 1
Accurate mass determination of novel steroid metabolites. UHPLC-MS/MS retention times, accurate mass, formulae and fragment ions are specified.

Steroid	Retention time (min)	Theoretical mass $(M + H)^+$	Observed accurate mass $(M + H)^+$	Formula	Fragments $(M + H)^+$
11,16diOHP4	1.78	347.22	347.22	$C_{21}H_{30}O_4$	121.064; 311.2
16OH-DHP4	2.89	333.24	333.24	$C_{21}H_{32}O_3$	213.2; 159.1; 145.1
11,16OH-DHP4	1.87	349.24	349.24	$C_{21}H_{32}O_4$	313.4; 97.1
3,16diOH-DHP4	3.09	335.26	335.26	$C_{21}H_{34}O_3$	145.2; 118.7; 104.6

Although CYP11B2 was able to hydroxylate ±35% 16OHP4 after 24 hrs, hydroxylation by CYP11B1 was negligible (Fig. 2 A; data is presented as substrate remaining after 24 hrs). A comparative analysis of the metabolism of P4 (1 μM) by CYP11B1 and CYP11B2 showed 60% and 50% substrate conversion, respectively. To the best of our knowledge, this is the first time that the conversion of P4 has been shown to catalysed by CYP11B1 and 2 (Fig. 2 B).

It is thus clear from the data that 16OHP4 is converted by CYP11B2, but not by CYP11B1. The zonal distribution of these enzymes — with CYP11B2 restricted to the glomerulosa (Gomez-Sanchez et al., 2014), and CYP17 not expressed in this zone (Sasano et al., 1989), questions the physiological relevance of this reaction. However, the depletion of sodium resulted in the elevated expression of CYP11B2 in the zona glomerulosa in rats and in the migration of these cells expressing CYP11B2 to neighbouring zona (Ogishima et al., 1992). It could thus be possible that the 11β-hydroxylation of 16OHP4 may occur at the interface cells of zonae glomerulosa and fasciculata, where CYP17A1 is highly expressed in the latter. Similarly, high levels of 11β-hydroxyandrostenedione (11OHA4) are produced in the adrenal with A4 mainly biosynthesised from DHEA in the zona reticularis, bordering the zona fasciculata which expresses CYP11B1 (Gomez-Sanchez et al., 2014). It has been shown that potassium and salt depletion up-regulated the expression of CYP11B2 in human vascular endothelial cells and in rats thus suggesting that in conditions such as salt-wasting CAH, the biosynthesis of 11,16diOHP4 may be possible (Bassett et al., 2004; Ogishima et al., 1992; Takeda et al., 1996). Moreover, CYP11B2 expression has also been detected in human hearts, with levels elevated in myocardial fibrosis samples, suggesting a role in chronic heart failure (Dhayat et al., 2015; Kayes-Wandover and White, 2000). Increased adrenal production of 16OHP4 under conditions such as CAH may lead to elevated circulating levels and subsequent hydroxylation by CYP11B2 in the heart. In the case of 21OHD in CAH, the traditional CYP11B1 and 2 substrates, cortisol and deoxycortisol, are absent, and with feedback mechanisms in place, stimulation of these enzymes by ACTH and AngII, may support the hydroxylation at C11 of the elevated C_{19} and C_{21} steroids associated with 21OHD.

3.3. The 5α-reduction of 16HOP4 by SRD5A1 and SRD5A2

The 5α-reductase enzymes, SRD5A1 and SRD5A2 are ubiquitously expressed within the metabolic/cardiorespiratory-, neurological-, immune-, sensory- and reproductive systems as well as in bone and skin. These isozymes play a major role in steroid modification regarding receptor interaction — where 5α-reduction yields active androgens, with these enzymes converting testosterone (T), 11β-hydroxytestosterone (11OHT) and 11keto-testosterone (11 KT) to DHT, 11β-hydroxydihydrotestosterone (11OHDHT) and 11keto-dihydrotestosterone (11KDHT), respectively (Bloem et al., 2013; Penning, 2014; Storbeck et al., 2013). Glucocorticoids, however, are reduced by 5α-reductase to inactive ligands in terms of glucocorticoid receptor interaction associated with gene activation (Nixon et al., 2012). The reduction of P4 to DHP4 has been observed upon incubation of P4 with rat brain slices (Compagnone and Mellon, 2000) and it has been shown to be a better substrate for

5α-reductase compared to T (Frederiksen and Wilson, 1971). Since Frederiksen and Wilson, (1971) assayed the metabolism of P4 and 17OHP4 and showed these P4 metabolites to be readily metabolised by SRD5A, 16OHP4 may follow the same metabolic pathway and produce active downstream metabolites. We thus assayed the reduction of 16OHP4 by SRD5A 1 and 2 and quantified the 5α-reduced metabolites (Fig. 1 B).

16OHP4 (1 μM) was efficiently reduced to 16OH-DHP4 (90–100%) by both isoforms (Fig. 3) with 50% conversion being achieved after 30- and 45 min by SRD5A1 and SRD5A2, respectively. We have shown comparable conversion of A4 and T to 5α-androstenedione (5α-dione) and DHT, respectively, under the same experimental conditions (50% conversion at 25- and 30 min). While the data suggests that the hydroxyl group at C16 does not hinder the 5α-reduction, the conversion of the C11-oxy C_{19} steroids on the other hand appears to be less efficient. A 50% conversion of these steroids to their respective 5α-reduced products was detected as follows: 11keto-androstenedione (11KA4), 65 min; 11OHA4, 75 min; 11 KT, 5 hrs; and 11OHT, 7½ hrs (Quanson, 2015). In addition, the oxidation of the C17 hydroxyl group by 17β-hydroxysteroid dehydrogenase (17βHSD) results in the 5α-reduction being even less efficient. These rapid conversions suggest that in tissue expressing SRD5A, especially in tissue expressing SRD5A such as in the liver (Thigpen et al., 1993), kidney (Quinkler et al., 2003), elevated in breast cancer (Lewis et al., 2004), PCa (Zhu and Imperato-McGinley, 2009) and the brain (Normington and Russell, 1992), 16OHP4 would be readily reduced as is the case for A4 and T. However, the impact on downstream physiological effects would depend on receptor interaction, whether the 5α-reduction of C_{21} steroids yields more active ligands, or whether the steroid metabolites are further metabolised.

Furthermore, both P4 and 17OHP4 are reduced by SRD5A to yield DHP4 and 5α-pregnan-17α-ol-3,20-dione (Pdione), respectively (Gupta et al., 2003; Sinreih et al., 2015). Subsequent reduction of Pdione by AKR1C2 produces 3α-hydroxy-5α,17α-pregnan-20-one (Pdiol), a better substrate for the CYP17A1 lyase activity than the natural substrates, leading to the production of DHT in the backdoor pathway (Gupta et al., 2003). The pathway yields potent androgens able to activate receptors which have physiological effects in steroidogenic peripheral tissue. As mentioned previously, since 16OHP4 inhibits the lyase activity of CYP17A1 (Inano and Tamaoki, 1978), high levels of the steroid may impede the reactions in the backdoor pathway during male sexual development. Both the classical and backdoor pathways lead to the production of DHT and have been shown to be actively involved in foetal androgen biosynthesis during male sexual differentiation and development (Flück et al., 2011). Elevated 17OHP4 and 16OHP4 levels are linked to clinical conditions and detected in testicular feminising patients (Griffiths et al., 1963; Sharma et al., 1965). High 16OHP4 levels are also present in testes tissue from PCa patients, levels which were decreased upon estrogen treatment (Oshima et al., 1967). The use of estrogen in clinical strategies inhibit tumour growth, with estradiol suppressing tumour androgen levels — possibly inhibiting tumour steroidogenesis while not associated with the estrogen receptor (ERα/β) (Montgomery et al., 2010). With the detection of 16OHP4 and 5α-reduced metabolites
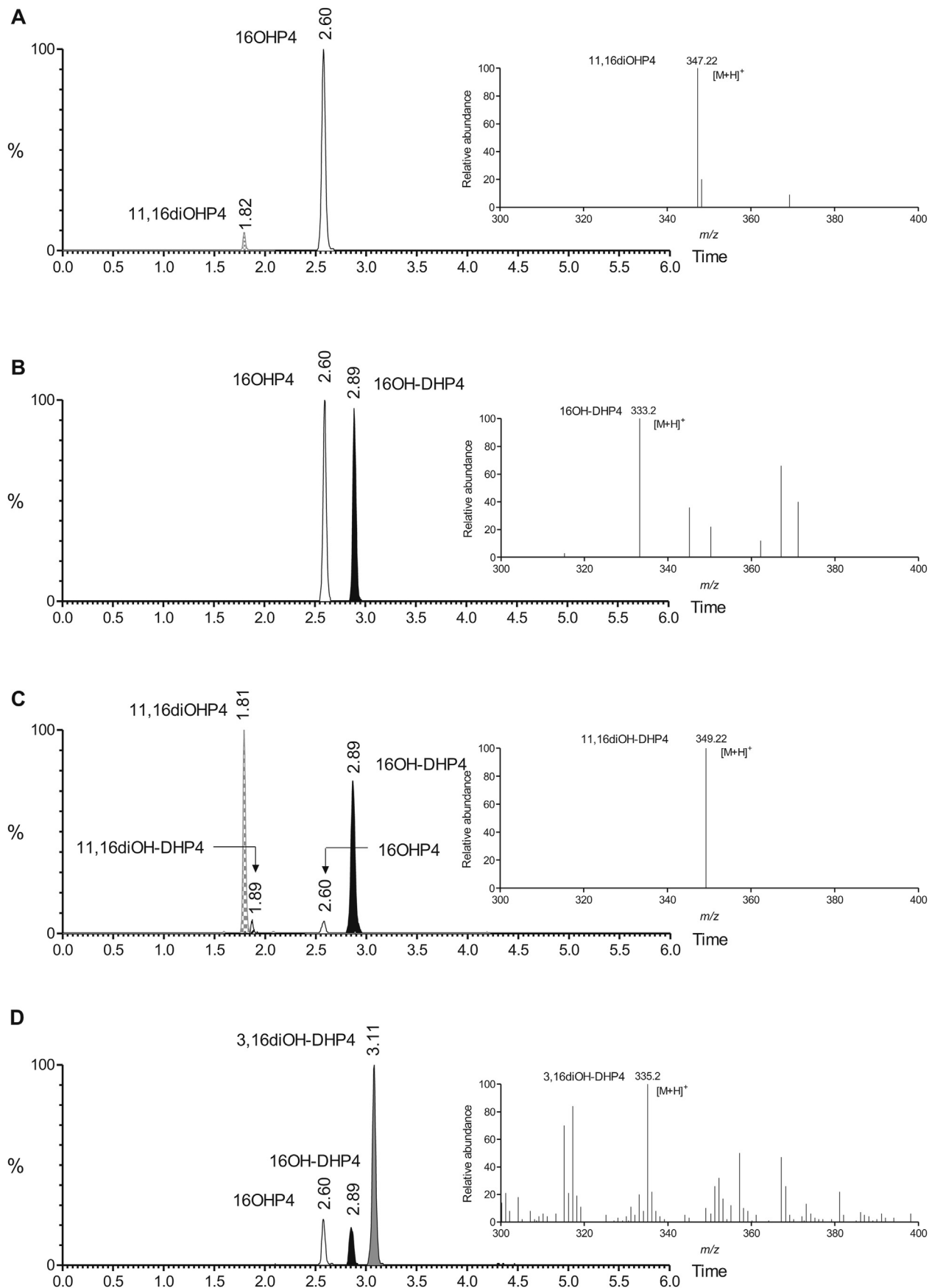
Fig. 1. UHPLC−MS/MS chromatographic separation of 16OHP4 metabolites with MS spectra of novel steroids [M+H]$^+$. (A) 16OHP4 and 11,16diOHP4 with 11,16diOHP4, m/z 347.22; (B) 16OHP4 and 16OH-DHP4 with 16OH-DHP4, m/z 333.2 (C) 16OHP4, 16OH-DHP4, 11,16diOHP4 and 11,16diOHP4 with 11,16diOH-DHP4, m/z 349.22; and (D) 16OHP4, 16OH-DHP4 and 3,16diOH-DHP4 with 3,16diOH-DHP4, m/z 335.2. Retention times are shown representing steroid compounds of a single injection shown in MRM mode. The steroids are indicated by relative abundance (%).
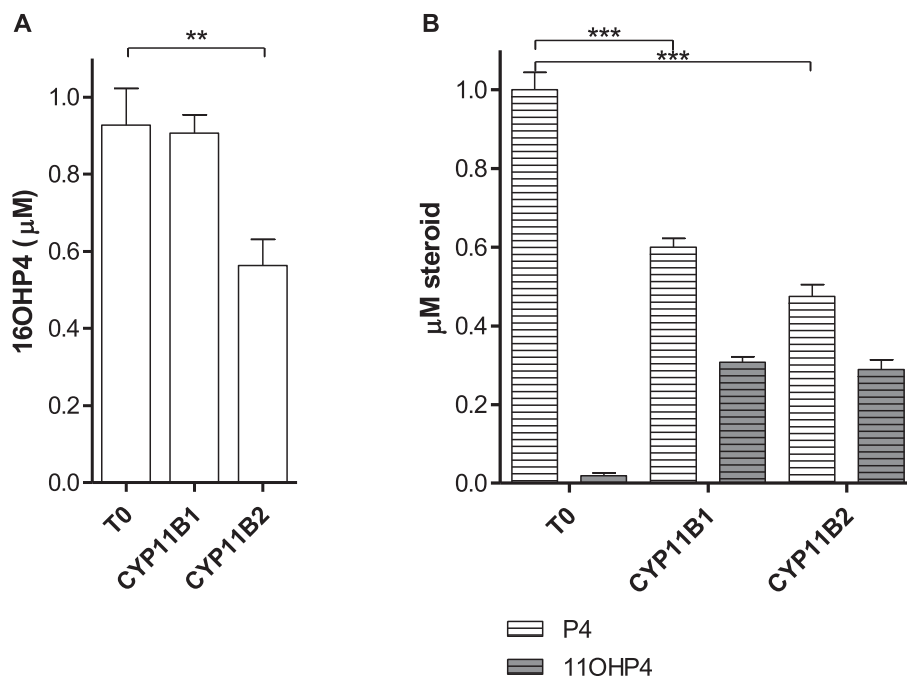
Fig. 2. CYP11B1 and CYP11B2 hydroxylation of C_{21} steroids (1 μM). Conversion after 24 hrs in transiently transfected HEK293 cells of (A) 16OHP4 and (B) P4. Results are expressed as the mean ± SEM of four independent experiments performed in triplicate (**$P \leq 0.01$, n = 4) for 16OHP4 and of three independent experiments performed in triplicate (***$P \leq 0.001$, n = 3) for P4.
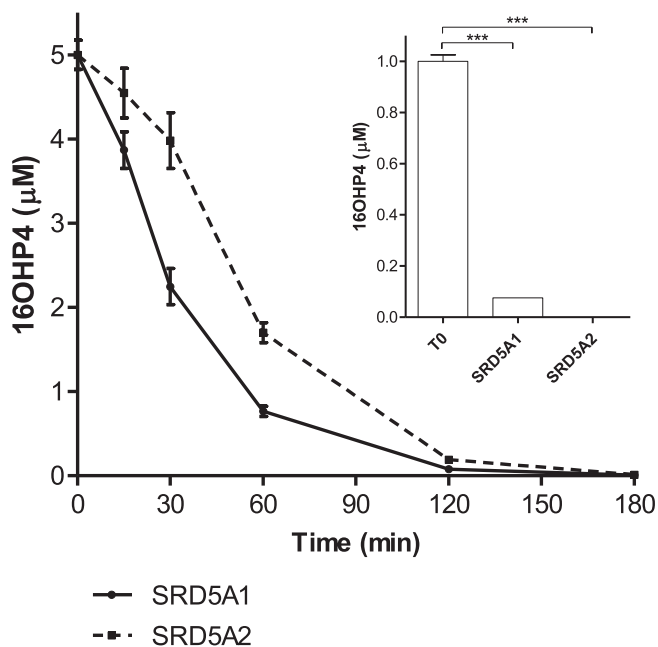
Fig. 3. 5α-reduction of 16OHP4 by SRD5A1 and SRD5A2. Conversion after 3 hrs in transiently transfected HEK293 cells. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate (***$P \leq 0.001$, n = 3).

