Corticosteroid Hormone Action in the Cardiovascular System

by

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Abstract

The cardiovascular system (CVS) has emerged as an important target of corticosteroid hormones. Mineralocorticoid receptor antagonists provide cardiovascular protection and are now routinely used in disorders such as primary hyperaldosteronism, resistant hypertension and congestive heart failure (CHF) but the underlying molecular mechanisms of corticosteroid hormone action remain unclear.

We have characterised corticosteroid hormone action and metabolism by 11β-hydroxysteroid-dehydrogenases (11β-HSDs) in isolated adult rat cardiomyocytes (CM) and cardiac fibroblasts (cFb). We have detected 11β-HSD1 expression and activity in CM and cFb where it facilitates glucocorticoid hormone action, whereas 11β-HSD2 was absent. We have shown differential gene regulation by aldosterone (Aldo) and corticosterone in CM and identified novel Aldo target genes which may provide insights into the molecular mechanisms of Aldo action.

We have also studied the role of corticosteroids in essential hypertension and the effect of spironolactone (Spiro) upon their secretion and metabolism in patients with chronic kidney disease. We have shown that mineralocorticoids but not glucocorticoids are involved in elevated blood pressure in essential hypertension and that Spiro treatment results in compensatory activation of the renin-angiotensin-aldosterone system (RAAS), whereas glucocorticoid secretion and metabolism remain unchanged.

In summary, these data provide novel molecular and clinical insights into corticosteroid hormone action in the CVS.

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I am most grateful to my parents and grandmother for all their support over the years. To acknowledge my deepest gratitude in relation to this journey, I would like to dedicate this thesis to my mother, Mechtild Hammer.

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"The journey not the arrival matters." – T.S. Eliot

"Man reist ja nicht, um anzukommen, sondern um zu reisen." – J.-W. v. Goethe

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Abbreviations

11-DHB	11-dehydrocorticosterone
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
24h ABP	24h ambulatory blood pressure
A	11-dehydrocorticosterone
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
ACR	Albumine creatinine ratio
ACTH	Adrenocorticotropic hormone
Aldo	Aldosterone
AME	Apparent mineralocorticoid excess
Ang I	Angiotensin I
Ang II	Angiotensin II
ARR	Aldosterone renin ratio
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ARB	Angiotensin receptor blockers
B	Corticosterone
BMI	Body mass index
bp	Base pair
cAMP	Cyclic adenosine-5’-monophosphate
cDNA	Complementary deoxyribonucleic acid
cFb	Cardiac fibroblast
CHF	Congestive heart failure
conc	Concentration
CTRL	Controls
CVS	Cardiovascular system
cRNA	Complementary ribonucleic acid
CTP	Cytosintriphosphate

CVS	Cardiovascular system
DBD	DNA binding domain
DHB	11-dehydrocorticosterone
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco’s modified eagles medium
DNA	Deoxyribonucleic acid
DOC	11-deoxycorticosterone
DOCA	11-deoxycorticosterone acetate
dNTP	Deoxynucleotide triphosphate
E	Cortisone
ECM	Extracellular Matrix
eGFR	Estimated glomerular filtration rate
ENaC	Epithelial sodium channel
EPL	Eplerenone
ER	Endoplasmic reticulum
F	Cortisol
FCS	Fetal calf serum
GC/MS	Gas chromatography / mass spectrometry
GE	Glycyrrhetinic acid
GR	Glucocorticoid receptor
H6PDH	Hexose-6-phosphate dehydrogenase
HPA	Hypothalamo-pituitary-adrenal
HRE	Hormone response element
HSD	Hydroxysteroid dehydrogenase
HSP	Heat shock protein
HF	Heart failure
HRE	Hormone response element
HSD11B1	Gene encoding 11β-HSD1
HSD11B2	Gene encoding 11β-HSD2
IGF-I	Insulin growth factor I
kb	Kilo base
K_m	Michaelis-Menten constant
LB	Luria Bertani broth
LBD	Ligand binding domain

LDL	Low density lipoprotein
LH	Luteinising hormone
LV	Left ventricle
MCR	Melanocortin receptor
MI	Myocardial infarction
MR	Mineralocorticoid receptor
MyoFb	Myofibroblast
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADH	Dihydronicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
nrCM	Neonatal rat cardiomyocytes
NYHA	New York Heart Association
PAC	Plasma aldosterone concentration
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRA	Plasma renin activity
RAAS	Renin angiotensin aldosterone system
RNA	Ribonucleic acid
RT	Reverse transcription
StAR	Steroid acute regulatory protein
TH-Aldo	Tetrahydro-aldosterone
THE	Tetrahydro-cortisone
THF	Tetrahydro-cortisol
THS	Tetrahydro-11-desoxycortisol
TLC	Thin layer chromatography
U	Unit
UFE	Urinary free cortisone
UFF	Urinary free cortisol

Chapter 1 - General Introduction

1.1 The clinical problem

Heart disease is a broad term describing a wide spectrum of diseases of different aetiologies affecting the heart such as ischaemic and hypertensive heart disease, cardiomyopathies, inflammatory and valvuar heart disease. All these conditions affect the performance of the heart and can ultimately result in CHF which is defined by the inability of the heart to supply sufficient blood flow to meet the body's needs.

CHF is a common, costly, disabling and deadly condition (McMurray & Pfeffer, 2005). It has been estimated that around 2% of adults in developing countries suffer from heart failure. However, over the age of 65 the prevalence increases to 6 - 10% (Dickstein et al., 2008; McMurray & Pfeffer, 2005). Heart failure is the reason for at least 20% of all hospital admissions among persons older than 65 years and moreover, the rate of hospitalisations for heart failure has increased by 159% over the past decade. Associated costs have been estimated to amount for 2% of the total budget of the National Health Service (NHS) in the United Kingdom (Stewart et al., 2002), and more than $35 billion in the United States (Lloyd-Jones et al., 2009). Furthermore, quality of life is impaired in heart failure patients and deteriorates with progression of the condition (Juenger et al., 2002). CHF usually worsens with time unless the underlying cause is reversible. Although some patients survive many years, progressive disease is associated with an overall annual mortality rate of 10% (Neubauer, 2007).

Over the years, many studies have explored the underlying pathophysiological mechanisms of heart failure and consequently drugs that interfere or modulate these processes have been proven to be effective treatments in slowing down disease progression and thereby reducing morbidity and mortality. Two landmark clinical trials, the Randomised ALdactone Evaluation Study (RALES) and the Eplerenone

Post-acute myocardial infarction Heart failure Efficacy and Survival Study (EPHESUS), have highlighted the adverse effects of corticosteroids in CHF and the incremental clinical benefit of mineralocorticoid receptor antagonists (MRA) in patients with heart failure (Pitt et al., 2003b; Pitt et al., 1999) (Figure 1-1). However, despite these important clinical insights and therapeutical advances, very little is known about the underlying cellular and molecular mechanisms.

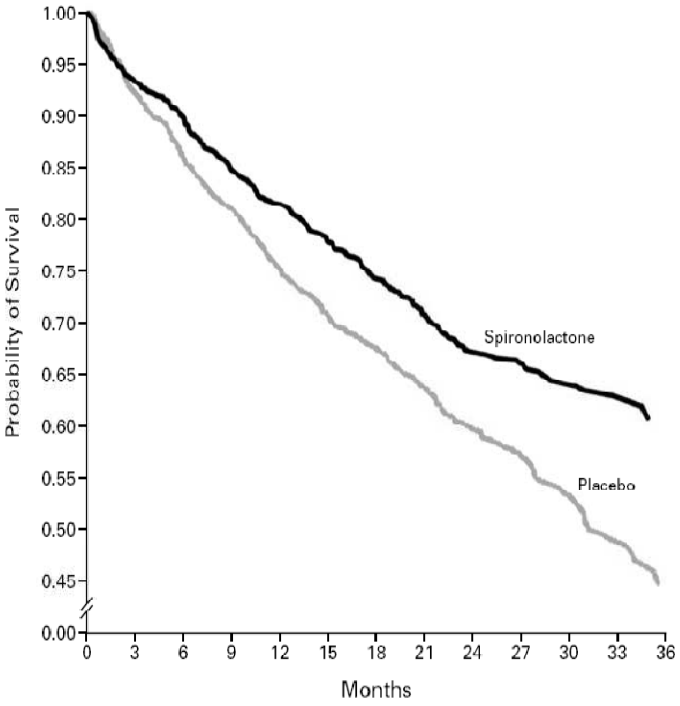

Figure 1-1 Kaplan-Mayer curves showing survival of patients with advanced congestive heart failure according to treatment group (placebo vs spironolactone) in the RALES study (Pitt et al., 1999).

1.2 The adrenal gland

The adrenal glands are located in the retroperitoneum on top of the kidneys. Both anatomically and functionally the adrenal gland can be divided into an outer layer, the adrenal cortex, and a central core, the adrenal medulla. The medulla can be considered as specialised ganglia of the sympathetic nervous system which releases

catecholamines (adrenaline, noradrenaline) into the circulation. By contrast, the adrenal cortex secretes steroid hormones and consists of three histologically distinct layers (zones) which produce three distinct classes of steroid hormones. The outer layer (*zona glomerulosa*) is located beneath the capsule (Figure 1-2) and is the main site of mineralocorticoid hormone production, namely aldosterone (Aldo). Adjacent to the *zona glomerulosa* lies the *zona fasciculata* which is the main site of glucocorticoid hormone production (cortisol in humans, corticosterone in rodents). The most inner zone of the adrenal cortex is the *zona reticularis* which predominantly produces the sex steroid precursor hormone dehydroepiandrosterone (DHEA) and its sulfated ester DHEAS. However, only humans and higher primates exhibit this zone and are capable of producing adrenal sex steroids whereas all other vertebrates only produce glucocorticoids and mineralocorticoids in the adrenal.

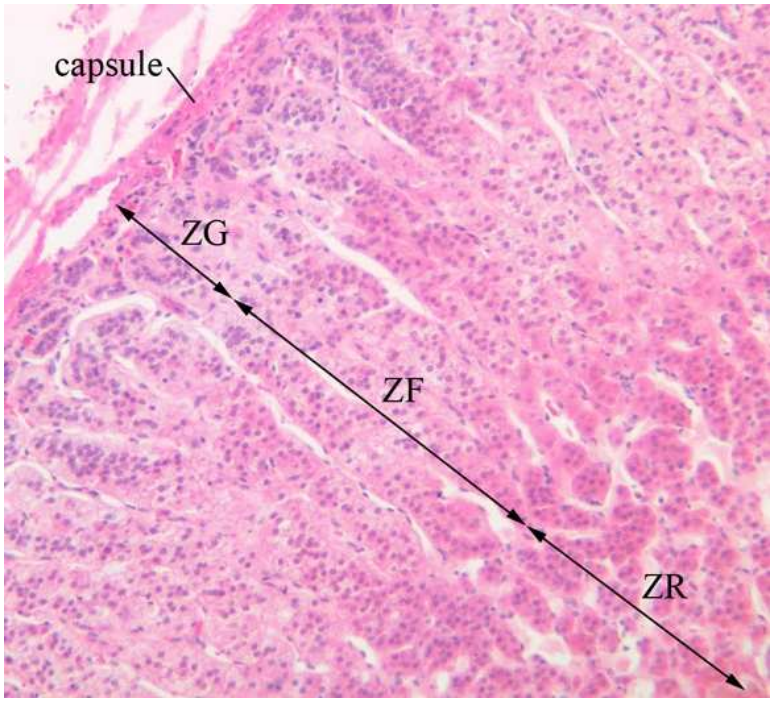

Figure 1-2 Histological section through the cortex of the human adrenal gland. Mineralocorticoids are synthesised in the zona glomerulosa (ZG), glucocorticoids in the zona fasciculate (ZF) and sex steroids the zona reticularis (ZR).