in healthy testes and the up-regulated expression of SRD5A1 in castration resistance prostate cancer (CRPC) (Montgomery et al., 2008), the metabolism of 16OHP4 to 16OH-DHP4 by the SRD5A isozymes may be relevant in PCa, as it may follow the same fate as 17OHP4, and contribute to the backdoor pathway and the production of active androgens.

3.4. The 5α-reduction of 11,16diOHP4 to 11,16diOH-DHP4

With several studies reporting high levels of 16OHP4 in steroidogenic tissue, including in the testes from PCa patients, and having established that it can be hydroxylated at C11, it is feasible that the 11β-hydroxylation may take place in tissue other than the adrenal. Although studies have shown both CYP11B1 and CYP11B2 are expressed in human primary prostate carcinomas and metastatic primary prostate carcinomas (Mitsiades et al., 2012) while CYP11B2 was shown to be present in androgen dependent prostate cancer cells, LNCaP cells, in the presence of dutasteride, a SRD5A inhibitor (Biancolella et al., 2007) protein function has, however, not been shown. Nevertheless, as the presence of SRD5A may result in the 5α-reduction of the 11,16diOHP4 in peripheral tissue, we investigated whether the steroid acts as a substrate for SRD5A1 and SRD5A2.

11,16diOHP4 was prepared in HEK293 cells using excess substrate (16OHP4) which would yield adequate amounts of the metabolite and allow the analyses of its downstream 5α-reduction. Analyses identified four metabolites confirming that 16OHP4 was not fully converted to 11,16diOHP4 after 24 hrs with the remaining 16OHP4 being converted to 16OH-DHP4 by SRD5A (Fig. 1 C). The high concentration required for the Q-TOF analysis and the moderate conversion of 16OHP4 by CYP11B2, resulted in ±70% substrate remaining (7.273 μM) and only ± 30% converted to 11,16diOHP4 in the first assay (Fig. 4 A). The media was subsequently transferred and incubated with SRD5A1 and SRD5A2 and analysed after 6 hrs, showing the efficient 5α-reduction of the remaining 16OHP4 to 16OH-DHP4, 0.061 μM and 0.030 μM, respectively (Fig. 4 B). Although analysis of ionisation signals/integrated peak areas of metabolites (Fig. 4 C and D) is by no means a quantitative measure as such it confirmed the presence of the novel steroids. In the first reaction, catalysed by CYP11B2, 16OHP4 was detected at the onset of the reaction (indicated at time point zero [T_0]) while both 16OHP4 and 11,16diOHP4 were detected after 24 hrs (Fig. 4 C). The 5α-reduced products were subsequently identified when first
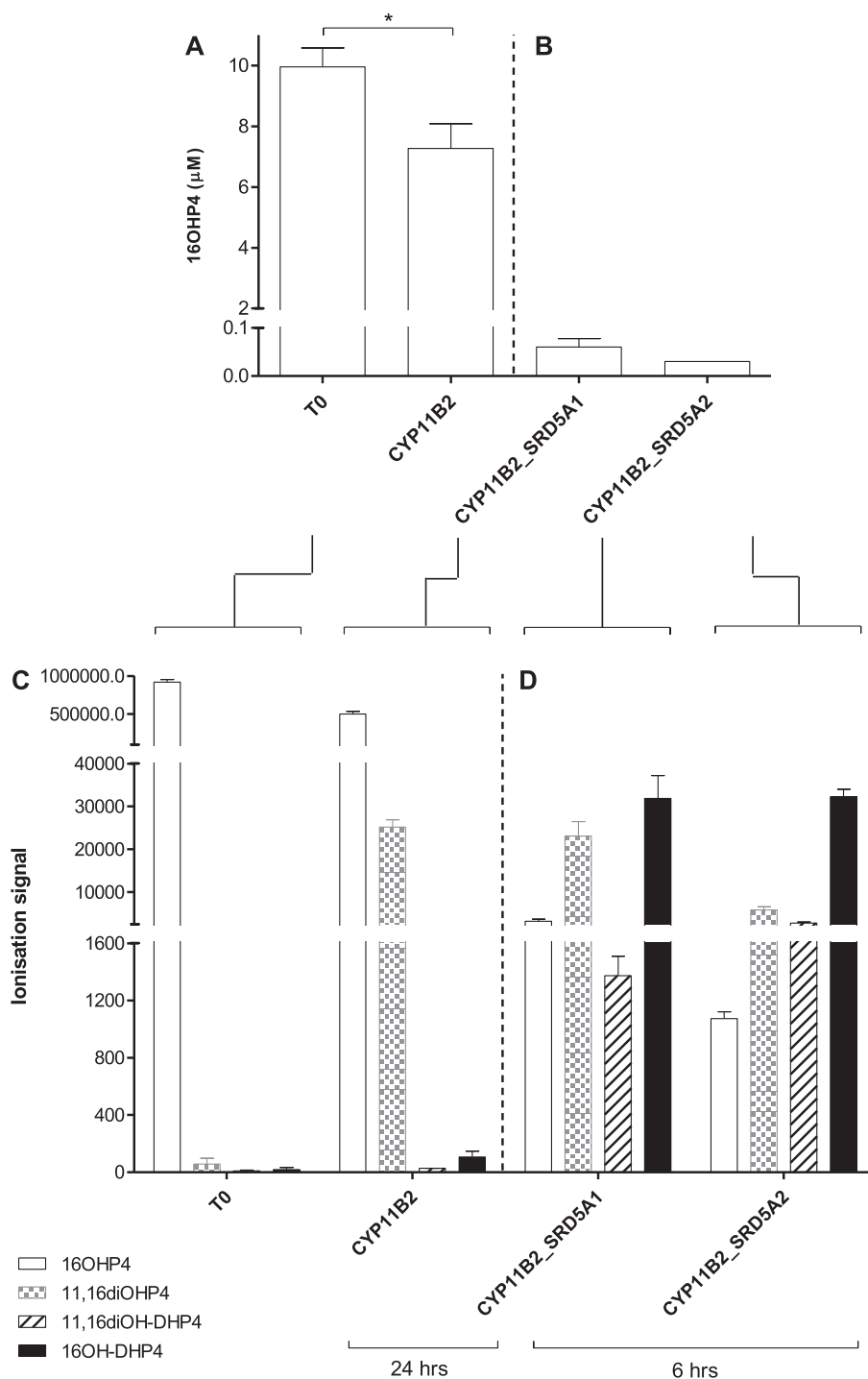
Fig. 4. Metabolism of 16OHP4 (10 μM) by CYP11B2 and SRD5A in transiently transfected HEK293 cells. (A) Conversion of 16OHP4 (10 μM) by CYP11B2 after 24 hrs; (B) Conversion of 16OHP4 by SRD5A1 and SRD5A2 after 6 hrs; (C) Ionisation signal/integrated peak area of 16OHP4, 11,16diOHP4 and 16OH-DHP4 in the CYP11B2 conversion assay; and (D) Ionisation signal/integrated peak area of 11,16diOH-DHP4, 16OH-DHP4, 11,16diOHP4 and 16OHP4 in the SRD5A conversion assay. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test. Results are expressed as the mean ± SEM of an experiment performed in triplicate (*$P \leq 0.05$, n = 2).

reaction steroids were transferred to cells expressing SRD5A, with the control samples once again confirming conversion. The 5α-reduced metabolites, 16OH-DHP4 and 11,16diOH-DHP4 were detected and identified together with their substrates 16OHP4 and 11,16diOHP4 (Fig. 4 D). The data suggests that the catalytic activity of the SRD5A isoforms are similar towards 16OHP4, while the 5α-reduction of 11,16diOHP4 by SRD5A2 was greater than that by SRD5A1 — however, as mentioned above, based on comparisons of

ionisation signals, product levels can only be determined by the inclusion of steroid reference standards. It is thus possible that 11,16diOHP4, once produced, is more likely to be reduced by SRD5A2 in the normal physiological milieu. The low rate of conversion of 16OHP4 to 11,16diOHP4 compared to the more rapid 5α-reduction of 16OHP4 calls into question the C11-hydroxylation reaction *in vivo*. Nevertheless, the hydroxylation reaction cannot be excluded, as increased levels of 16OHP4 and CYP11B expression in

peripheral tissue other than the adrenal, associated with disease conditions, have been reported.

3.5. The reduction of 16OH-DHP4 to 3,16diOH-DHP4 by AKR1C2

In the subsequent metabolism of SRD5A reduced C_{21}- as well as C_{19} steroids, the 3αHSD isoforms of the aldo-keto reductase (AKR) superfamily catalyse the conversion of functional groups at C3, 17 and 20. The reductive direction of AKR1C2 converts 5α/β-reduced steroid hormones to the C3-hydroxy steroids, generally considered to be inactive hormones regarding steroid receptor transactivation. Since 17OHP4 is reduced by SRD5A and AKR1C2 in the backdoor pathway in steroidogenic tissue it is possible that 16OHP4, being readily converted by both the SRD5A isozymes, may yield a potential substrate for AKR1C2 expressed in peripheral tissue such as the liver, prostate, uterus, testes, kidney and breast, particularly relevant in disease and adrenal disorders characterised by elevated 16OHP4 (Lewis et al., 2004; Penning et al., 2000; Quinkler et al., 2003; Rizner et al., 2003). 16OH-DHP4 was prepared and the metabolism by AKR1C2 assayed in HEK293 cells. Steroid metabolites were analysed using UHPLC-MS/MS (Fig. 1 D), and the novel steroid, 3,16diOH-DHP4 identified and daughter ions characterised. Due to the lack of standards and the similar molecular mass of 16OH-DHP4 metabolites reduced at either C3 or C20, the site of reduction is uncertain. However, in the reduction of 5β-reduced P4 by AKR1C2 a 66:33 ratio for C3:C3,C20 steroid metabolites has been reported. Although the reduction of the C3 keto group would depend on the position of the steroid in the active pocket, which is very similar if substrates are docked in the same orientation (Jin et al., 2011), this would also depend on the influence of the C16-hydroxy moiety on substrate orientation. Nevertheless, 16OHP4, 10 μM, was rapidly converted to 16OH-DHP4 after 6 hrs by SRD5A1 and SRD5A2 with negligible substrate remaining (0.524 and 1.060 μM, respectively) (Fig. 5 A and C). In the subsequent conversion by AKR1C2, analyses after 24 hrs showed the remaining substrate had been converted to 0.11- and 0.621 μM respectively (Fig. 5 B and D). Fig. 5 C and D illustrates the ionisation signal/integrated peak area of the compounds confirming the presence of 16OH-DHP4 and the formation of 3,16diOH-DHP4. Although AKR1C2 reduces the keto moiety at C3, it is also possible that the keto group at C20 may be reduced resulting in the formation of pregn-4-ene-16α,20α-diol-3one, however, it would appear to be unlikely as 16OHP4 has been shown to inhibit the 20α-hydroxysteroid dehydrogenase activity non-competitively (Inano and Tamaoki, 1978). While the intensities of 16OHP4 remained unchanged in the control reaction, a low intensity signal was detected possibly representing a reduced product indicating conversion by endogenous enzymes, possibly contributing to the decrease detected in 16OHP4 levels. Similar reductive conversions have been reported in endometrial carcinoma cell models expressing SRD5A1 and AKR1C2, in which the reduction of the C20-keto group was detected without the prior 5α-reduction of P4 (Sinreih et al., 2015). Elevated levels of Pdiol have been detected in the urine of 21OHD patients indicating metabolism via the backdoor pathway and together with Pdiol being readily converted by CYP17A1 in order to produce androsterone (Gupta et al., 2003; Kamrath et al., 2012), it may be that 16OHP4 follows a similar route in the production of active androgens. However, 16OHP4 has been shown to be a competitive inhibitor of the 17,20 lyase activity of CYP17A1 and its contribution to the adrenal disorder may thus be favourable.

While the 3α-reduction of steroids is considered to act as an inactivation reaction of potent steroid hormones an inverse effect is observed in the nervous system. DHP4 and its 3α-reduced metabolite (allopregnanolone) were the major metabolites produced when labelled P4 was incubated with rat brain tissue slices (Korneyev et al., 1993). Allopregnanolone, a potent neurosteroid elevated at pre-parturition, exerts its effect by interacting with the $GABA_A$ receptor (Concas et al., 1998; Majewska et al., 1986). A shift in the sensitivity of the $GABA_A$ receptor towards neurosteroids is observed between late-pregnancy and after birth when the receptor becomes insensitive to allopregnanolone (Koksma et al., 2003). The sensitivity of the receptor towards neurosteroids was found to be dependent on intracellular calcium levels, phosphorylation and to the activation status of the oxytocin receptor. Oxytocin administration to rats resulted in a rapid desensitisation of the $GABA_A$ receptor to allopregnanolone. Similarly, 16OHP4 levels increased during pregnancy but declined rapidly upon parturition (Den et al., 1979) suggesting a possible association with oxytocin. Furthermore, as it has been shown that oxytocin increased SRD5A expression levels in human and rat prostate and as well as up-regulating endometrial SRD5A expression (Assinder, 2008; Assinder et al., 2004). The reduction of 16OHP4 levels associated with parturition may be attributed to the rapid reduction to 16OH-DHP4 possibly triggered by oxytocin.

3.6. The interaction of DHP4, 16OHP4 and 16OH-DHP4 with the PR and AR

Putative physiological effects of DHP4 and 16OH-DHP4 would be mediated by their binding to intracellular steroid receptors. While it has been reported that DHP4 binds to the PR (Jewgenow and Meyer, 1998), the study did not, however, distinguish between PR isoforms. We thus investigated whether DHP4 and 16OH-DHP4 would exhibit agonist activity via both PR isoforms, PR-A and PR-B. In addition, since ovarian 16OHP4 levels are increased in patients with PCOS, and excess androgen production is associated with PCOS, we also investigated the putative androgenic properties of these metabolites. HEK293 cells were transiently transfected with human PR-A, PR-B or AR cDNA expression vectors together with a P4 response element (PRE) -containing promoter-reporter construct and treated with 100 nM ligand. Note that due to the high degree of conservation within the DNA binding domain of steroid receptors, most steroid receptors bind the same DNA response element, and thus the PRE also serves as a response element for the AR. Results show that DHP4 is a weak partial agonist for transactivation via both PR-A (Fig. 6 A) and PR-B (Fig. 6 B), while 16OHP4 and 16OH-DHP4 are weak partial agonists for PR-B, but not PR-A. We previously reported that at 10 μM 16OHP4 displayed similar PR agonist activity to P4 in COS-1 cells (Storbeck et al., 2011), suggesting that the effects are concentration-dependent while the cell model systems may also impact data due to the expression of endogenous enzymes metabolising steroid metabolites.

To the best of our knowledge, this is the first study showing that DHP4 is a weak partial agonist for both PR isoforms, suggesting that this metabolite would exert distinctive physiological effects via either the PR-A or PR-B as it is widely reported that these isoforms display different physiological functions in different target tissues (Conneely et al., 2002; Hung et al., 1994). In addition, our data show that the 5α-reduction of P4 results in a significant decrease ($p \leq 0.001$) in activity via the PR, while the 5α-reduction of C_{19} steroids lead to more potent androgens in terms of the AR, as in the case of DHT and 11KDHT. In the present study the C_{21} steroids exhibited very weak androgenic activity irrespective of the 5α-reduction of P4 and of 16OHP4 (Fig. 6 C). We have previously shown, also in COS-1 cells, that P4 displays weak partial agonist activity via the AR and is in fact a relatively potent AR antagonists (Africander et al., 2014). Our data suggest that 16OHP4 and 16OH-DHP4 may elicit PR-B-mediated biological activities comparable to DHP4 in target tissues, while these metabolites may also exhibit
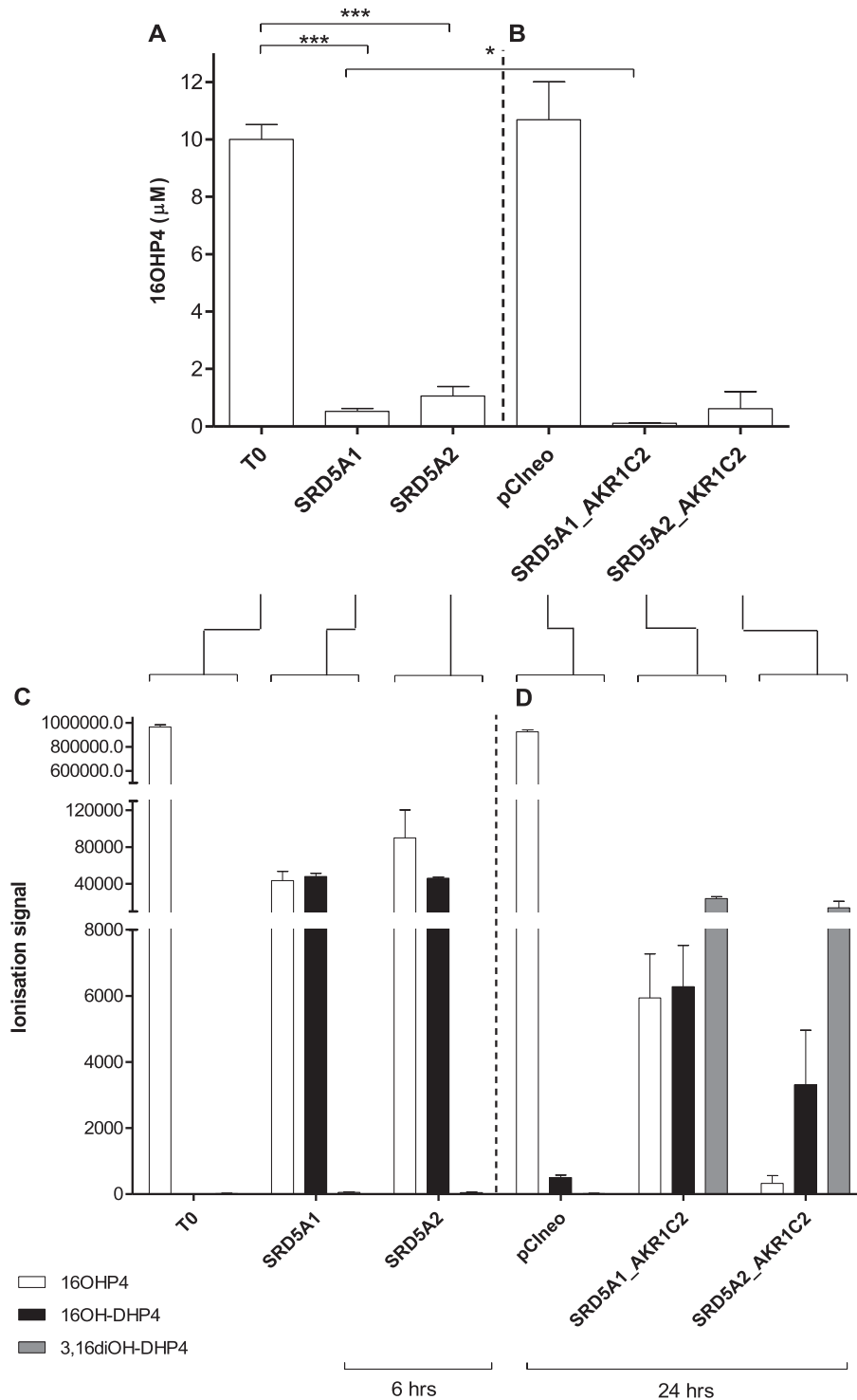
Fig. 5. Metabolism of 16OHP4 by SRD5A and AKR1C2 in transiently transfected HEK293 cells. (A) Conversion of 16OHP4 (10 μM) by SRD5A1 and SRD5A2 after 6 hrs; (B) Conversion of 16OHP4 by AKR1C2 after 24 hrs; (C) Ionisation signal/integrated peak area of 16OHP4 and 16OH-DHP4 in the SRD5A conversion assay; and (D) Ionisation signal/integrated peak area representing 16OHP4, 16OH-DHP4 and 3,16diOH-DHP4 in the AKR1C2 conversion assay. Statistical analysis was done using a one-way ANOVA followed by a Dunnett's multiple comparison test and a *t*-test. Results are expressed as the mean ± SEM of two independent experiments performed in triplicate (*P ≤ 0.05, ***P ≤ 0.001, n = 2).

physiological effects via PR-A and the AR, albeit weak.