1.3 Adrenal steroidogenesis

1.3.1 Biosynthesis and uptake of cholesterol

Cholesterol is the common precursor of all steroid hormones and therefore adrenal cells require a constant supply of cholesterol. Three mechanisms contribute to the cholesterol pool within the adrenal gland. Firstly, cholesterol can be synthesised *de novo* from acetyl coenzyme A. The key and rate limiting enzyme involved in this process is hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase which converts β-hydroxy-β-methylglutaryl-CoA to mevalonic acid. HMG-CoA reductase is expressed in the adrenal and is up-regulated by the adrenocorticotropic hormone (ACTH) (Mason & Rainey, 1987). Secondly, cholesterol-esters can be taken up from circulating low density lipoproteins (LDL) through receptor mediated endocytosis involving apolipoprotein B and E or LDL receptors and are subsequently hydrolysed by cholesterol ester hydrolase to yield cholesterol. Finally, a high density lipoprotein receptor, scavenger receptor class B type 1 (SR-B1), has been identified on adrenocortical cells (Acton et al., 1996). In contrast to LDL-mediated delivery of cholesterol, HDL apolipoproteins are not internalised but binding of apolipoprotein A1 (apoA1) to SR-B1 leads to formation of channels by which cholesterol esters can enter the cell. In rodents this mechanism may account for up to 90% of adrenal cholesterol supply (Kraemer, 2007).

1.3.2 Biosynthesis of steroid hormones

The structure of steroid hormones is depicted in Figure 1-3. The first step of steroidogenesis is catalysed by the side chain cleavage enzyme (P450scc) which is responsible for the scission of the cholesterol side chain between carbon atoms C20 and C22 resulting in the formation of pregnenolone (Figure 1-3). P450scc is located

in the inner mitochondrial membrane and requires the steroid acute regulatory protein (StAR) which is embedded in the outer mitochondrial membrane in order to facilitate cholesterol translocation across the mitochondrial membranes. The exact mechanism underlying mitochondrial cholesterol transport remains unresolved but other proteins such as the translocator protein have been shown to bind cholesterol and directly interact with StAR (Rone et al., 2009). In keeping with the pivotal role of StAR in adrenal steroidogenesis, mutations in its gene result in an almost complete inability for steroid hormone synthesis which presents clinically as congenital lipoid adrenal hyperplasia (Stocco, 2002).

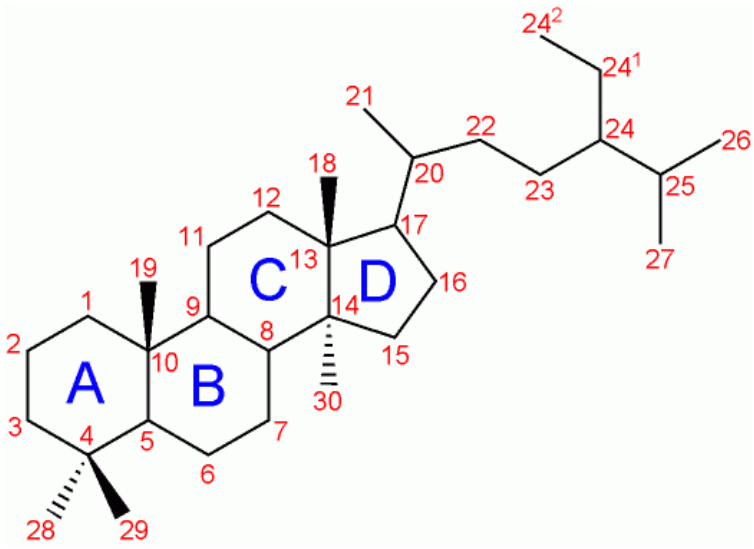

Figure 1-3 Schematic view of the steroid backbone with its classical nomenclature. Letters A-D identify rings, numbers the carbon atoms.

Pregnenolone serves as a substrate for all subsequent downstream steroids. Its fate is determined by the spatial expression of steroidogenic enzymes which are distinct in each zone (Figure 1-4).

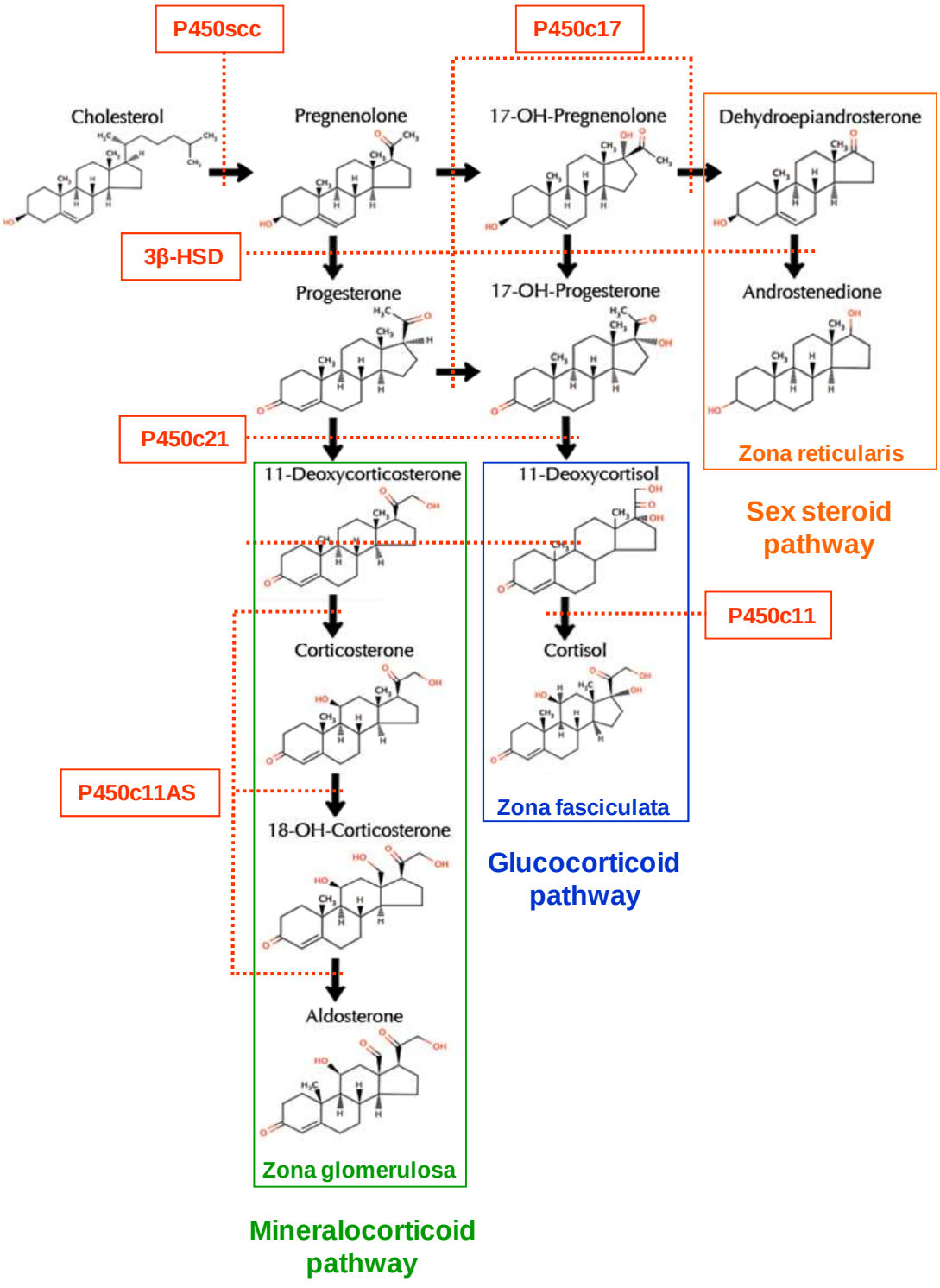

Figure 1-4 Pathways of adrenal mineralocorticoid, glucocorticoid and sex steroid synthesis. P450scc: side chain cleavage enzyme; 3β-HSD: 3β-hydroxysteroid dehydrogenase; P450c17: 17α-hydroxylase/17-20 lyase, P450c21: 21-hydroxylase; P450c11: 11-hydroxylase; P450c11/AS: aldosterone synthase.

1.3.2.1 Cortisol synthesis

In humans, the *zona fasciculata* expresses the enzyme 3β-HSD2, 17α-hydroxlase (P450c17), 21-hydroxylase (P450c21) and 11β-hydroxylase (P450c11) which results in synthesis of the glucocorticoid cortisol (Figure 1-5). Following side chain scission of cholesterol by P450scc in the mitochondria, pregnenolone is released from the mitochondria into the cytoplasm where it serves as a substrate for the enzyme 3β-hydroxysteroid dehydrogenase 2 (3β-HSD2) resulting in dehydrogenation of the 3-hydroxyl group and isomerisation of the double bond at C5 to form progesterone. Progesterone is subsequently hydroxylated at the position C17 by the enzyme 17α-hydroxlase (P450c17) located in the endoplasmic reticulum to form 17-hydroxyprogesterone. The final two modifications resulting in cortisol production occur in the mitochondria. First 17-hydroxyprogesterone is hydroxylated at the C21 position by the enzyme P450c21 to form 11-deoxycortisol which is then hydroxylated at the C11 position by the enzyme P450c11 resulting in cortisol (Arlt & Stewart, 2005). In contrast to humans and higher primates, rodents do not express the enzyme P450c17 in the adrenal gland and therefore corticosterone is the principal glucocorticoid secreted by the *zona fasciculata*.

1.3.2.2 Aldosterone synthesis

Aldo production occurs in the *zona glomerulosa* which expresses the steroidogenic enzymes 3β-HSD2, 11β-hydroxylase (P450c11), 21-hydroxylase (P40c21) and aldosterone synthase (P450c11AS) but not P450c17. Similar to the synthesis of cortisol pregnenolone is firstly converted to progesterone in the endoplasmic reticulum. All further biochemical modifications occur in the mitochondria where progesterone is hydroxylated at the C21 position to form 11-deoxycorticosterone (DOC). The final reactions in Aldo synthesis are carried out by aldosterone synthase,

which hydroxylates 11-deoxycorticosterone at C11 to corticosterone then at the C18 position to form 18-hydroxycorticosterone followed by dehydrogenation at C18 to form Aldo which is the principal mineralocorticoid.

1.3.2.3 Adrenal androgen synthesis

Adrenal androgen production only occurs in humans and higher primates through the expression of P450c17 in the *zona reticularis* of the adrenal gland. Following pregnenolone synthesis form cholesterol in the mitochondria, pregnenolone serves as a substrate for P450c17 located in the endoplasmic reticulum. P450c17 harbors two distinct enzymatic activities, namely 17α-hydroxylase and 17, 20 lyase. Both enzymatic steps rely on the presence of P450 oxidoreductase (POR) which serves as an electron donor whereas the lyase reaction in addition also relies on the presence of cytochrome b5 as a co-factor. Cytochrome b5 is only expressed in the *zona reticularis* but not the *zona fasciculata* and thus, the lyase reaction is restricted to the *zona reticularis*. These two P450c17 reactions result in the production of the sex steroid hormone precursor DHEA.