It is also possible that 16OHP4 and/or 16OH-DHP4 may activate the ER and the MR. ERα levels together with those of the PR have been shown to be increased in tissue obtained from boys with unilateral cryptorchidism when compared to normal tissue and it was suggested that these receptors play an important role in

normal testicular descent (Przewratil et al., 2004). While 16OHP4 does not interact with the MR its 5α-reduced form may. The expression of the MR has been shown in the ovary — both at mRNA level and protein level together with that of both 11β-hydroxysteroid dehydrogenase (11βHSD) isoforms (Gomez-Sanchez et al., 2009). Activation of the MR by 16OH-DHP4 may indeed lead to
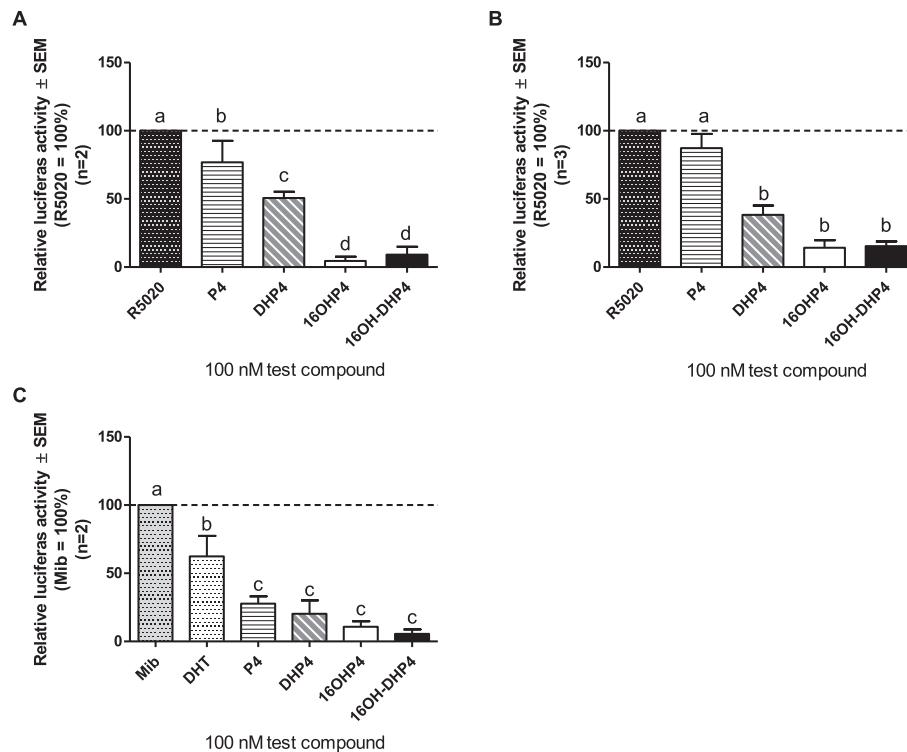
Fig. 6. Receptor interaction of P4, 16OHP4, DHP4 and 16OH-DHP4 with the hPR isoforms and the hAR. HEK293 cells expressing a luciferase promoter-reporter construct (pTAT-PRE-E1b-luciferase) and human receptors (A) hPR-A (pSG5-hPR-A), (B) bPR-B (pSG5-hPR-B) or (C) hAR (pSG5-hAR), were treated with the ligands (100 nM) for 24 hrs. Induction is shown as % luciferase activity, with the synthetic receptor-selective agonists R5020 (A and B) or Mib (C) set as 100%. Results shown are the averages (±SEM) of 2 independent experiments performed in triplicate. Statistical analysis was done using one-way ANOVA followed by a Newman-Keuls post-test (comparing all pairs of columns). Differences in statistical significance are designated by the letters a, b and c.

the induction of specific target specific genes relevant to PCOS while the expression of 11βHSD 1 and 2 may play a critical role in the balance of C11-oxy C_{21} and C_{19} steroids in the ovary.

The roles of SRD5A and AKR1C2 are very specific in terms of steroid activation/inactivation, thereby regulating receptor interaction and occupancy. However, sex steroids in circulation crossing the blood-brain-barrier and acting as neurosteroids have very specific effects via the membrane-bound chloride-ion gated channel $GABA_A$ receptor. Downstream progesterone metabolism entails either the reduction of the C3-keto group or the 5α-reduction by SRD5A yielding DHP4 and subsequently the reduction at C3 to yield allopregnanolone, depending on the level of expression of the relevant enzymes. While it is known that DHP4 mediates effects through the $GABA_A$ receptor, while P4 mediates effects only via the PR and allopregnanolone only via the $GABA_A$ receptor, we show that DHP4 also mediates effects via the PR. It has been shown that AKR1C1 and AKR1C2 are the most abundant isoforms catalysing the production of neuroactive tetrahydrosteroids in the brain, and these tetrahydrosteroids also modulate the $GABA_A$ receptor. P4, once reduced by SRD5A and subsequently at C3 to form allopregnanolone, is able to enhance a $GABA_A$-induced response at 10—100 nM, with data suggesting that the modulatory effects of the steroid are partly regulated by the enzymatic reduction of the keto moiety at C3 (Belelli and Lambert, 2005; Mellon, 2007; Penning et al., 2000). Our study showed that DHP4 can activate both PR isoforms and to a lesser degree the AR and that the interaction of 16OHP4 with these steroid receptors was weak. Although we did not investigate effects via the $GABA_A$ receptor, these potential effects cannot be ruled out. Increased 16OHP4 levels associated with parturition and possible *de novo* biosynthesis of the steroid - CYP17A1 has been shown to be expressed in the human fetal

nervous system (Schonemann et al., 2012), may suggest that upon reduction by SRD5A as well as AKR1C2, non-genomic effects via the $GABA_A$ receptor may ensue and/or, may have other physiological effects as has been shown in the case of 17OHP4 which impacts cervical ripening ensuring parturition once it's reduced to Pdiol (Mahendroo et al., 1999).

4. Conclusion

The present study provides evidence of the downstream metabolism of 16OHP4 by steroidogenic enzymes producing novel steroid metabolites. Both 16OHP4 and its 5α-reduced metabolite, which would be rapidly formed upon reaching peripheral tissue, activated the PR-B as weak partial agonists while DHP4 activated both the PR-A and PR-B as a partial and a weak partial agonist, respectively, exhibiting significantly lower activity than P4. A similar trend was observed regarding the AR, with all steroids assayed exhibiting only weak partial agonist activity. Our data clearly indicates an inactivating role for SRD5A regarding P4 and its interaction with both PR isoforms. AKR1C2 would also contribute further to the inactivation of DHP4, as well as 16OH-DHP4, once the C3 keto group is reduced. However, in terms of the $GABA_A$ receptor, the role for SRD5A may be the opposite thus modulating $GABA_A$ receptor-induced responses.

Although the clinical relevance of 16OHP4 is uncertain, the steroid has been described in normal physiological processes, in disease conditions and in cancers. That the steroid has a role to play in the foetal adrenal, in pregnancy and in the birth processes is clear with 16OHP4 being elevated during pregnancy and declining rapidly with the onset of parturition. Our data to date indicate that steroid receptor interaction is concentration dependent, however,

the induction of target genes upon activation remains to be determined. That 16OHP4 homeostasis is critical to normal reproduction and development is evident from the significantly elevated levels thereof in testicular feminization syndrome and cryptorchidism together with abnormal levels having being linked to other clinical conditions and disorders such as CAH, PCOS and 21OHD. The clinical significance of 16OHP4 and 16OH-DHP4 has not been previously considered and our data show, for the first time, comparable activation of the AR and PR isoforms. Their activation of the ER and the MR by 16OH-DHP4 remains to be determined − given the well-established contribution of steroid receptors to clinical conditions it is fair to speculate that the steroid and its metabolites may contribute towards the development and progression of the aforedescribed diseases. Should 16OHP4 and 16OH-DHP4 be present at high concentrations within the steroid pool and be inefficiently inactivated and conjugated, they may very well exert effects comparable to the more potent steroid ligands. What thus remains is the extensive clinical profiling of these steroids, and the investigation into their potential induction of receptor-activated genes and their elimination from target tissue.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.09.025.

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CHAPTER 5

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Experimental protocols

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This chapter provides a detailed description of the experimental protocols employed in this study. Protocols include tissue culturing of the non-steroidogenic HEK-293 cells and LNCaP cells, experimental design, and steroid identification and analysis using Q-TOF MS, UHPLC-MS/MS and UPC^2-MS/MS.

5.1   Materials

The materials were described in detail in chapter 3 and 4. In addition, a complete list of steroids is provided in the section below. Steroids (A4; T; 5α-dione; AST; DHT; P4; 17OHP4; Pdione; DOC; CORT; ALDO; deoxycortisol, cortisol and cortisone) were purchased from Sigma-Aldrich (St. Louis, USA). 16OHP4; DHP4; 11OHP4; 11KP4; 11K-DHP4; alfaxalone; Pdiol; pregnanetriol; DOF; 21-desoxycortisone, DOE; DHEA, 11OHAST; 11KAST; 11OHA4; 11OHT; 11KA4; 11KT; 11KDHT; and DHT-G, were obtained from Steraloids (Wilton, USA).

5.2   Plasmid constructs and preparation

Plasmid constructs included: ovine pcDNA3.1-CYP17A1, human pCMV-CYP11B1, pCMV-CYP11B2, human pTrc99A-ADX, human pCMV7-SRD5A1 and pCMV7-SRD5A2, human pcDNA3-AKR1C2, pTAT-GRE-E1b-luc, human pSG5hPR-A and PR-B; pSG5hPR-B, human pSG5/hAR, pCI-neo (Promega).

Plasmid constructs encoding the cDNA of the respective enzymes used in the conversion assays were prepared as follow: Luria-Bertani (LB) medium (tryptone, 1%; yeast extract, 0.5%; NaCl, 1%; pH 7) was inoculated with plasmid *E. coli* freezer stocks (80% glycerol) and incubated at 37°C in an Innova shaking incubator (New Brunswick) at 230 rpm for 12-16 hrs. The plasmid was isolated and purified using either a Promega Wizard® Plus Midipreps DNA purification system (Madison, USA) or, in the case of ovine CYP17A1, a Macherey-Nagel Nucleobond® Maxiprep DNA isolation kit (Duren, Germany) according to the manufacturer instructions. Using the midiprep kit, 100 mL LB media was inoculated with 100 µL of a *E. coli* 5 mL overnight starter culture and using the maxiprep kit 300 mL LB media was inoculated with 300 µL of an overnight starter culture. All the plasmids used coded for ampicillin resistance, hence ampicillin (100 µg/mL) was added to the media prior to inoculation. The culture was centrifuged at 4°C after which the cells were lysed, the cDNA purified and dissolved in 200 µL nuclease-free H$_2$O. The plasmid yield (µg/µL) was determined using UV spectrophotometry measuring at 260 and 280 nm (Cary 60 UV-VIS; Agilent technologies). Appropriate dilutions of the purified cDNA were made with nuclease-free H$_2$O to allow the optimal density to range between 0.1 and 1.0 – the linear range. An absorbance (Abs) ratio (260/280 nm) of 1.8 ±0.1 was optimal. The DNA concentration was determined

using the following equation (1) and the measurements summarized in table 5.1. All plasmids were validated through restriction enzyme digest and agarose electrophoresis.

$$\left[\frac{\mu g}{\mu L}\right] = \frac{Average\ of\ Absorbance\ @260nm\ x\ dilution\ factor\ x\ 50\mu g/mL}{1000} \tag{1}$$

Table 5.1

Absorbance ratio, cDNA concentration (μg/μL) and yields together with the culture volume and purification prep kit used.

Plasmid	260/280 ratio	[μg/μL]	Yield (μg)	Culture volume	Kit
pCMV7/SRD5A1	1.8551	0.9525	190.5	100 mL	Promega
pCMV7/SRD5A2	1.8369	1.3358	267.2	100 mL	Promega
pCI-neo	1.8560	2.697	539.4	100 mL	Promega
pCMV/CYP11B1	1.8316	1.709	341.8	100 mL	Promega
pCMV/CYP11B2	1.7077	0.8515	170.3	100 mL	Promega
pTrc99A ADX	1.8364	2.222	444.4	100 mL	Promega
pcDNA3/AKR1C2	1.8858	0.714	142.8	100 mL	Promega
pcDNA3.1/CYP17A1	1.7654	4.595	1,378.5	300 mL	NucleoBond

5.3   Steroid conversion assays in HEK-293 cells

HEK-293 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 0.0015% NaHCO$_3$ (m/v) (pH 7.0), FBS (10%; v/v) and antibiotics (1%) consisting of penicillin-streptomycin (10 000 U/mL and 10 mg/mL, respectively). Cells from freezer stocks, previously prepared in DMEM containing 5% DMSO and stored in liquid nitrogen according to ATCC instructions, were incubated until 80% confluent (2-3 days) at 37°C, 5.0% CO$_2$ and 90% relative humidity prior to collecting cells by trypsinization (1 mL/100 mm, trypsin (0.1%) and EDTA (0.04%)) and centrifugation (5 min at 600 x g). The cell pellet was resuspended in DMEM, filtered through a cell culture sieve, and the cells counted. Cell count and viability (%) were determined by adding trypan blue stain to the cell solution in a 1:1 ratio prior to using a Countess® automated cell counter (Invitrogen, Eugene, USA). Cell were subsequently

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replated, 1 x 10^5 live cell/mL, in Corning® CellBIND® 75 cm^2 flasks and allowed to reach 80% confluency. The protocol was repeated for 3-5 passages. Once confluent, the cells were replated in Corning® CellBIND® 100 mm dishes (10 mL/dish), or 24 well plates (0.5 mL/well) at a concentration of 2 x 10^5 live cells/mL. Cells which were replated in Corning® CellBIND® 12 well plates were seeded at a concentration of 1 x 10^5 live cells/mL. After a 24 hr incubation period the cells were transiently transfected with plasmid constructs encoding the appropriate enzymes by using the XtremeGene HP® DNA transfection reagent according to the manufacturer instructions. The transfection reaction mixture contained unsupplemented DMEM (0.1% of the well volume), plasmid DNA (1 µg/µL) and transfection reagent (in a 1:3 ratio, DNA:reagent) and was incubated for 15-20 min prior to addition to cells. The cells were incubated with the transfection reagent for 48-72 hrs after which the media was replaced with fresh media supplemented with the appropriate steroid substrate at the desired concentrations.

Steroid stocks of 2 mg/mL were made in absolute ethanol and added at appropriate concentrations to experimental media. After the incubation period, on completion of assay, an aliquot (500 µL) of steroid containing media was sampled and internal steroid standards added. Deuterated steroid standards were prepared in absolute ethanol (50 ng/µL). Per experiment, an internal standard master mix (0.15 ng/µL) containing the appropriate deuterated steroids was prepared in deionized H$_2$O. The internal standard mixture, 100 µL, was added to each sample aliquot prior to extraction.

Steroids were extracted from the media by liquid-liquid extraction using a 3:1 ratio *(v/v)* MTBE:culture media. Samples were vortexed for 15 min, and incubated for 20 min at -80°C, after which the organic phase was transferred to a clean test tube prior to being dried at 40°C under nitrogen. The dried steroids were redissolved in 150 µL HPLC-grade 50% methanol in deionised water and analysed by UHPLC-MS/MS. For all enzyme assays, pCI-neo (Promega, Madison, USA), a vector containing no insert DNA, was included as a negative control. Time course conversion assays were conducted in a similar manner with multiple 500 µL aliquots taken from the 100mm dish.

5.3.1   The conversion of 11K-DHP4 to alfaxalone by AKR1C2

Analyses of the conversion assays investigating the reduction of 11K-DHP4 by AKR1C2 (as described in chapter 3.3.6) were initially conducted without alfaxalone. Therefore, the decrease in substrate (11K-DHP4) was quantified and the product presented by the ion signal (Fig. 5.1). After obtaining alfaxalone, these samples were re-analyzed, quantified and presented as in chapter 3.3.6; fig. 3.12.
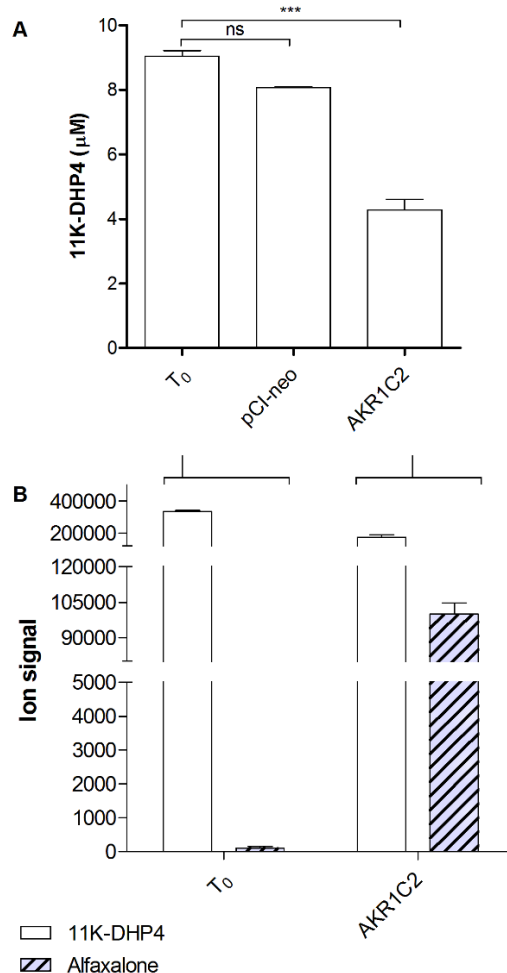
Figure 5.1   Metabolism of 10 µM 11K-DHP4 by AKR1C2 HEK293 cells. (A) Steroid substrate concentration and (B) ion signal at time zero and after 24 hrs.

5.4   Steroid metabolism in LNCaP cells

LNCaP cells were cultured at 37°C, 90% relative humidity and 5% CO_2 in RPMI-1640 media (pH 7). The media was supplemented with $NaHCO_3$ (0.0015%), D-(+)-Glucose (0.0025%), penicillin-streptomycin (1%), HEPES (1%), sodium pyruvate (1%) and FBS (1%). The cells were cultured and, when confluent, were counted as described in section 5.3 prior to replating in Corning® CellBIND® T75 flasks. In all experiments cells were seeded at a density of 2×10^5 live cells/mL in Corning® CellBIND® 12 well plates (1 mL/well). The cells were allowed to attach and grow for 48 hrs prior to substrate addition. Steroid substrate (11OHA4, 11OHP4 and 11KP4), 1 µM, was prepared by adding the appropriate volume from the steroid stocks (2 mg/mL) to RPMI-1640 culture media and incubating the cells for 48 hrs. Due to the presence of UGT enzymes, aliquots (500 µL) were collected in duplicate. One set of the duplicated aliquots was treated with β-glucuronidase prior to extraction. Testosterone-D2/D2-T (1.5 ng), progesterone-D9/D9-P4 (15 ng), 17-hydroxyprogesterone-D8/D8-17OHP4 (15 ng), androstenedione-D7/D7-A4 (15 ng) and 11β-hydroxy-androstenedione-D7/D7-

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11OHA4 (15 ng) were added to each sample as internal standards and steroid extracted as previously described. Steroid metabolism was determined by analyzing steroid metabolites using a UPC^2-MS/MS.

5.4.1 The deconjugation of glucuronidated steroids

β-glucuronidase (*E. coli* Type VII-A; 5 292 000 units/g) was dissolved in deionized H$_2$O (1 mg/mL) and stored at -20°C. One of the duplicate sets of aliquots (500 µL) collected on completion of experiments investigating the metabolism of 11OHP4 and 11KP4 in LNCaP was subjected to a glucuronidase digestion. The pH of the samples was determined using MColorpHast™ non-bleeding pH-indicator strips (Merck Millipore, Germany) followed by the addition of ±20 µL acetic acid (1%) to lower the pH to 6.5. The acidified samples were treated with β-glucuronidase (400 units; 76 µL;) and incubated for 24 hrs at 37°C to allow the deglucuronidation of steroids. Following the incubation period, internal standards were added and the steroids extracted as previously described (section 5.3).

As controls, DHT and DHT-G, 1 µM, were dissolved in RPMI-1640 and aliquots (500 µL) taken in duplicate. One set of duplicates was treated with β-glucuronidase while the other remained untreated. Steroids were extracted and DHT quantified in the treated and non-treated samples using the UPC^2-MS/MS (Fig. 5.2).
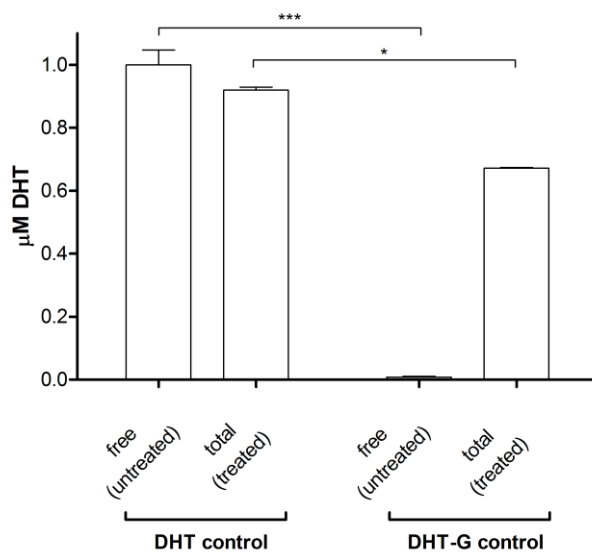
Figure 5.2   UPC^2-MS/MS analysis of DHT representing free and total DHT in β-glucuronidase control reactions.

5.5   Accurate mass determination and steroid analysis

Stock solutions of the steroids used in this study were prepared in absolute ethanol to a concentration of 2 mg/mL and stored at -20°C. Steroid standards were prepared to range from 0.001 to 1.00 ng/µL for UPC^2-MS/MS analysis and a set to range from 0.002 to 2.00 ng/µL for UHPLC-MS/MS analysis.

The standards were prepared in the same medium as the experimental medium, internal standards added followed by liquid-liquid extraction as described in section 5.3. The data was collected using Masslynx Software 4.1 software (Waters Corporation).

5.5.1 *Identification and accurate mass determination of steroid using Q-TOF-MS.*

A Waters Synapt G2 Q-TOF mass spectrometer (Milford, USA) was used to determine the accurate mass and daughter ions of the novel and/or unidentified steroids using positive ionisation (ESI+). An ACQUITY UPLC (Waters, Milford, USA) using a Waters BEH C18 column (2.1 mm x 100 mm) was used to separate the steroids. The solvents used were formic acid (1%) and acetonitrile. The method was adapted from previous published methods briefly summarized in Table 5.2 (Swart *et al.*, 2013).

Table 5.2

Q-TOF-MS settings

Capilarry voltage	Cone coltage	Source temperature	Desolvation temperature	Desolvation gas	cone gas
2.5 kV	15 V	120 °C	275 °C	650 L h^{-1}	50 L h^{-1}

5.5.2 *Quantification of steroid assays using UHPLC-MS/MS.*

All the steroids recovered during conversion assays (excluding metabolism in LNCaP cells) were quantified referenced to internal standards on an UHPLC-MS/MS on a ACQUITY UHPLC (Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP column (2.1 mm x 100 mm 2.6 µm) (Torrence, CA) and Xevo triple quadrupole mass spectrometer (Waters, Milford, USA). Analyses were carried out in MRM mode using an electrospray probe in the positive ionization (ESI+) mode. The mobile phases consisted of analytical grade deionised water containing (1%) formic acid (A) and 49%:49%:2% methanol:acetonitrile:isopropanol (B). The sample was applied in an injection volume of 5 µL. The UHPLC-MS/MS conditions were adapted according to the steroids being separated and are summarized in the tables below. Briefly, mass spectrometry settings are described in table 5.3. The UHPLC conditions (gradients, flow rates and elution curves) and steroid ion species, retention times, cone voltage (V), collision energy (CE), calibration range and linearity are provided in tables 5.4 - 5.9. The conditions described in table 5.4 and 5.5 were used to analyse steroid metabolites in the SRD5A and AKR1C2 catalysed conversions in chapter 3, and the conversion- and time course assays in chapter 4. CYP11B conversion- and time course assays in chapter 3 were analysed with the setting described in table 5.6 and 5.7 whereas the CYP17A1 conversion assays were analysed with the conditions described in table 5.8 and 5.9.

5.5.3 *Quantification of steroid metabolism in LNCaP cells using UPC²-MS/MS.*

Steroids extracted from the samples collected in the metabolic investigations carried out in LNCaP cells were analysed by ACQUITY UPC²–MS/MS (Waters, Milford, USA) and separated using an ACQUITY UPC² BEH column (3 mm x 100 mm, 1.7 µm). The steroids were detected and quantified using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) set to the conditions described in table 5.3, 5.10 and 5.11. CO_2 (A), manipulated to a supercritical fluid, is the primary mobile phase used which is mixed with methanol (Sigma) containing 1% formic acid (B) using a make-up pump. The run time was 6.5 min with a flow rate of 2 mL/min and an injection volume of 2 µL.

Table 5.3

Settings for the UHPLC-MS/MS and the UPC²-MS/MS analyses.