1.4 Steroid hormone action

1.4.1 Steroid hormone receptors

Nuclear hormone receptors such as the glucocorticoid (GR) and mineralocorticoid (MR) receptor are ligand activated transcription factors and belong to the nuclear receptor superfamily. The GR and the MR derive from a gene duplication of an ancestral corticoid receptor deep in the vertebrate lineage (Bridgham et al., 2006). Both receptors are composed of different domains with different functions (Figure 1-5). At the N-terminus of steroid hormone receptors (A/B region) resides the activation function (AF1)/tau1 domain which plays a critical role in the regulation of

target gene transcription (Figure 1-5). The DNA binding domain (DBD) (C region) has the most conserved amino acid sequence among members of the steroid hormone receptor family, and consists of two zinc-finger motifs, each containing four highly conserved cysteine molecules which coordinate binding of a zinc atom. This results in the formation of a tertiary structure containing helices that interact with specific DNA sequences, so called hormone response elements (HRE). The ligand binding domain (LBD) (E region) participates in several activities including hormone binding, homo- and/or heterodimerisation, formation of the heat-shock protein (HSP) complex and transcriptional activation and repression. Between the DBD and LBD lies the hinge region (D region) which is important for conformational changes upon ligand binding.

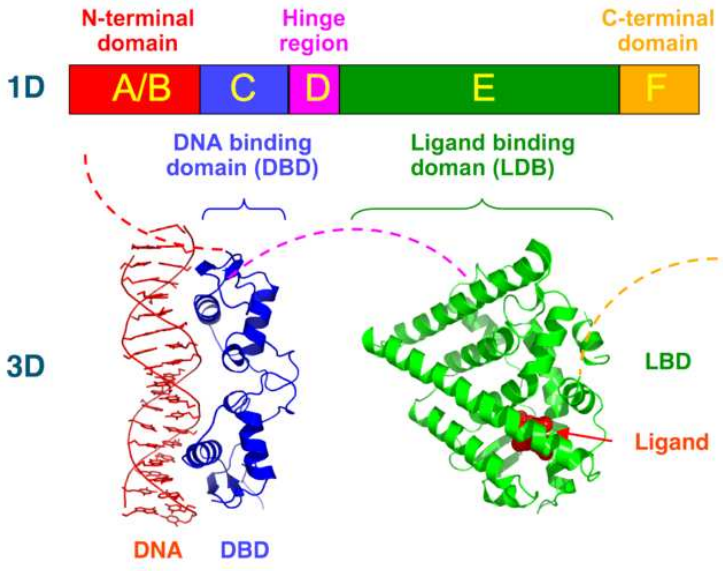

Figure 1-5 Schematic diagram of the steroid hormone receptor structure. The 1d model shows the different functional domains. The 3d model shows the interaction of the DNA binding domain with DNA and the interaction of the ligand binding domain (LBD) with a steroid ligand.

The hinge region allows the DBD and LBD to adopt different conformations without creating a steric hindrance. The hinge region also harbors a nuclear localisation signal or elements of a functional nuclear localisation signal.

1.4.2 Co-regulators of steroid hormone receptors

Following ligand binding, steroid hormone receptors undergo a conformational change which increases their affinity for co-regulator proteins. Co-regulators function as adaptors in a signaling pathway that transmits transcriptional responses from the DNA bound ligand-receptor complex to the basal transcriptional machinery. Numerous co-regulator proteins have been identified that interact with steroid hormone receptors such as the glucocorticoid (GR) and mineralocorticoid receptor (MR) to regulate transcription of target genes (Lonard & O'Malley, 2006). Depending on their effect on target gene transcription, co-regulators are divided in two different classes, co-activators and co-repressors. Co-activator proteins such as members of the p160 steroid receptor co-activators (SRCs) possess intrinsic histone acetyltransferase activity (HAT) and contain distinct regions and motifs for the recruitment of other proteins with enzymatic activities, including CREB-binding protein CBP/p300 and co-activator associated arginine methyltransferase 1 (CARM-1) (Stallcup et al., 2003; Xu & Li, 2003). Histone acetylation is important in maintaining an open chromatin structure, which facilitates the access of the ligand-activated steroid hormone receptor to the proximal promoter of a target gene. By contrast, co-repressors such as nuclear co-repressor (N-CoR) and silencing mediator of the retinoid and thyroid hormone receptor (SMRT) proteins have intrinsic histone deacetyltransferase activity that catalyse chromatin deacetylation and thereby form surfaces for the recruitment of additional components of co-repressor complexes, resulting in reduced transcriptional activity of target genes (Deroo & Archer, 2001).

1.4.3 Gene regulation by steroid hormones

Steroids are lipophilic hormones and can diffuse through plasma membranes. Classically steroid hormones exert their effects by binding to their cognate nuclear hormone receptor which in their unbound form reside in the cytoplasm where they form a multimeric protein complex (Figure 1-6). Chaperones such as HSPs are an integral part of this protein complex and ensure that the steroid hormone receptor remains in a high affinity conformation. Upon binding of the steroid to the LBD, HSPs dissociate from the receptor-ligand complex. Classically, the activated receptor then forms a homodimer and translocates to the nucleus where the steroid-nuclear receptor complex binds the HRE via its DBD. Following DNA binding, co-regulator proteins are recruited and interact with the transcriptional machinery to modulate target gene expression (described in section 1.4.2).

Besides their role in regulating transcription of target genes which typically occurs within minutes to hours, steroids can also exert more rapid effects which occur within seconds. To distinguish this alternative mode of steroid hormone action from classical transcriptional events, it is referred to as non-genomic or non-genotropic effects (Losel et al., 2004; Wehling et al., 2006). These rapid effects of steroid hormones were first observed by Hans Selye more than 60 years ago when he noticed that steroid infusions within a few minutes led to anaesthesia of laboratory animals (Selye, 1941). Since then these rapid effects have been studied on a cellular level and various classes of steroids including glucocorticoids, mineralocorticoids, estrogens and progestins have been shown to exert rapid effects in the cytoplasm resulting in activation of signal transduction pathways including the mitogen activated protein kinase (MAPK) / extracellular activated kinase (ERK), protein kinase A (PKA) and protein kinase C (PKC) pathway (Boldyreff & Wehling, 2003; Marino et al., 2006).

Not all of these rapid effects can be blocked by classical steroid hormone receptor antagonists suggesting that nuclear hormone receptors may not always be involved in mediating these effects.

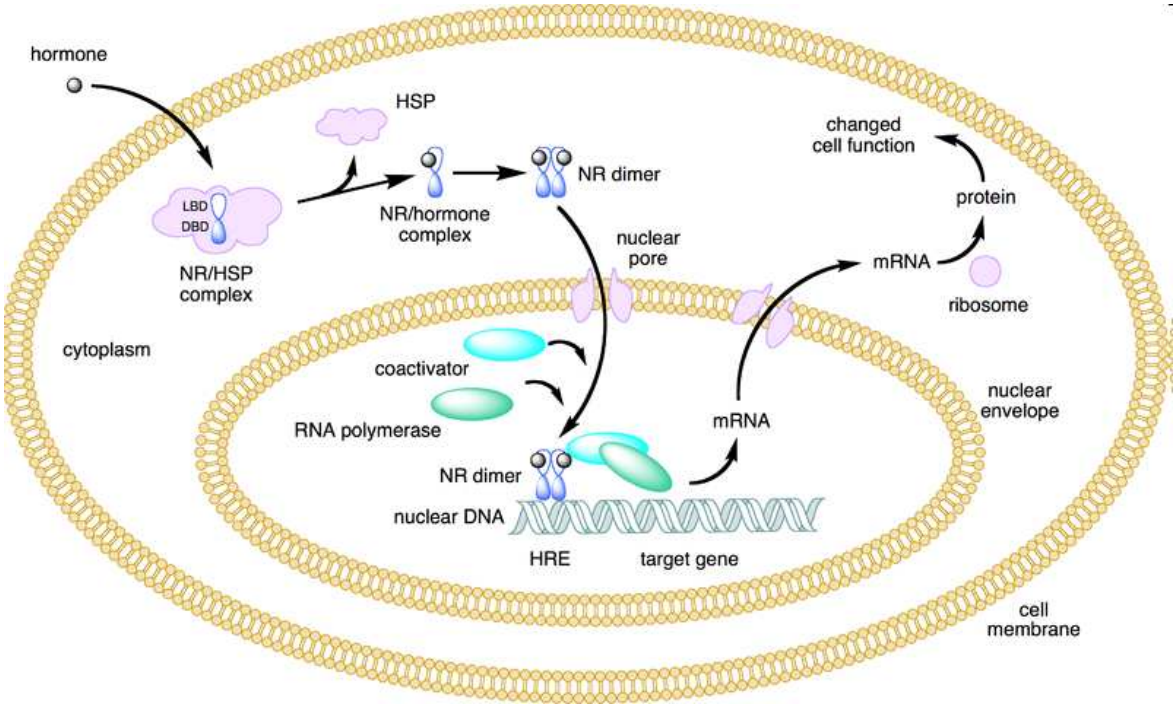

Figure 1-6 Schematic illustration of steroid hormone action. Steroid hormones bind to their cognate nuclear receptor (NR) which results in release of heat shock proteins (HSP). Two receptor monomers form a homodimer and translocate to the nucleus to bind to hormone responsive elements (HRE) and recruit co-activators and co-repressors to modulate target gene transcription.

1.5 Glucocorticoids

1.5.1 Regulation of glucocorticoid production

Adrenal glucocorticoid secretion is regulated by the hypothalamo-pituitary-adrenal (HPA) axis (Figure 1-7). The hypothalamus links the nervous and the endocrine system and in response to various stimuli releases corticotrophin releasing hormone (CRH) into the hypothalamo-hypophyseal portal vein. CRH then travels to the

pituitary gland to stimulate the release of the adrenocorticotropic hormen (ACTH) from the corticotroph cells located in the anterior lobe of the pituitary gland. In addition, ACTH release can be augmented by hypothalamic release of arginine vasopressin (AVP). By binding to the melanocortin 2 receptor (MC2R) ACTH stimulates adrenal *zona fasciculata* cells to release cortisol which exert a negative feedback upon CRH release in the hypothalamus and ACTH release in the pituitary gland (Figure 1-7).

HPA drive is tightly regulated by a variety of stimuli. Under physiological conditions HPA activity and consequently cortisol levels follow a circadian rhythm with highest levels in the morning reaching a nadir around midnight. Circadian regulation of hypothalamic CRH release is complex but the suprachiasmatic nucleus (SCN) of the hypothalamus appears to play a pivotal role as destruction of the SCN in rats results in the loss of corticosteroid periodicity (Moore & Eichler, 1972). Besides circadian rhythm, HPA drive is determined by a variety of factors such as food intake, mood, nutritional status, physical and psychological stress, and circulating cytokines. The dynamic range of the HPA axis is exemplified by the wide range of 24h cortisol secretion rates. Normal daily cortisol production adds up to approximately 8-12mg (Esteban et al., 1991) whereas under situations of severe stress, such as trauma or systemic infections, production rates can increase by up to 5 fold (Salem et al., 1994).