Settings	UHPLC-MS/MS	UPC²-MS/MS
Column temperature	50°C	60°C
ESP mode	positive	positive
MRM mode	yes	yes
Capillary voltage	3.5 kV	3.8 kV
Cone voltage (CV)	15–35 V	15-35 V
Collision energy (CE)	15–40 eV	8-30 eV
Source temperature	120 °C	120 °C
Desolvation temperature	400 °C	500 °C
Desolvation gas flow	900 L.h^{-1}	1000 L.h^{-1}
Cone gas flow	50 L.h^{-1}	150 L.h^{-1}
Automated back pressure regulator (ABPR)	-	2000 psi

Table 5.4

UHPLC-MS/MS gradient 1 applied to the separation of steroids listed in table 5.5.

	Time (min)	Flow rate	%A[*]	%B[**]	Curve
1	initial		85.0	15.0	Initial
2	1.00		60.0	40.0	6
3	3.50		45.0	55.0	6
4	3.60	0.400 mL/min	0.0	100.0	6
5	4.00		0.0	100.0	6
6	4.01		85.0	15.0	6
7	5.00		85.0	15.0	6

Table 5.5

UHPLC-MS/MS separation and quantification of steroids. The gradient described in table 5.4 was used to separate the steroids listed. Each steroid is presented with its retention time (RT), parent and daughter ion species together with the respective mass spectrometry properties such as the cone voltage (CV), collision energy (CE), calibration range and the linearity. Limit of quantification (LOQ) is 0.02 ng/µL.

Steroid	RT (min)	Mass transitions			CV (V)		CE (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Daughter ion 1	Daughter ion 2	Daughter ion 3						
Internal standards										
D2-T	3.17	291.00 > 99.10	291.00 > 111.25	-	25	25	30	30	-	-
D8-17OHP4	3.22	340.10 > 100.00	340.10 > 114.00	-	26	26	25	28	-	-
D9-P4	4.23	324.20 > 100.00	324.20 > 113.00	-	30	30	20	25	-	-
C_{21} steroids										
11KP4	2.98	329.21 > 121.00	329.21 > 84.80	-	15	15	20	20	0.02 - 2	0.9916
11K-DHP4	3.31	331.20 > 105.00	331.20 > 147.00	-	25	25	30	30	0.02 - 2	0.9939
Unknown	2.81	333.20 > 84.80	333.20 > 297.10	-	15	15	20	15	0.02 - 2	-
Alfaxalone	3.48	333.20 > 147.20	333.20 > 159.27	-	25	25	30	30	0.02 - 2	0.9903
11OHP4	3.03	331.20 > 121.00	331.20 > 295.00	-	20	20	25	15	0.02 - 2	0.9916
11OH-DHP4	3.34	333.10 > 84.80	333.10 > 173.00	-	30	30	30	20	0.02 - 2	-

16OHP4	2.60	331.20 > 97.00	331.20 > 108.90	-	30	30		15	15		0.02 - 2	0.9911
16OH-DHP4	2.89	333.20 > 145.10	333.20 > 159.10	333.20 > 213.20	30	30	30	15	15	15	0.02 - 2	-
11,16diOHP4	1.78	347.22 > 121.06	347.22 > 269.20	347.22 > 311.20	30	30	30	20	20	15	0.02 - 2	-
11,16diOH-DHP4	1.87	349.20 > 97.10	349.20 > 313.40	-		15	15	25	20		0.02 - 2	-
3,16diOH-DHP4	3.09	335.20 > 104.60	335.20 > 118.70	335.20 > 145.20	30	30	30	35	40	40	0.02 - 2	-

Table 5.6

UHPLC-MS/MS gradient 2 applied to the separation of steroids listed in table 5.7.

	Time (min)	Flow rate	%A	%B	curve
1	initial		85.0	15.0	Initial
2	0.50		60.0	40.0	6
3	3.50		35.0	55.0	6
4	3.70	0.400 mL/min	0.0	68.0	6
5	3.71		0.0	100.0	6
6	4.00		85.0	100.0	6
7	4.01		85.0	15.0	6
8	5.00		85.0	15.0	6

Table 5.7

UHPLC-MS/MS separation and quantification of steroids. The gradient described in table 5.6 was used to separate the steroids listed. Each steroid is presented with its retention time (RT), parent and daughter ion species together with the respective mass spectrometry properties such as the cone voltage (CV), collision energy (CE), calibration range and the linearity. Limit of quantification (LOQ) is 0.02 ng/µL.

Steroid	RT (min)	Mass transitions		CV (V)		CE (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Daughter ion 1	Daughter ion 2						
Internal standards									
D2-T	3.68	291.00 > 99.10	291.00 > 111.25	30	30	20	20	-	-
D7-11OHA4	2.59	310.20 > 147.20	310.20 > 99.80	25	25	25	30	-	-
D7-A4	3.78	294.30 > 100.00	294.30 > 113.00	25	25	25	25	-	-
D8-17OHP4	4.14	340.10 > 100.00	340.10 > 114.00	26	26	25	28	-	-
D9-P4	4.37	324.20 > 100.00	324.20 > 113.00	30	30	20	25	-	-
D4-Cortisol	2.02	367.00 > 121.00		35		25		-	-
C_{19} steroids									
A4	3.81	287.20 > 96.90	287.20 > 108.80	30	30	15	15	0.02-2	0.9965
11OHA4	2.62	303.20 > 267.20	303.20 > 121.20	30	30	15	30	0.02-2	0.9883
T	3.69	289.20 > 97.20	289.20 > 109.00	30	30	22	22	0.02-2	0.9967
11OHT	2.46	305.30 > 121.00	305.30 > 269.00	35	35	20	15	0.02-2	0.9833

C$_{21}$ steroids

P4	4.38	315.20 > 96.90	315.20 > 297.20	30	30	15	15	0.02-2	0.9924
17OHP4	4.15	331.10 > 97.00	331.10 > 108.90	30	30	15	15	0.02-2	0.9811
16OHP4	2.98	331.20 > 97.00	331.20 > 108.90	30	30	15	15	0.02-2	0.9899
11,16diOHP4	1.63	347.22 > 121.06	347.22 > 311.20	30	30	25	15	0.02-2	-
11OHP4	3.97	331.20 > 121.00	331.20 > 295.20	30	30	20	20	0.02-2	0.9944
DOF	2.65	347.10 > 121.00	347.10 > 269.20	25	25	20	20	0.02-2	0.9873
CORT	2.80	347.00 > 121.00	347.00 > 329.10	30	30	15	15	0.02-2	0.9981
DOC	3.86	331.20 > 97.00	331.20 > 108.90	30	30	15	15	0.02-2	0.9944
ALDO	1.68	361.40 > 97.00	361.40 > 343.20	30	30	32	18	0.02-2	0.9944
Deoxycortisol	2.89	347.00 > 97.00	347.00 > 108.90	30	30	15	15	0.02-2	0.9863
Cortisol	2.03	63.00 > 121.00		30		20		0.02-2	0.9973

Table 5.8

UHPLC-MS/MS gradient 3 applied to the separation of steroids listed in table 5.9.

	Time (min)	Flow rate	%A	%B	curve
1	initial		85.0	15.0	Initial
2	0.04		70.0	30.0	6
3	3.12		65.0	35.0	5
4	4.67		62.0	38.0	6
5	5.45		61.0	39.0	6
6	7.10	0.400 mL/min	40.0	60.0	6
7	7.20		0.0	100.0	6
8	7.70		0.0	100.0	6
9	7.80		85.0	100.0	6
10	10.00		85.0	100.0	6

Table 5.9

UHPLC-MS/MS separation and quantification of steroids. The gradient described in table 5.8 was used to separate the steroids listed. Each steroid is presented with its retention time (RT), parent and daughter ion species together with the respective mass spectrometry properties such as the cone voltage (CV), collision energy (CE), calibration range and the linearity. Limit of quantification (LOQ) is 0.02 ng/µL.

Steroid	RT (min)	Mass transitions		CV (V)		CE (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Daughter ion 1	Daughter ion 2						
Internal standards									
D2-T	4.23	291.00 > 99.10	291.00 > 111.25	25	25	30	30	-	-
D9-P4	7.13	324.20 > 100.00	324.20 > 113.00	30	30	20	25	-	-
C_{19} steroids									
A4	4.57	287.20 > 96.90	287.20 > 108.80	30	30	15	15	0.02-2	0.9961
11KA4	2.66	301.20 > 257.00	301.20 > 241.20	35	35	30	25	0.02-2	0.9986
AST	6.15	273.20 > 105.30	291.30 > 147.00	30	30	30	25	0.02-2	0.9796
11KAST	3.11	305.00 > 147.00	305.00 > 107.00	15	15	30	25	0.02-2	0.9889
C_{21} steroids									
P4	7.13	315.20 > 96.90	315.20 > 297.20	30	30	15	15	0.02-2	0.9872
17OHP4	5.07	331.10 > 97.00	331.10 > 108.90	30	30	15	15	0.02-2	0.9932
Pdiol	6.83	317.40 > 111.00	317.40 > 299.00	15	15	20	15	0.02-2	0.9723

Alfaxalone	4.84	333.20 > 147.20	333.20 > 159.27	25	25	30	30	0.02-2	0.9718
DOE	3.05	345.20 > 162.80	345.20 > 121.10	25	25	25	20	0.02-2	0.9899

Table 5.10

UPC^2-MS/MS gradient 1 gradient 3 applied to the separation of steroids listed in table 5.11.

	Time (min)	Flow rate	%A	%B	curve
1	initial		98.0	2.0	Initial
2	2.70		90.0	10.0	7
3	3.80		88.0	12.0	6
4	4.30	2.00 mL/min	82.0	18.0	6
5	5.40		75.0	25.0	6
6	5.50		98.0	2.0	1
7	6.50		98.0	2.0	1

Table 5.11

UHPLC-MS/MS separation and quantification of steroids. The gradient described in table 5.10 was used to separate the steroids listed. Each steroid is presented with its retention time (RT), parent and daughter ion species together with the respective mass spectrometry properties such as the cone voltage (CV), collision energy (CE), calibration range and the linearity. Limit of quantification (LOQ) of all steroids is 0.001 ng/µL with the exception of 11OHAST and 11KAST with a LOQ of 0.01 ng/µL.

Steroid	RT (min)	Mass transitions		CV (V)		CE (eV)		Calibration range (ng/µL)	Linearity (r^2)
		Quantifier	Qualifier						
Internal standards									
D2-T	2.91	291.00 > 99.10	291.00 > 111.25	30	30	20	20	-	-
D7-11OHA4	3.05	310.20 > 147.20	310.20 > 99.80	25	25	25	30	-	-
D7-A4	1.82	294.30 > 100.00	294.30 > 113.00	25	25	25	25	-	-
D8-17OHP4	2.71	340.10 > 100.00	340.10 > 114.00	26	26	25	28	-	-
D9-P4	1.68	324.20 > 100.00	324.20 > 113.00	30	30	20	25	-	-
C_{19} steroids									
A4*	1.83	287.20 > 96.90	287.20 > 108.80	30	30	15	15	0.001-1	0.9976
11OHA4*	3.05	303.20 > 267.20	303.20 > 121.20	30	30	15	30	0.001-1	0.9992
11KA4*	2.43	301.20 > 257.00	301.20 > 265.20	35	35	25	25	0.001-1	0.9991
T*	2.91	289.20 > 97.20	289.20 > 109.00	30	30	22	22	0.001-1	0.9981

11OHT*	4.25	305.30 > 121.00	305.30 > 269.00	35	35	20	15	0.001-1	0.9994
11KT*	3.57	303.20 > 121.00	303.20 > 267.00	30	30	20	20	0.001-1	0.9988
5α-dione*	1.11	289.18 > 253.14	289.20 > 97.20	22	30	16	22	0.001-1	0.9964
3α-Adiol*	3.27	275.20 > 257.00	275.20 > 175.00	15	15	15	15	0.001-1	0.9982
DHEA	2.30	271.21 > 253.2	271.21 > 243	30	30	15	15	0.001-1	0.9958
DHT*	2.20	291.20 > 255.00	291.20 > 273.00	25	25	15	20	0.001-1	0.9960
11KDHT*	3.22	305.20 > 243.00	305.20 > 269.00	30	30	20	20	0.001-1	0.9996
AST*	2.24	273.20 > 105.30	291.30 > 273.30	30	18	30	8	0.001-1	0.9971
11OHAST*	3.44	289.00 > 271.00	289.00 > 213.00	15	15	15	15	0.01-1	0.9988
11KAST*	3.01	305.00 > 147.20	305.00 > 173.10	30	30	30	30	0.01-1	0.9992
C_{21} steroids									
P4	1.68	315.20 > 97.00	315.20 > 109.00	28	28	20	26	0.001-1	0.9947
DHP4	0.98	317.00 > 105.20	317.00 > 95.00	30	30	30	30	0.001-1	0.9978
17OHP4	2.72	331.10 > 97.00	331.10 > 109.00	26	26	22	28	0.001-1	0.9982
Pdiol	3.32	317.40 > 111.00	317.40 > 299.00	20	20	25	15	0.001-1	0.9898
Pdione	2.03	333.40 > 159.00	333.40 > 137.00	20	20	25	25	0.001-1	0.9953
Pregnanetriol	4.20	301.20 > 135.00	301.20 > 81.00	25	25	15	25	0.001-1	0.9915