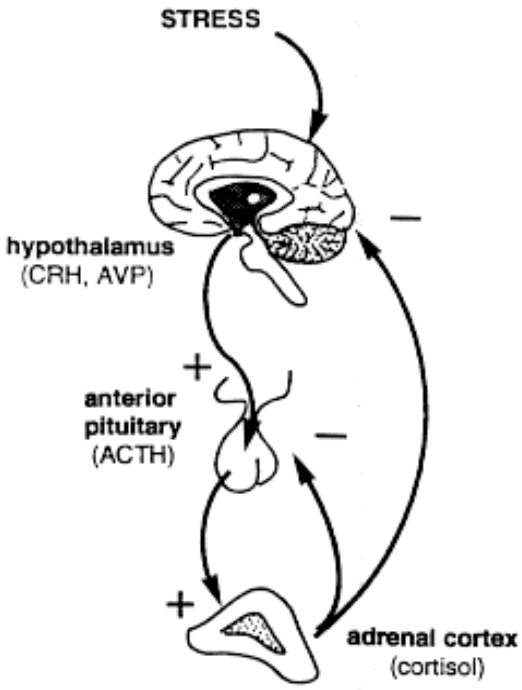

Figure 1-7 The hypothalamo-pituitary-adrenal (HPA) axis (taken from http://www.cns.med.ucla.edu).

1.5.2 Glucocorticoid hormone action

Virtually all cells in the body express the glucocorticoid receptor (GR) and are therefore glucocorticoid targets. Two splice variants of the GR exist, GRα and GRβ, but GRβ has a low affinity for glucocorticoids and may therefore function as a dominant negative regulator of GRα activation (Oakley et al., 1999). Following binding of cortisol (corticosterone in rodents) to the GR, the steroid-receptor complex forms a homodimer, translocates to the nucleus and binds to HREs which are organised as inverted repeats of the hexanucleotide motif TGTTCT with a spacer of 3 base pairs (ie AGAACAnnnTGTTCT). Following DNA binding, co-activators and co-repressors are recruited to activate or repress gene transcription (described in section 1.4.2)

Besides binding to HREs as a dimer, the GR can also directly interact with other transcription factors as a monomer and thereby modulate gene expression of target genes. These transcription factors include the nuclear factor κB (NF-κB), activating protein 1 (AP-1), interferon regulatory factor 3 (IRF-3), nuclear factor of activated T-cells (NFAT) and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and are typically involved in activation of inflammatory genes (De Bosscher et al., 2008). Direct interaction of monomeric GR with these transcription factors, however, represses expression of these inflammatory genes. It is therefore believed that the classical mode of action of GR by binding as a dimer to HREs is predominantly responsible for transactivation of target genes whereas monomeric interaction with other transcription factors is the main mode for transrepression (Reichardt et al., 2001).

1.5.2.1 Metabolic effects

Glucocorticoids play a pivotal role in carbohydrate, lipid and amino acid metabolism. In the liver, glucocorticoids increase gluconeogenesis and facilitate glucose release (Rooney et al., 1993). In muscle tissue, they induce protein catabolism resulting in release of amino acids. In adipose tissue, glucocorticoids induce both hormone-sensitive lipase and lipoprotein lipase activity which causes release of free fatty acids and glycerol into the circulation (Arner, 2005). Glucocorticoids impair insulin mediated glucose uptake which results in insulin resistance of peripheral tissues including the liver, muscle and adipose tissue (van Raalte et al., 2009).

In the skin, glucocorticoids reduce collagen synthesis which causes loss of connective tissue and as a result thinning and vulnerability, whereas in bone glucocorticoids suppress osteoblast function and thereby favour bone catabolism over bone formation.

1.5.2.2 Immune modulatory effects

Glucocorticoids have inhibitory effects on a broad range of specific immune responses mediated by T cells and B cells as well as potent suppressive effects on the effector functions of monocytes and neutrophils. Not only do glucocorticoids reduce the number of circulating lymphocytes and monocytes, they also suppress the activity of B cells and proliferation of cytotoxic T cells by interacting with macrophages and helper T cells. For example, glucocorticoids decrease the synthesis of interleukin 1 (IL-1) by macrophages (MacDermott & Stacey, 1981) and the synthesis of interleukin 2 (IL-2) by T helper cells (Gillis et al., 1979). These cytokines increase the activity of B cells and cytotoxic T cells, as well as those of other leukocytes including macrophages and helper T cells.

1.5.3 Glucocorticoid excess

Glucocorticoid excess most commonly results from pharmacological treatment with synthetic glucocorticoids whereas endogenous glucocorticoid excess (Cushing's syndrome) is a rare condition of excessive cortisol production. The phenotypic pattern of glucocorticoid excess can be predicted from the physiological effects of cortisol. One of the most striking features associated with glucocorticoid excess is weight gain which is caused by accumulation of adipose tissue in defined regions including visceral, dorsal and facial fat depots resulting in the clinical appearance of an "apple shape" body composition, a "buffalo hump" and the appearance of a "moon face", respectively (Figure 1-8). In contrast, glucocorticoid excess leads to muscle atrophy resulting in sarcopenia and proximal myopathy. In the skin, glucocorticoid excess results in thinning, easy bruising and development of purple striae on the abdominal skin. Glucocorticoid excess results in a reduction of bone mineral density

and development of osteoporosis. In addition, patients have high blood pressure and show insulin resistance which may manifest as type II diabetes mellitus.

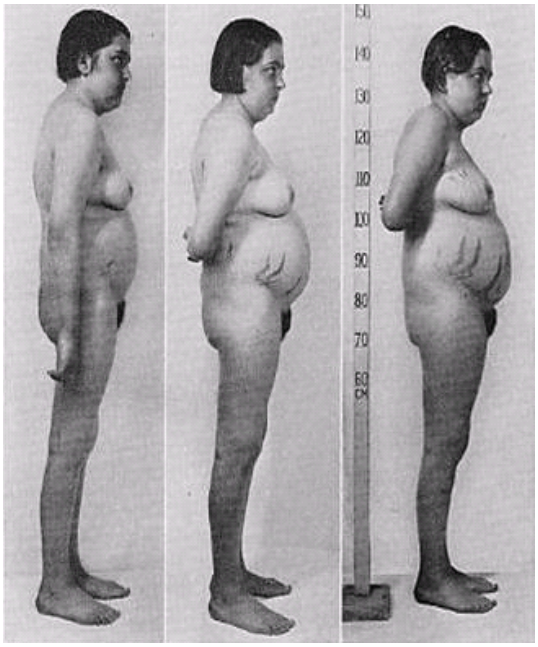

Figure 1-8 Depiction of the clinical signs of Cushing's syndrome. This picture shows the progression of classical clinical signs of overt cortisol excess (Cushing's syndrome) including "moon face", "buffalo hump", central obesity, abdominal stretch marks, proximal myopathy, loss of height due to osteoporotic fractures.

In order to prevent these detrimental consequences of glucocorticoid excess, exogenous glucocorticoid treatment should be at the lowest acceptable dose and for as short duration as possible. Whereas in patients with Cushing's syndrome the underlying condition (i.e. pituitary adenoma, ectopic ACTH producing tumour or adrenal adenoma/carcinoma) requires surgically removal.

1.5.4 Glucocorticoid metabolism

Plasma cortisol is bound with high affinity to corticosteroid-binding globulin (CBG), which protects it from degradation. The normal plasma half-life of cortisol is

approximately 80-100 minutes (Brien, 1981). Increased metabolism of cortisol results in a shortened half life. In order to prevent a fall in circulating cortisol, HPA drive and consequently adrenal cortisol production increases, compensating for increased cortisol clearance. Historically the liver has been regarded as the predominant site of cortisol metabolism but a wide variety of extra hepatic tissues express enzymes involved in cortisol metabolism. The major enzymatic reactions contributing to cortisol metabolism are depicted in Figure 1-9. Cortisol can be converted to cortisone by the enzyme 11β-HSD2 which is predominantly found in the kidney. The opposite reaction, namely the reactivation of cortisol from cortisone, is catalysed by 11β-HSD1 which is predominantly expressed in the liver and adipose tissue. Global activities of both isozymes determine the ratio of cortisol to cortisone in the circulation and consequently, alterations of enzymatic activities results in an altered equilibrium. Cortisol and cortisone can be metabolised by reduction of the double bond of the A ring by the action of 5α- and 5β-reductases resulting in the respective 5α- and 5β-dihydro steroids. These are subsequently dehydrogenated by 3α-hydroxysteroid dehydrogenases (3α-HSDs) leading to the generation of tetrahydro-cortisone (THE), 5β-tetrahydro-cortisol (5β-THF) and 5α-tetrahydro-cortisol (5α-THF). In normal human physiology global 5β-reductase activity is twice as high as that of 5α-reductase resulting in a 5β-THF / 5α-THF ratio of approximately 2. To a lesser extent cortisol and cortisone are also metabolised by hydroxylation at positions C6, C20 (Shackleton et al., 1985).

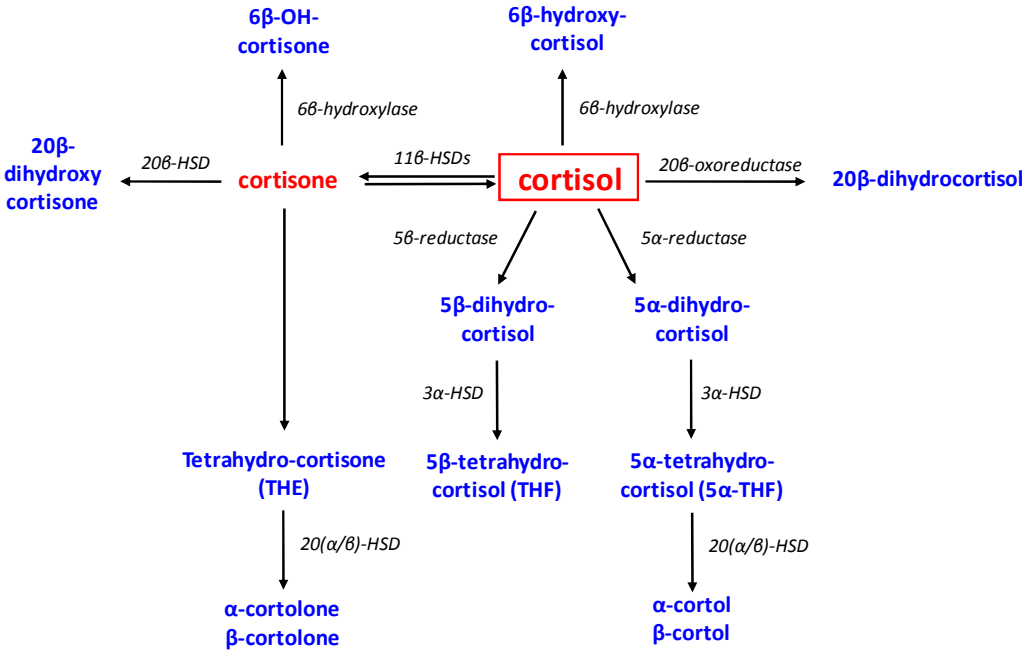

Figure 1-9 Depiction of the main enzymatic steps involved in cortisol metabolism (3α-, 11β-, 20α/β-hydroxysteroid dehydrogneases (HSDs), 5α-, 5β-reductases).

1.6 Mineralocorticoids

1.6.1 Regulation of mineralocorticoid production

Aldo, the principal mineralocorticoid hormone, plays a pivotal role in the regulation of electrolyte and fluid homeostasis by regulating sodium reabsorption and potassium excretion in mineralocorticoid target tissues such as the kidney, salivary glands and colon. Aldo secretion from the *zona glomerulosa* of the adrenal gland is regulated by distinct mechanisms, the most important being the RAAS (Figure 1-10). Sodium depletion, low blood pressure and/or hypovolaemia are key stimuli, sensed by cells of the *macula densa* of the juxtaglomerular apparatus in the kidney, to release renin which is a protease that converts angiotensinogen into Ang I. Ang I then undergoes a second proteolytic step catalysed by the angiotensin converting enzyme (ACE) resulting in angiotensin II (Ang II). In the *zona glomerulosa* of the adrenal gland, Ang

II by binding to angiotensin II type 1 receptors stimulates Aldo production and release. In mineralocorticoid target cells Aldo binds to its cognate receptor, the MR, resulting in target gene regulation which ultimately leads to increased urinary sodium reuptake, potassium excretion and water retention. Fluid and sodium retention in turn results in expansion of the extracellular space and as a consequence blood pressure increases. By contrast, high blood pressure and/or fluid overload inhibits renin secretion and as a consequence Ang II and Aldo levels decrease.

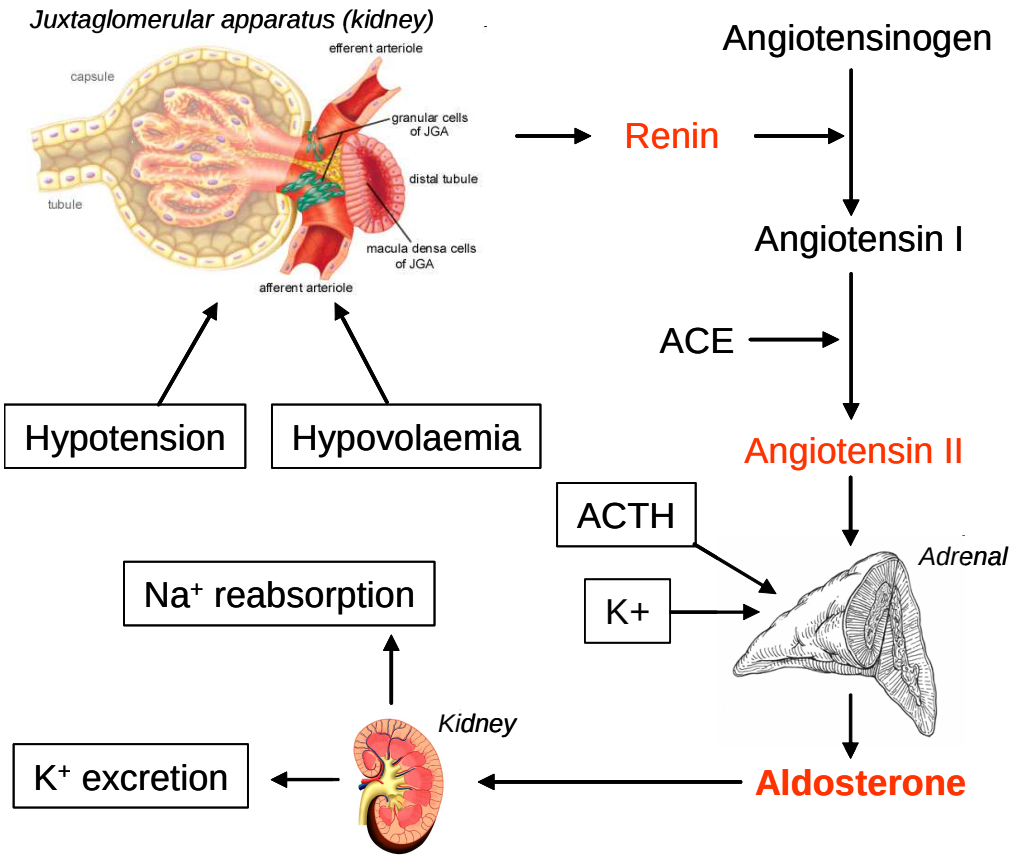

Figure 1-10 Schematic illustration of the renin-angiotensin-aldosterone system (RAAS).

Beside the RAAS, Aldo synthesis and secretion is also directly stimulated by high potassium levels (hyperkalaemia) and by ACTH. Consequently, Aldo levels similarly

to cortisol also exhibit a circadian rhythm with highest levels in the morning and lowest levels before midnight.

1.6.2 Mineralocorticoid action

Classical mineralocorticoid responsive cells which are characterised by the concomitant expression of the MR and the enzyme 11β-HSD2 (described in section 1.7.2.2), encompass epithelial cells of the distal nephron (distal tubule and collecting duct) of the kidney, ductal cells of sweat glands and enterocytes of the distal colon. After entering the cytoplasm, Aldo binds to the MR which homodimerises and translocates into the nucleus where it binds to HREs, which have an identical DNA sequence to HREs bound by the GR. Target genes for the MR in the kidney include the serum and glucocorticoid induced kinase 1 (SgK1), glucocorticoid-induced leucine zipper (GILZ), the α subunit of the epithelial sodium channel (α-ENaC), the renal outer medullary potassium channel (ROMK), and the sodium/potassium adenosine triphosphatase (ATPase) (Bens et al., 2006).

ENaC is expressed at the apical membrane and constitutes the rate-limiting step in transepithelial Na^+ transport. The abundance of functional ENaC is dynamically regulated both at the level of ENaC production and integration into the cell membrane, as well as ENaC endocytosis and degradation (Figure 1-11). The ubiquitin ligase neural precursor expressed, developmentally down-regulated protein 4-2 (Nedd4-2), is associated with ENaC at the cell surface and facilitates degradation and endocytosis by ubiquitination of N-terminal lysines of the α and γ subunit of ENaC (Kamynina & Staub, 2002; Snyder, 2005).

MR mediated Aldo action increases ENaC abundance and thereby transepithelial Na^+ transport by two separate mechanisms. Firstly, Aldo directly stimulates expression of α-ENaC, which in turn facilitates ENaC release from the endoplasmic

reticulum resulting in the formation of new ENaC complexes. Secondly, it also up-regulates the serine/threonine kinase SgK-1 which phosphorylates Nedd4-2 resulting in reduced degradation of ENaC.

Other direct target genes of Aldo action include that of the renal outer medullary potassium (ROMK) channel, which is expressed at the apical membrane and facilitates potassium excretion, and the Na^+/K^+ ATPase which is expressed at the basolateral membrane to exchange intracellular sodium and potassium.

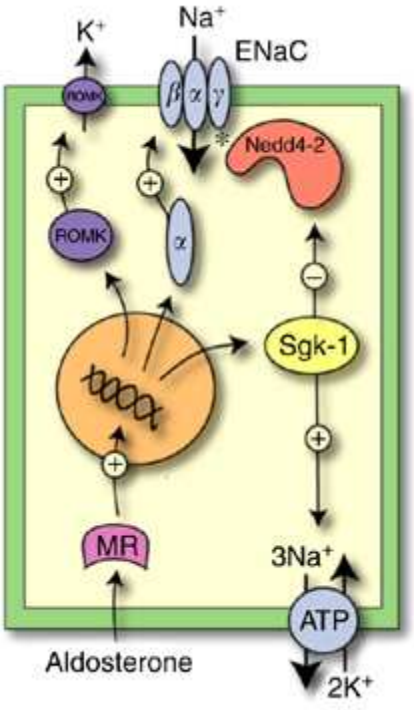

Figure 1-11 Schematic illustration of aldosterone action in cells of the distal nephron of the kidney. ENaC: epithelial sodium channel; ROMK: renal outer medullary potassium channel; SgK-1: serum and glucocorticoid induced kinase 1; Nedd4-2: neural precursor expressed, developmentally down-regulated protein 4-2 (Kiryluk & Isom, 2007).

1.6.3 Primary hyperaldosteronism

Primary hyperaldosteronism (PHA) is now considered the most frequent cause of secondary hypertension accounting for 5-10% of all hypertensives. Primary

hyperaldosteronism is either caused by an Aldo producing adenoma (APA) (about 1/3 of all patients) or by excessive bilateral Aldo production from hyperplastic adrenals and is then referred to as idiopathic hyperaldosteronism (IHA). Aldo excess invariably leads to increased salt and fluid retention, reflected by suppressed renin levels and high blood pressure but hypokalaemia develops only in 50% of APA and 20% of IHA patients (Rossi et al., 2006). Patients with APAs may be amenable for surgery, whereas all other patients are treated medically with the overriding goal to normalise blood pressure to prevent co-morbidities secondary to high blood pressure levels (Young, 2007).

Familial hyperaldosteronism (FH) is a rare heritable form of primary hyperaldosteronism and two types have been described. FH type I, or glucocorticoid-remediable aldosteronism (GRA), is autosomal dominant in inheritance and caused by a fusion of the promoter region of the gene for CYP11B1 and the coding sequences of CYP11B2, resulting in ACTH-dependent activation of the aldosterone synthase gene in the *zona glomerulosa*. Suppression of ACTH drive by exogenous glucocorticoids results in down-regulation of aldosterone synthase activity and ameliorates Aldo excess in FH type I. FH type II refers to the familial occurrence of APA or IHA or both but so far the underlying cause has not been identified (So et al., 2005).

1.6.4 Secondary hyperaldosteronism

Secondary hyperaldosteronism is characterised by the hypersecretion of Aldo as a consequence of increased activation of the RAAS and can be divided into two categories depending on the presence of hypertension. In the absence of hypertension RAAS activation is a result of homeostatic attempts to maintain sodium or circulatory volume which occurs in diarrhea, excessive sweating, low cardiac

output states, and hypoalbuminemia due to liver or renal disease. Renovascular hypertension is the most common cause of secondary hyperaldosteronism associated with high blood pressure. It is caused by ischemia of the renal parenchyma secondary to atherosclerotic renal artery stenosis or fibromuscular dysplasia. By contrast, renin secreting juxtaglomerular cell tumours of the kidney and Wilms tumours are rare forms of elevated renin and Aldo levels in the presence of hypertension.

1.6.5 Mineralocorticoid receptor antagonists

MR antagonists are steroid based compounds (Figure 1-12) that competitively inhibit the binding of Aldo to the MR and thereby prevent Aldo induced MR activation. Given the classical role of Aldo in blood pressure regulation, both Spiro and EPL are licensed as anti-hypertensive drugs and exhibit a particularly good blood pressure lowering effect in patients with so called “resistant hypertension” (Chapman et al., 2007) and patients with primary hyperaldosteronism (Karagiannis et al., 2008). In addition, Spiro has also been used for a long time in patients with chronic liver disease (Moore et al., 2003) and heart failure (Weber & Villarreal, 1993), conditions characterised by avid salt and fluid retention as a result of RAAS activation. More recently, the RALES and EPHESUS study have shown beneficial effects of MR antagonists in patients with chronic and acute post-MI heart failure as low doses of Spiro and EPL, respectively, dramatically improved survival in these patients (Pitt et al., 2003b; Pitt et al., 1999). The use of these drugs, however, may be limited in some patients given their side effects. Spiro is an unselective MR antagonist as it exhibits also anti-androgenic and progestional effects which can result in gynecomastia and erectile dysfunction in men and irregular menstrual bleeding in females. By contrast, EPL does not interfere with the androgen or progesterone

receptor and is therefore considered a selective Aldo receptor antagonist (SARA). Both drugs, however, even when used at low doses have the risk to cause hyperkalaemia, particularly in patients who are already treated with an angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) and patients with chronic renal impairment. Therefore in these patients potassium levels need to be monitored regularly.