16OHP4	3.41	331.20 > 97.00	331.20 > 108.90	30	30	15	15	0.001-1	0.9939
11OHP4	3.22	331.20 > 121.00	331.20 > 295.00	30	30	20	20	0.001-1	0.9943
11αOHP4	3.53	331.20 > 295.20	331.20 > 121.00	30	30	15	30	0.001-1	0.9909
11KP4	2.45	329.21 > 121.00	329.21 > 84.80	15	15	20	20	0.001-1	0.9920
11K-DHP4	1.91	331.20 > 105.00	331.20 > 147.00	25	25	30	30	0.001-1	0.9981
Alfaxalone	3.20	333.20 > 147.27	333.20 > 159.27	25	25	30	30	0.001-1	0.9856
DOE	3.17	345.20 > 162.80	345.20 > 121.10	25	25	25	20	0.001-1	0.9980
DOF	3.80	347.10 > 121.00	347.10 > 269.20	20	20	25	15	0.001-1	0.9963

* Limit of detection (LOD; Signal/Noise(S/N) ratio > 3) was previously determined by Du Toit *et al.* (2017).

5.6   Luciferase reporter assay

The assay was described in detail in chapter 3. Additional experimental details are included in sections described above.

5.7   Statistical analysis

Experiments were carried out in triplicate and expressed as means ±SEM. One-way ANOVA followed by Dunnett's multiple comparison test was carried out using GraphPad Prism 5 (GraphPad Software, Inc, CA, USA). The P value ≤ 0.05 was considered statistically significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). A one-way ANOVA followed by a Newman-Keuls post-test was were performed in the analyses of the luciferase reporter data, and the letters a, b, c, d and e were used to indicate differed statistical significance.

CHAPTER 6

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Conclusions and future perspectives

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This study hypothesized that 11OHP4, 11KP4 and 16OHP4, produced in the adrenal, may be metabolised by peripheral steroidogenic enzymes to yield metabolites which may exert biological effects by activating steroid receptors. It was also hypothesized that these C11-oxy C_{21} steroids may contribute to the C11-oxy C_{19} steroid pool in adrenal disorders and diseases. Current steroidogenic pathways describing the metabolism of these steroids are incomplete, partly due to limited research and partly due to shortcomings in analytical tools. In addition, receptor studies investigating the physiological relevance are also limited. This study was therefore conducted and provides evidence for the downstream metabolism of 11OHP4, 11KP4 and 16OHP4 and the interaction of these steroids and their metabolites with the PR and AR. These steroids have been associated with clinical conditions such as PCOS, of which the etiology is unclear or where therapies fail to correct the hormone related disorder, and diseases such as 21OHD and CRPC.

This study presents the first evidence for the biosynthesis of 11OHP4 from P4 catalysed by CYP11B1 and CYP11B2 *in vitro*. Both isozymes display an equal conversion rate of 11OHP4. Nevertheless, *in vivo* the metabolism is greatly influenced by specific stimulants such as ACTH or AngII, affecting the expression levels of the catalysing enzymes. As ACTH stimulates the release of free cholesterol via cAMP, regulates ZF proliferation and upregulates the expression of CYP11B1, the reaction would mainly be catalysed by CYP11B1 expressed in the adrenal and abnormal testes. Therefore, elevated levels of 11OHP4 are more often associated with ACTH stimulation, particularly in the dysregulated stimulation of the HPA axis. Moreover, the reversible conversion of 11OHP4 and 11KP4 is catalysed by 11βHSD1 and 11βHSD2 which adds a layer of complexity to the peripheral metabolism of 11OHP4.

Although the CYP11B assays described in this study were reported as single experiments, similar assays conducted in our laboratory and the controls which were included *viz*. the natural substrates, and androgens, A4 and T, aligned with the previous results obtained in our laboratory (published and unpublished). These assays served to establish only whether CYP11B1 and/or CYP11B2 would catalyse the 11β-hydroxylase reaction. In addition, investigations into the conversion of 11OHP4 and 11KP4 by SRD5A and the subsequent metabolism by ARK1C2 were conducted firstly using 10 μM substrate in order to determine the accurate mass of the metabolites. Although single data sets were depicted for these experiments, the conversions were subsequently repeated. Similarly, three independent SRD time course assays, investigating the metabolism of 11OHP4 and 11KP4, were conducted and are represented in a single data set. The lack of standards have hampered the investigation of product formation which was later corrected in the case of 11K-DHP4 upon obtaining the steroid commercially.

These studies clearly identified metabolites showing the conversion of 11OHP4 and 11KP4 by the SRD5A, AKR1C2 and CYP17A1 in a novel C11-oxy backdoor pathway describing the peripheral metabolism of C11-oxy C_{21} steroids (Fig. 6.1). To date, this study is the first to report the metabolism of 11OHP4 by SRD5A and AKR1C2 yielding novel steroid metabolites yet to be considered in diseases. This study is also the first to report the metabolism of 11KP4 by SRD5A and the subsequent reduction by AKR1C2 yielding alfaxalone as well as the metabolism of alfaxalone by CYP17A1.
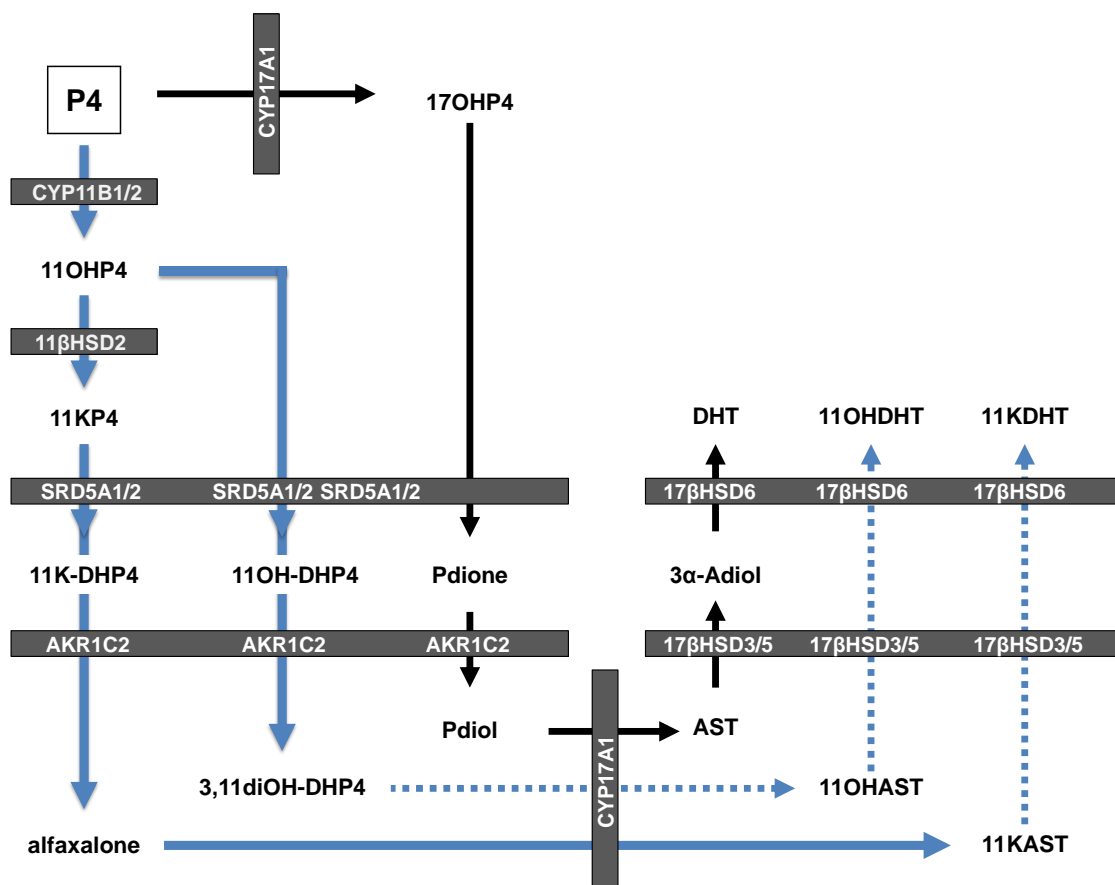
Figure 6.1   P4 metabolism in the novel C11-oxy backdoor pathway and classic backdoor pathway. Novel pathways, blue arrows (confirmed reactions →, unconfirmed, -----); backdoor pathway, black arrows.

The production of 11KAST in the latter reaction supports the hypothesis that C11-oxy C_{21} steroids are converted to androgens and thus have the potential to contribute to the C11-oxy androgen pool in conditions such as 21OHD, PCOS and PCa. These steroids would contribute to the biosynthesis of 11β-hydroxy- and 11-keto androgens −androgens that have been implicated in the progression of androgen-dependent diseases with these C11-oxy C_{19} steroids having been shown to be potent androgens, as well as remaining active for longer periods due to decreased conjugation in comparison to C_{19} steroids. The current findings provide evidence of a novel C11-oxy backdoor pathway in which P4 is metabolised by CYP11B to 11OHP4 and subsequently oxidized to 11KP4 by 11βHSD2. Both 11OHP4 and 11KP4 are excellent substrates for SRD5A enzymes yielding 11OH-DHP4 and 11K-DHP4, which in turn are converted to 3,11diOH-DHP4 and alfaxalone, respectively, by AKR1C2,

similar to 17OHP4 in the backdoor pathway (Fig. 6.1). These conversions were subsequently verified in the LNCaP cell model suggesting the reaction would occur in CRPC. The conversion of alfaxalone to 11KAST by CYP17A1 was confirmed in this project, whereas the investigation regarding the metabolism of 3,11diOH-DHP4 by CYP17A1 was hampered by the lack of standards. However, 11OHAST was shown to be the main androgen detected in the urine of 21OHD children (Kamrath *et al.*, 2016), which would support findings in the present study as this metabolite is a product of 11OHA4 as well as 11OHP4. Further investigations are required to fully characterise the biosynthesis of 11OHAST.

Furthermore, this study reports on the interaction of C11-oxy C_{21} steroids with the PR isoforms and with the AR. This study is the first to report agonist activity towards the AR and PR by the C11-oxy C_{21} steroids and their metabolites. The super-agonistic activity observed for 11OHP4 towards the AR emphasized the importance of considering this steroid as well as other C11-oxy C_{21} steroids in AR positive tissue such as the prostate and the ovary, which has to date not been the focus of research in the clinical setting. It was also shown that the androgenicity of 11OHP4 is far greater than that of DHT or Mib. In addition, the 11KP4 metabolite is a full agonist at 1nM. The androgenicity of both 11OHP4 and 11KP4 decreases once the steroid has undergone 5α-reduction with 11K-DHP4 still exhibiting partial agonist activity. Interestingly, in terms of the PR, while the activity of the C11-hydroxyl steroid is reduced once converted to the 5α-reduced metabolite, the C11-keto steroid is activated and now exhibits partial agonist activity.