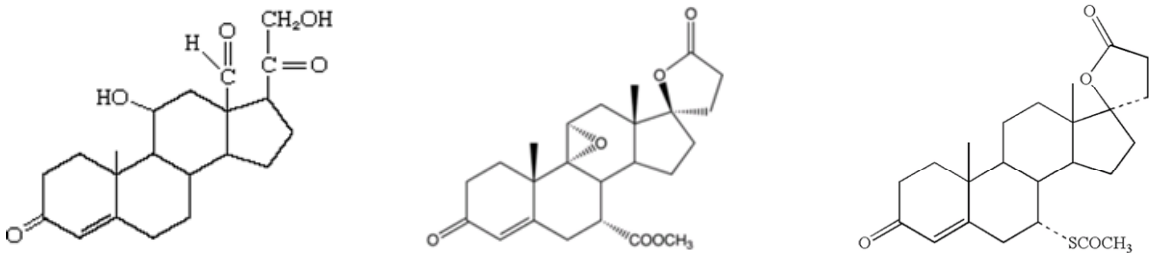

Figure 1-12 Structures of aldosterone and its antagonists eplerenone and spironolactone.

1.6.6 Mineralocorticoid metabolism

The half life of Aldo in the circulation is approximately 30 minutes (Coppage et al., 1962). The liver is the main site of Aldo metabolism. Only small amounts of Aldo appear in the urine whereas the majority is excreted as biologically inactive, water-soluble Aldo metabolites. The main urinary Aldo metabolite is 3α,5β-tetrahydro-aldosterone (TH-Aldo) which results from A ring reduction by 5β-reductase and subsequent dehydrogenation by 3α-HSDs (Figure 1-13). Other Aldo metabolites include 3α-hydroxy-5β-pregnane-(11β-18),(18-20)-dioxide and aldosterone-18-glucuronide. By contrast, only trace amounts of Aldo appear as 5α-reduced metabolites in the urine.

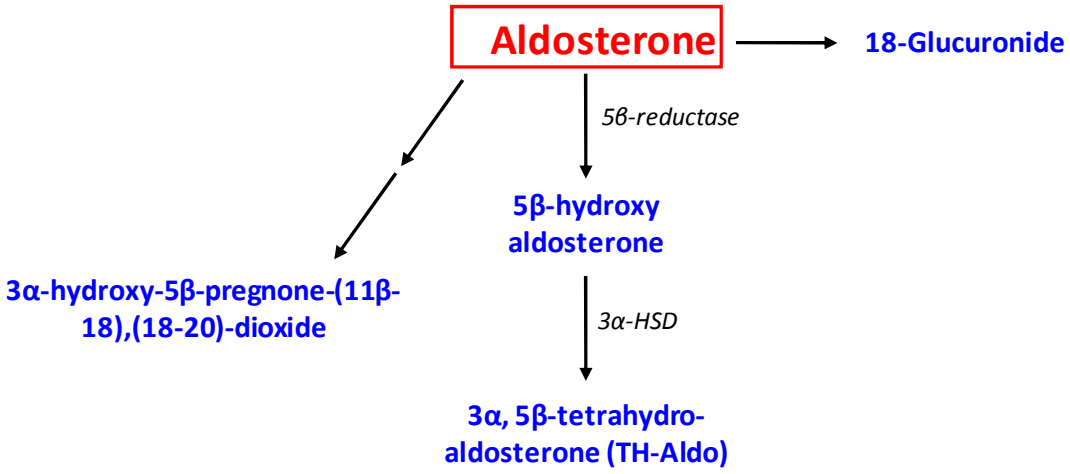

Figure 1-13 Metabolism of aldosterone.

1.7 11β hydroxysteroid dehydrogenases

The interconversion of active glucocorticoids cortisol (Kendall's compound F) in man and corticosterone (Kendall's compound B) in rodents into their biologically inactive metabolites (cortisone in man, 11-dehydrocorticosterone in rodents (Kendall's compounds E and A, respectively)) is catalysed by two 11β-HSD isoenzymes (Figure 1-14). 11β-HSD type 1 was initially purified from rat liver microsomes and showed only dehydrogenase activity in tissue homogenates (conversion of cortisol to cortisone) (Lakshmi & Monder, 1988). This was a surprising finding because *in vivo* the liver showed oxoreductase activity (conversion of cortisone to cortisol) suggesting the existence of a second isoform (Monder et al., 1989). Later on it became clear that 11β-HSD1 indeed possesses both oxoreductase and dehydrogenase activities *in vitro* but *in vivo* and in intact cells it predominantly functions as an oxoreductase.

Evidence for an additional isoenzyme that was active in mineralocorticoid target tissues was initially obtained from histochemical studies on kidney sections. A few years after purification and cloning of 11β-HSD1, this isoenzyme was identified by expression cloning and named 11β-HSD2 (Albiston et al., 1994). The two

isoenzymes are products of two separate genes, possess only 14% identity, exhibit different enzymatic kinetics and have different expression patterns.

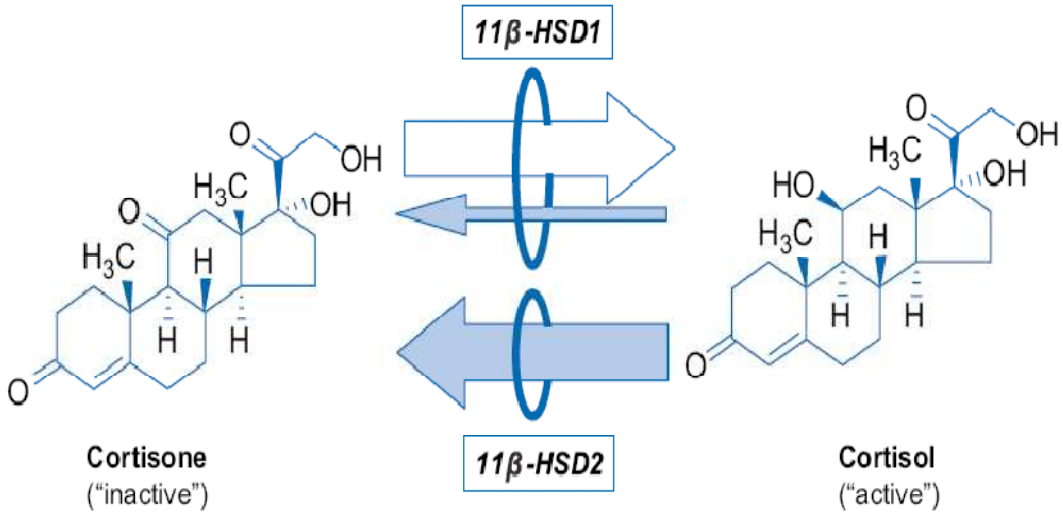

Figure 1-14 Interconversion of cortisol and cortisone by 11β-hydroxysteroid dehydrogenases (11β-HSDs) (Hammer & Stewart, 2006).

1.7.1 11β-hydroxysteroid dehydrogenase type 1

1.7.1.1 Characterisation of 11β-HSD1

The human 11β-HSD1 enzyme is encoded by the HSD11B1 gene located on chromosome 1q32.2-41 spanning 30 kbp. The gene consists of 6 exons encoding a messenger ribonucleic acid (mRNA) of approximately 1400 nucleotides which is translated into a protein of 292 amino acid with a molecular weight of 30 kDa (Tomlinson et al., 2004). The enzyme belongs to the family of the short-chain dehydrogenases/reductases and predominantly utilises NADPH/$NADP^+$ as a co-factor.

Enzymatic studies of purified homogenous enzyme revealed Michaelis-Menten (K_m) constants of 1.83 - 0.06 µM for cortisol and 17.3 - 2.24 µM for corticosterone. Although first-order rate constants are one order of magnitude lower for cortisol than corticosterone, maximal velocities are similar (Lakshmi & Monder, 1988).

1.7.1.2 Function and expression

Initially, kinetic assessment of 11β-HSD1 revealed only dehydrogenase activity in tissue homogenates but subsequent studies in intact tissues (Duperrex et al., 1993) as well as whole cells (Low et al., 1994) showed oxoreductase activity. It is now well accepted that although 11β-HSD1 is a bidirectional enzyme, capable of catalysing both oxoreductase as well as dehydrogenase reactions, *in vivo* almost exclusively behaves as an oxoreductase. The enzyme is attached to the membrane of the endoplasmic reticulum (Monder & Lakshmi, 1988) with its catalytic domain facing the lumen of the endoplasmic reticulum (Odermatt et al., 1999; Ozols, 1995). Although the endoplasmic reticulum as a whole is regarded an oxidative environment, it harbors the enzyme hexose-6-phosphate dehydrogenase (H6PDH) which regenerates NAPDH from $NADP^+$ by converting glucose-6-phosphate into 6-phospho-gluconolactone (Figure 1-15). It has recently been demonstrated that NADPH production by H6PDH is indispensable for 11β-HSD1 oxoreductase activity as mice which with a disruption of the H6PDH gene show a complete loss of oxoreductase activity and reversal of its enzymatic activity from an oxoreductase to a dehydrogenase (Lavery et al., 2006).

11β-HSD1 facilitates tissue specific GR-mediated hormone action and is widely expressed in both man (Ricketts et al., 1998) and rodents (Gomez-Sanchez et al., 2008) with highest expression levels and activity in liver and adipose tissue as well as gonads and the brain (Tomlinson et al., 2004).

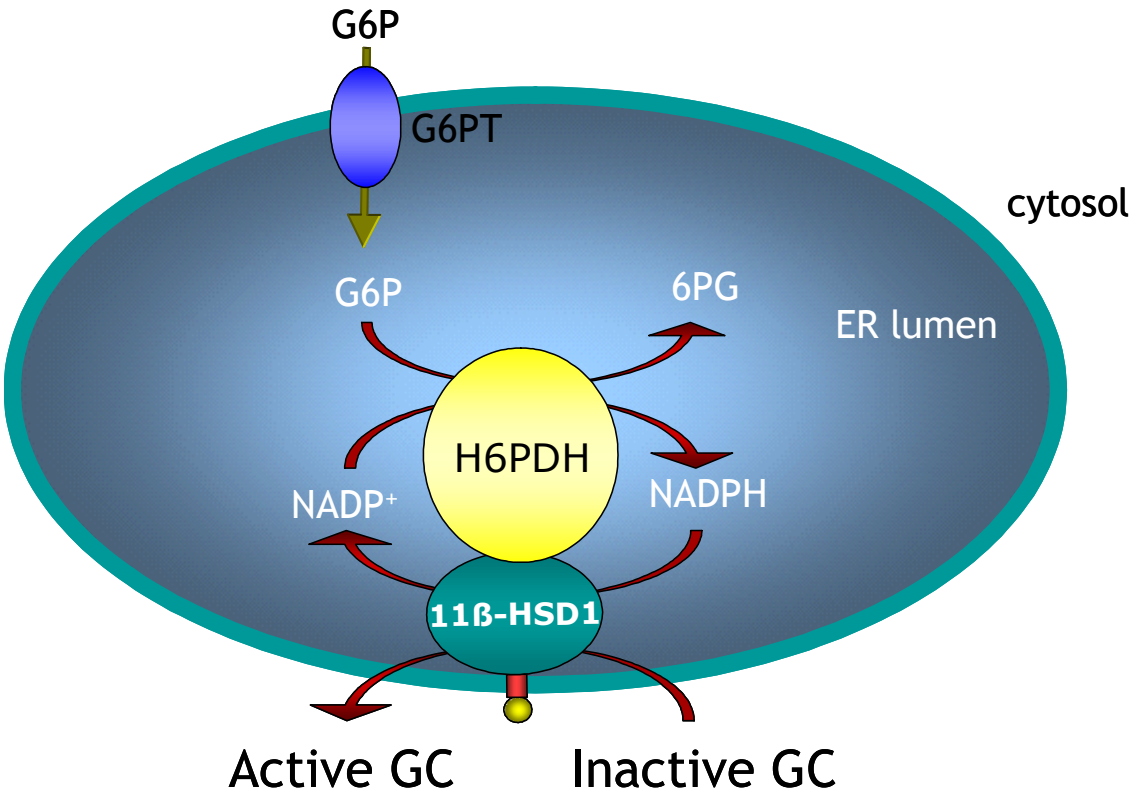

Figure 1-15 Schematic illustration of 11β-HSD1 function in the endolasmic reticulum. 11β-HSD1 faces the lumen of the endoplasmic reticulum where it reduces inactive into active glucocorticoids (GC) at the expense of NADPH which is oxidised to $NADP^+$. NADPH supplies are regenerated from $NADP^+$ by the microsomal enzyme hexose-6-phosphate-dehydrogenase (H6PDH) which converts glucose-6-phosphate (G6P) to 6-phospho-gluconolactone (6PG). G6P enters the endoplasmic reticulum via G6P translocase (G6PT).

1.7.1.3 Cortisone reductase deficiency

Cortisone reductase deficiency (CRD) is characterised by a failure to regenerate cortisol from cortisone via 11β-HSD1. To date only heterozygote mutations resulting in 11β-HSD1 haploinsufficiency have been described in humans. A putative phenotype may be predicted from genetically engineered mice with a disruption of the gene encoding for 11β-HSD1. These mice are unable to convert inert 11-dehydrocorticosterone to corticosterone resulting in a reduced half life of corticosterone (Kotelevtsev et al., 1997). This is compensated for by an increased

HPA drive with increased adrenal corticosterone production reflected by hyperplastic adrenal glands. Phenotypically, these mice exhibit mainly metabolic changes. On starvation they show attenuated activation of the key hepatic gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase and are resistant to hyperglycemia provoked by obesity or stress. Thus, attenuation of hepatic 11β-HSD-1 may provide a novel approach to modulate hepatic gluconeogenesis and thereby improve glucose homeostasis.

In man, mutations in the gene encoding for H6PDH have been described which result in a functional loss of 11β-HSD1 oxoreductase activity (Draper et al., 2003; Lavery et al., 2008). Biochemically these patients show very low ratios of urinary cortisol (THF+5α-THF) to cortisone (THE) metabolites of 0.05 or lower (reference range, 0.7–1.2) which are in agreement with loss of 11β-HSD1 oxoreductase and gain of dehydrogenase activity. Clinically, female patients show a mild phenotype of androgen excess which is a result of increased HPA drive resembling that of polycystic ovarian syndrome (PCOS). The male phenotype is precocious puberty which presumably reflects increased adrenal androgen production (Lavery et al., 2008).

1.7.1.4 11β-HSD1 and the metabolic syndrome

The metabolic syndrome describes an aggregation of cardiovascular risk factors and according to the World Health Organisation (WHO) is defined by the presence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance and presence of two of the following criteria: elevated blood pressure (≥ 140/90 mmHg), dyslipidaemia (triglycerides: ≥ 1.695 mmol/L and high-density lipoprotein cholesterol (HDL-C) ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female)), central obesity (waist : hip ratio > 0.90 (male); > 0.85 (female), or body mass index > 30

kg/m^2) and microalbuminuria (urinary albumin excretion ratio ≥ 20 µg/min or albumin creatinine ratio (ACR) ≥ 30 mg/g).

Although patients with the metabolic syndrome and Cushing's syndrome share many clinical features, circulating cortisol levels in patients with the metabolic syndrome are normal whereas in Cushing's syndrome they are elevated. The enzyme 11β-HSD1 plays a pivotal role in determining tissue specific glucocorticoid concentrations by regenerating active from inactive glucocorticoids, and thus, it has been hypothesised that increased 11β-HSD1 activity may be involved in the pathogenesis of the metabolic syndrome. To further explore this theory, transgenic mice strains have been created which overexpress 11β-HSD1 in adipose tissue (Masuzaki et al., 2001; Masuzaki et al., 2003) and the liver (Paterson et al., 2004). Mice overexpressing 11β-HSD1 in adipocytes exhibit visceral obesity, insulin-resistant diabetes, hyperlipidaemia and hyperphagia (Masuzaki et al., 2001) and develop salt-sensitive hypertension (Masuzaki et al., 2003). By contrast, mice with liver specific overexpression of 11β-HSD1 remain lean but exhibit fatty liver, insulin-resistance and also develop hypertension as a result of liver glucocorticoid excess (Paterson et al., 2004). Taken together, increased 11β-HSD1 activity in these mouse models strikingly recapitulates many features of the metabolic syndrome suggesting that increased 11β-HSD1 activity may be a critical contributory factor in the development and pathogenesis of the metabolic syndrome.

1.7.1.5 Pharmacological inhibition of 11β-HSD1

11β-HSD1 activity is inhibited by the liquorice derivatives, glycyrrhizic acid, its hydrolytic product glycyrrhetinic acid (GE), and the hemisuccinate derivative carbenoxolone (CBX). Glycyrrhetinic acid is a potent competitive inhibitor of 11β-HSD1 (Monder et al., 1989; Whorwood et al., 1993), but it is not selective to 11β-

HSD1 as it also inhibits 11β-HSD2 with an inhibition constant (K_i) of 5–10 nM (Albiston et al., 1994; Stewart et al., 1994).

1.7.2 11β-hydroxysteroid dehydrogenase type 2

1.7.2.1 Characterisation of 11β-HSD2

The human 11β-HSD2 enzyme is encoded by the HSD11B2 gene located on chromosome 16q22 spanning 6.2 kbp (Agarwal et al., 1995). The gene consists of 5 exons encoding for a mRNA of approximately 1800 nucleotides which is translated into a protein of 405 amino acid with a molecular weight of approximately 40 kDa (Brown et al., 1996a). 11β-HSD2 belongs to the family of short-chain alcohol dehydrogenases. Sequence alignment shows highest similarity (37%) with placental 17β-hydroxysteroid dehydrogenase type 2 (Wu et al., 1993), whereas it is only 21% identical to the predicted sequence of human 11β-HSD1 (Tannin et al., 1991).

Similar to the 11β-HSD1 isoenzyme, 11β-HSD2 is bound to the membrane of the endoplasmic reticulum, but in contrast, its catalytical domain faces the cytoplasm (Odermatt et al., 1999; Ozols, 1995). Hydropathicity analysis suggests that 11β-HSD2 has three successive hydrophobic segments of approximately 20 amino acids each in the N-terminal region (Agarwal et al., 1994) which may function as transmembrane segments anchoring the enzyme to the membrane of the endoplasmic reticulum.

Kinetic studies of 11β-HSD2 show that in contrast to 11β-HSD1, it functions solely as a dehydrogenase (converting cortisol to cortisone) and has a high affinity for cortisol and corticosterone with an apparent Km of 47 and 5.1 nM, respectively, and relies on the presence of $NADH/NAD^+$ as a co-factor (Albiston et al., 1994; Brown et al., 1993; Stewart et al., 1994). Mutations (1.7.2.3) or pharmacological inhibition of 11β-HSD2

(1.7.2.4) results in reduced cortisol metabolism and as a consequence cortisol half life is prolonged.

1.7.2.2 Localisation and function – MR specificity

Expression of 11β-HSD2 has been examined by RNA blot hybridisation in various species including humans, sheep and rodents. In all species, this isoenzyme is highly expressed in the placenta and mineralocorticoid target tissues, particularly the kidney, sweat glands and the colon, whereas it is not detected in the liver, heart, or adult testis (Agarwal et al., 1994; Albiston et al., 1994; Cole, 1995; Zhou et al., 1995). 11β-HSD2 is also expressed in vascular smooth muscle cells (Hatakeyama et al., 1999) and the brain where its expression is restricted to cells in the commissural portion of the nucleus tractus solitarius, subcommissural organ, and ventrolateral and ventromedial hypothalamus (Roland et al., 1995).

The distinct tissue expression pattern of 11β-HSD2 in classical mineralocorticoid target tissues is closely related to its function. Circulating concentrations of cortisol and corticosterone are 100 to 1000-fold higher than those of Aldo but, paradoxically, the MR has similar affinities for both Aldo and cortisol *in vitro* (Edwards et al., 1988; Funder et al., 1988). Therefore, MR selectivity for Aldo in mineralocorticoid target tissues relies on efficient pre-receptor inactivation of cortisol to cortisone by 11β-HSD2 (Figure 1-16). In contrast to cortisol, Aldo cannot be metabolised by 11β-HSD2 because it forms a C11-C18 hemi-ketal group in aqueous solution.

Besides classical mineralocorticoid target tissues, 11β-HSD2 is also highly expressed in the placenta where it protects the embryo from maternal spill over of active glucocorticoids. Insufficient placental metabolism of active glucocorticoids results in intrauterine growth restriction (IUGR) (Shams et al., 1998). Furthermore, excess intrauterine glucocorticoid exposure leads to fetal programming of adult

disease including increased blood pressure in adulthood, glucose levels, HPA axis activity and anxiety-related behaviors (Seckl & Holmes, 2007).

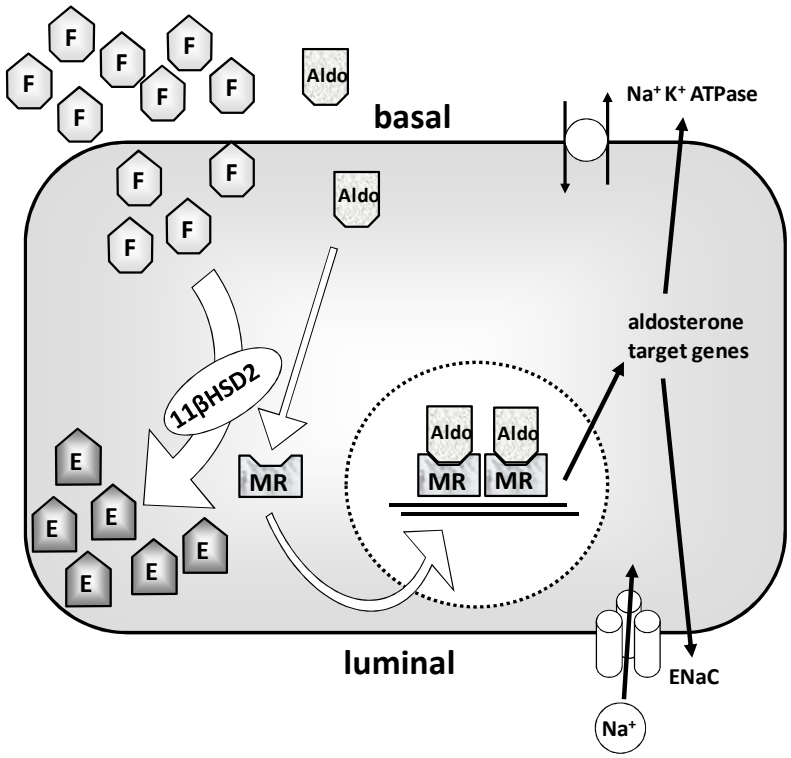

Figure 1-16 Schematic illustration of aldosterone action in the kidney. The mineralocorticoid receptor (MR) has equal affinities for cortisol and aldosterone. Binding of aldosterone (Aldo) in the kidney is ensured by 11β-HSD2 which converts cortisol (F) into inactive cortisone (E). Activation of the MR by Aldo results in target gene expression including the epithelial sodium channel (ENaC) (Hammer & Stewart, 2006).