It can therefore be concluded that the interplay between these C11-oxy C_{21} steroids and the steroid receptors is complex. The findings suggest that the 5α-reduction and C11 keto oxidation of 11OHP4 decreases the activity of the AR towards these ligands (Fig. 6.2). Nevertheless, the metabolites remain active with respect to the AR and would elicit cellular responses. The downstream conversion of these C11-oxy C_{21} steroids would result in the production of 11OHDHT and/or 11KDHT, suggesting that the C_{21} steroids would not only contribute to the androgen pool but would also, as C_{21} steroids, contribute to the androgenic effect prior to metabolism. It is thus possible that the activity of 11OHP4 towards the AR would secure the induction of AR target genes in ADT.
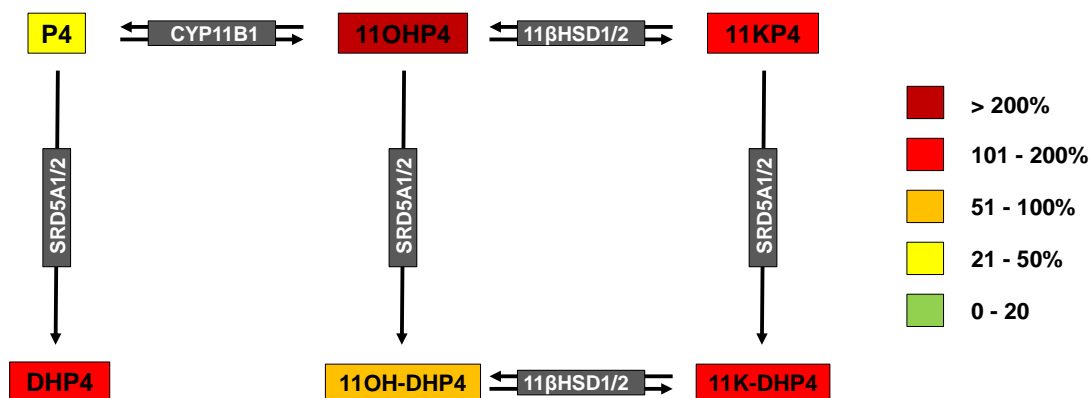

Figure 6.2   Metabolism of P4 catalysed by CYP11B1, 11βHSD and SRD5A, with the AR agonist activity of steroids indicated by colour as set relative to Mib.

Furthermore, the full agonistic activity displayed by 11OHP4 for the PR-A suggests that this steroid will regulate cellular machinery coupled to the PR-A and to a lesser degree PR-B. The agonistic activity exhibited by 11OHP4 would be significant during the menstrual cycle in which PR-A expression is higher than PR-B (Mangal *et al.*, 1997) and in conditions such as PR-A mediated breast cancer (Esber *et al.*, 2015). Conversion to 11OH-DHP4 and 11KP4 significantly reduced the activity towards the PR-A (Fig. 6.3) as well as PR-B (Fig. 6.4). In contrast, the 5α-reduction of 11KP4 yielding 11K-DHP4 increased activity significantly. The metabolism of 11OHP4 to 11KP4 by 11βHSD2 reflects the inactivation of cortisol to cortisone rather than the activation observed in the case of the C11-oxy androgens in which 11βHSD2 converts 11OHT and 11OHDHT to their potent 11keto derivatives. It is in this scenario that the co-expression of 11βHSD and/or SRD5A catalysing the reduction at C5 and the C11 hydroxyl/keto interconversion would regulate AR and PR activation in terms of both the C11-oxy C_{21} steroids and C11-oxy C_{19} steroids.
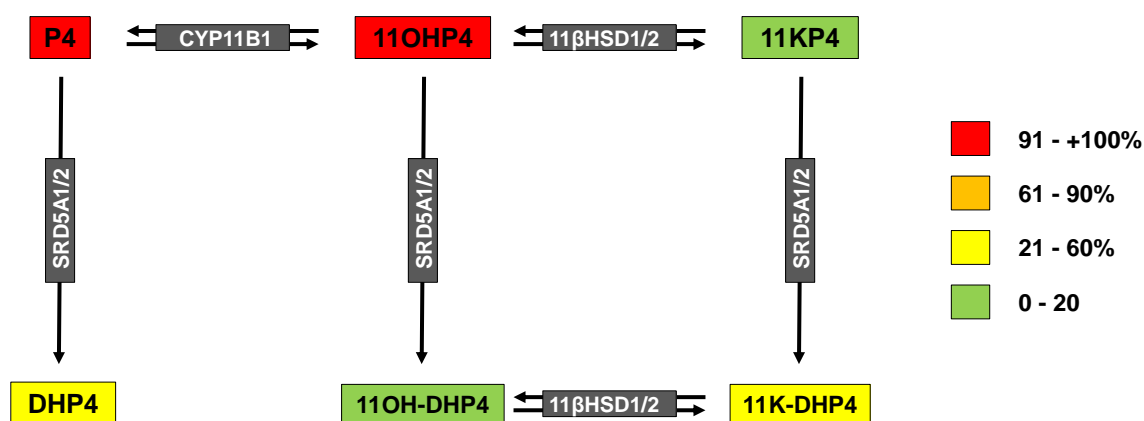
Figure 6.3   Metabolism of P4 catalysed by CYP11B1, 11βHSD and SRD5A, with the PR-A agonist activity of steroids indicated by colour as set relative to R5020.
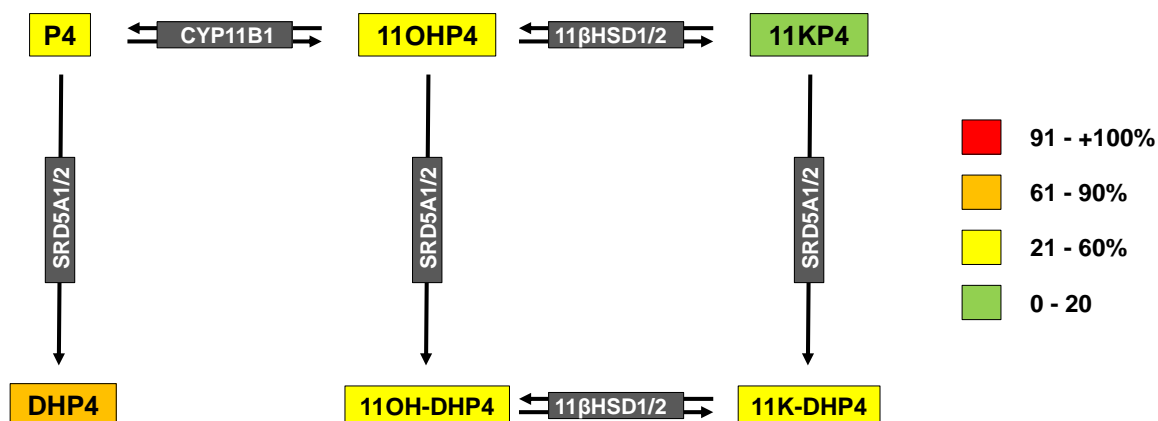
Figure 6.4   Metabolism of P4 catalysed by CYP11B1, 11βHSD and SRD5A, with the PR-B agonist activity of steroids indicated by colour as set relative to R5020.

Studies have shown antiandrogen therapies in CRPC to upregulate expression of a mutated AR and GR of which the latter stimulates AR-like genes resulting in GR-driven resistance and, as a consequence contributes to the progression of CRPC (Arora *et al.*, 2013). Interestingly 11OHP4, but not 11KP4, was shown to bind to the GR (Galigniana *et al.*, 1997) and would therefore be able to induce a response in CRPC. In addition, although limited studies have been conducted with the MR, it has also been shown that 11KP4 exhibited a higher affinity for the MR compared to 11OHP4. MR, SRD5A and both 11βHSD isozymes are expressed in the ovary (Gomez-Sanchez *et al.*, 2009; Graupp *et al.*, 2011) and together with the AR and PR also being expressed in the ovary (Horie *et al.*, 1992; Suzuki *et al.*, 1994) suggests that 11OHP4, 11OH-DHP4, 11KP4 and 11KP-DHP4 may have as of yet unexplored receptor mediated effects in the ovary.

Turning attention to 16OHP4, this study was the first to report the metabolism of 16OHP4 by CYP11B2, SRD5A and AKR1C2 yielding novel metabolites (Fig. 6.5). The biosynthesis of 16OHP4 and its metabolism by CYP11B2 *in vivo* in the adrenal is uncertain due to the adrenal cortex zonation. In clinical conditions such as salt wasting 21OHD, CYP11B2 expression is upregulated, merging cells between the ZG and ZF may thus allow the 11β-hydroxylation of 16OHP4. In addition, the increased 16OHP4 levels in circulation discussed previously would suggest peripheral conversion in tissue expressing CYP11B. Nevertheless 11,16diOHP4 is subsequently reduced to 5α-pregnan-11β,16α-diol-3,20-dione (11,16diOH-DHP4) by SRD5A and, due to the rapid conversion of 16OHP4 to 16OH-DHP4, similar to that of 17OHP4 in the backdoor pathway, the downstream metabolism by CYP11B2 appears unlikely. In addition, AKR1C2 catalyses the conversion of 16OH-DHP4 to 3α,16α-dihydroxy-5α-pregnan-20-one (3,16diOH-DHP4) as a second inactivation of 16OHP4 following SRD5A. 16OHP4 and 16OH-DHP4 showed little agonist activity toward the AR and PR of which the interaction was concentration dependent.
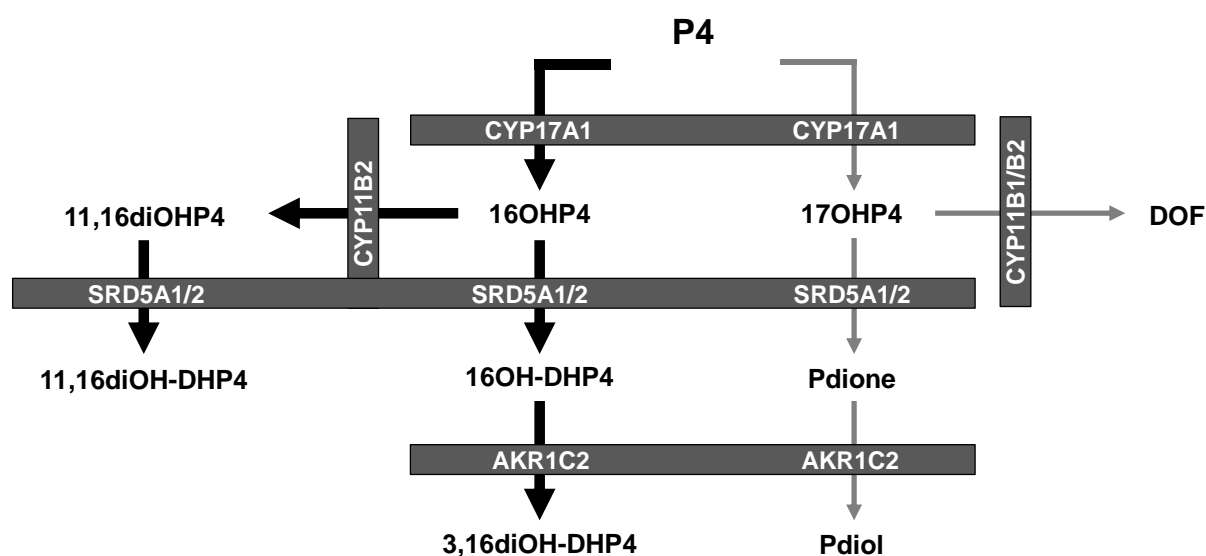

Figure 6.5   Schematic representation of the metabolism of 16OHP4. Novel 16OHP4 pathway, bold black arrows; backdoor pathway, grey arrows.

In summary, this study has clearly demonstrated the downstream metabolism of 11OHP4, 11KP4 and 16OHP4 together with the AR and PR interaction. In addition, evidence is provided for a novel C11-oxy backdoor pathway in which C11-oxy C_{21} steroids are metabolised and may contribute to the C11-oxy C_{19} pool. Furthermore, the data demonstrated significant agonistic activity for 11OHP4 and its metabolites towards the AR. The C11-oxy C_{21} steroids were also shown to activate the PR isoforms. Therefore, C11-oxy C_{21} steroids may exert physiological responses or may be readily incorporated into the novel pathway initiated by the metabolism by SRD5A. These findings are significant to a number of diseases (breast cancer, CRPC, 21OHD, PCOS) and would impact the physiological regulatory role in both male and female reproductive physiology, while the contribution of the kidney regarding the metabolism of 11OHP4 remains unknown. Future studies will identify PR and AR target genes induced by the C11-oxy C_{21} steroids and elucidate the interaction with the ER which is often expressed in association with the PR. Moreover, PCa tissue of patients receiving abiraterone treatment (CYP17A1 inhibitor) express a mutated AR which is activated by P4, indicating yet another area in which the activity of C11-oxy C_{21} steroids towards the mutated AR remains to be explored.

As the kidney expresses all the enzymes of the novel C11-oxy backdoor pathway including the PR and AR (Bumke-Vogt *et al.*, 2002; Quinkler *et al.*, 2003, 2002), it is the perfect cell model for validating the novel pathway in future studies.

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