1.7.2.3 Apparent Mineralocorticoid Excess

Apparent mineralocorticoid excess (AME) is an extremely rare form of hypertension; so far fewer than 100 cases have been described worldwide (Funder et al., 1988; Wilson et al., 2001). AME is inherited in an autosomal recessive fashion and more than 60 kindreds with affected siblings have been reported. Presentation is usually during neonatal life or childhood with low birth weight, failure to thrive, short stature, severe hypertension and hypokalaemic metabolic alkalosis. In addition, renal cysts

and nephrocalcinosis have been reported and may lead to renal insufficiency. Biochemical abnormalities comprise hypokalaemia, suppressed renin and undetectable Aldo levels, hence the term ‘apparent mineralocorticoid excess’. The molecular basis for this condition was only elucidated after cloning the HSD11B2 gene. All identified patients harbor bi-allelic HSD11B2 mutations resulting in a functionally defective enzyme (Figure 1-17). Lack of functional 11β-HSD2 leaves the MR unprotected from cortisol binding and activation. These findings in humans are reflected in mice with a genetically engineered disruption of the HSD11B2 gene which show a similar phenotype as AME patients (Kotelevtsev et al., 1999). At present, over 30 different mutations have been identified within the HSD11B2 gene to cause AME. Missense mutations have emerged as the most frequent genetic alteration in AME but frame shift and splice site mutations have also been described (Hammer & Stewart, 2006).

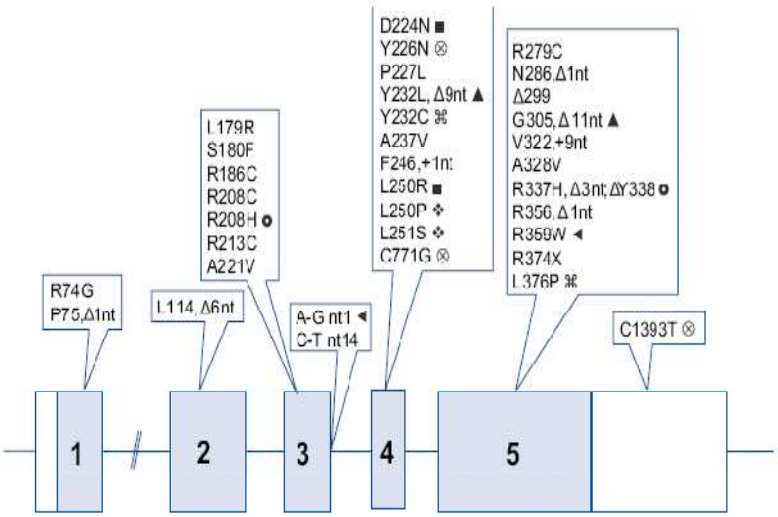

Figure 1-17 Schematic illustration of the HSD11B2 gene structure and all mutations that have been identified to cause AME (Hammer & Stewart, 2006).

1.7.2.4 Pharmacological inhibition of 11β-HSD2

Liquorice extract (Glycyrrhiza glabra) has been used for hundreds of years as a sweetener in confectionery and as a herbal remedy. Isolation of the active component, glycyrrhetinic acid and the observation that liquorice promotes healing of peptic ulcers led to the development of carbenoxolone, an 18β-hemisuccinate derivative of GA, which subsequently was used as a drug in the treatment of both duodenal and gastric ulcerations. However, both liquorice and carbenoxolone induce undesirable mineralocorticoid-like side effects including oedema, hypertension and hypokalaemia in up to 50% of patients (Card et al., 1953) which are explained by their potent ability to inhibit 11β-HSD2 with a K_i in the low nanomolar range. Therefore, liquorice intoxication is now well accepted as the acquired counterpart to the inherited syndrome of AME.

1.7.2.5 11β-HSD2 in essential hypertension

Although patients with essential hypertension do not exhibit overt signs of mineralocorticoid excess, blood pressure levels have been positively correlated with plasma sodium and negatively correlated with plasma potassium levels, suggesting that adrenal corticosteroids may play a role in the pathogenesis of essential hypertension (Beretta-Piccoli et al., 1983). Furthermore, in analogy to milder forms of AME, a proportion but not all patients with essential hypertension exhibit an increased cortisol half-life (Walker et al., 1993) and an elevated urinary free cortisol / cortisone (UFF/UFE) ratio suggesting that subtle changes in 11β-HSD2 activity may be involved in the pathogenesis of essential hypertension (Mariniello et al., 2005). A contributory role of 11β-HSD2 is further underlined by genetic studies which have reported associations of a polymorphism in the first intron of the HSD11B2 gene with reduced 11β-HSD2 activity and high blood pressure in patients with salt-sensitive

hypertension compared to controls (Agarwal et al., 2000; Carvajal et al., 2005). Additionally, in a recent report 4 polymorphisms in the promoter region of the HSD11B2 gene have been linked to the presence of salt-sensitive essential hypertension and reduced expression levels of the HSD11B2 gene *in vitro* which for the first time provides a mechanistic explanation for impaired 11β-HSD2 activity caused by polymorphisms in patients with salt-sensitive essential hypertension (Alikhani-Koupaei et al., 2007).

1.8 Heart disease

Heart disease is a broad term for a variety of disorders affecting the heart including ischaemic heart disease, hypertensive heart disease, cardiomyopathies and CHF. Heart disease remains the leading cause of death in England and Wales followed by cerebrovascular disease and cancer (News report 2006, UK National statistics) and the United States (National Vital Statistics Report 2007, Centers for disease control and prevention).

1.8.1 Ischaemic heart disease

Ischaemic heart disease is characterised by reduced blood supply to the heart muscle and is caused by atherosclerosis of the coronary arteries and is the most common cause of death in most Western countries, and a major cause of hospital admissions. Known risk factors include age, smoking, hypercholesterolaemia, diabetes mellitus, hypertension, male gender and a positive family history for ischaemic heart disease.

Atherosclerosis initially leads to narrowing of the coronary arteries, compromising blood supply to the heart, particularly during physical activity to cause shortness of breath and chest pain (angina) on exertion. Cession of blood supply caused by

complete blockage of coronary blood flow invariably results in myocardial infarction (MI). Following MI, the heart undergoes reorganisation of its architecture with scar formation of the infarcted area and changes to non-infarcted areas such as compensatory hypertrophy of myocardial tissue and cardiac fibrosis (Figure 1-18). These changes are referred to as 'cardiac remodelling' and may lead to the development of heart failure (1.8.3).

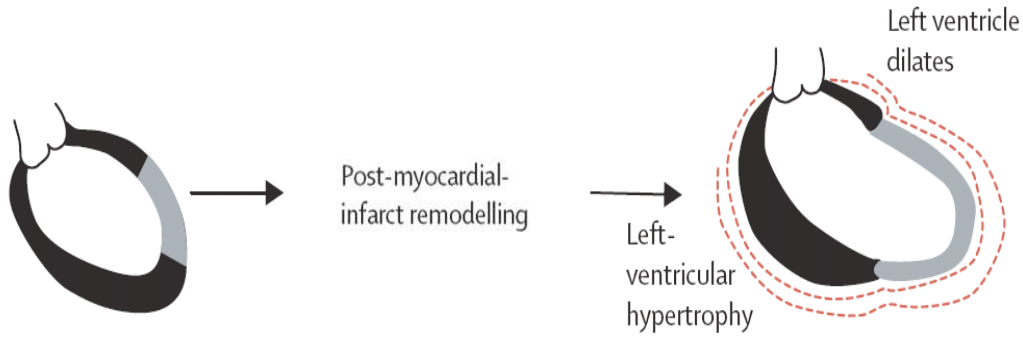

Figure 1-18 Schematic illustration of post infarct remodelling. The grey area depicts infarcted myocardium, the black area represents viable myocardium which undergoes remodelling post myocardial infarction (Opie et al., 2006).

1.8.2 Hypertensive heart disease

Hypertensive heart disease describes the changes inflicted by chronic elevated blood pressure levels. Besides the contributory effects of high blood pressure on the development and progression of ischaemic heart disease, long standing hypertension also directly affects the myocardium both structurally and functionally. High blood pressure causes compensatory left ventricular hypertrophy (LVH) (Figure 1-19) which eventually results in heart failure. In contrast to patients with ischaemic or idiopathic heart failure who usually have an enlarged and dilated left ventricle, patients with hypertensive heart disease initially develop a hypertrophied but normal-

sized left ventricular chamber characterised by myocyte hypertrophy (with increased width relative to length) and ventricular wall thickening without chamber dilatation, a response that may be a compensatory mechanism to normalise wall stress. LVH is accompanied by pathologic features including CM death, fibrosis and scarring. Cardiac fibrosis seems to be more widespread in hypertensive heart disease than in other causes of heart failure which contributes to myocardial stiffness, especially during diastole. Characteristically systolic function in these patients is preserved but myocardial hypertrophy impairs relaxation and filling of the left ventricle resulting in smaller stroke volumes which is referred to as diastolic heart failure (or heart failure with preserved ejection fraction) (Berk et al., 2007).

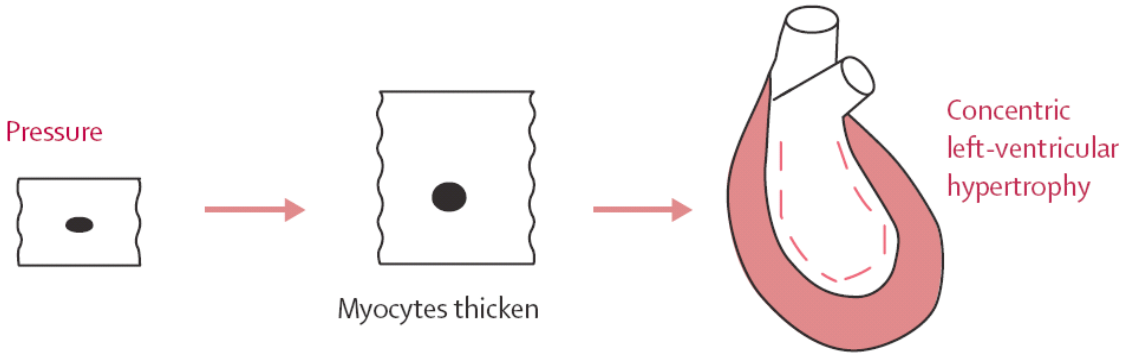

Figure 1-19 Schematic illustration of concentric left ventricular hypertrophy. Chronic pressure overload results in thickening of cardiomyocytes and the left-ventricular wall (Opie et al., 2006).

1.8.3 Heart failure

Besides idiopathic cardiomyopathy and valvular heart disease, ischaemic and hypertensive heart disease are the most frequent underlying conditions leading to CHF (Jessup & Brozena, 2003). CHF may be secondary to either systolic or diastolic dysfunction or a combination of both. To date, no gold standard has been agreed on but different criteria which are predominantly or exclusively based on signs and

symptoms have been proposed (Framingham, Boston or Duke criteria). Clinical signs of CHF include reduced exercise tolerance, shortness of breath, peripheral and/or pulmonary oedema and cyanosis. Severity of CHF has been classified by the New York Heart Association (NYHA) into four classes (I-IV) according to clinical signs and symptoms and more recently by the American Colleague of Cardiology (ACC) / American Heart Association (AHA) into four stages (A-D).

1.8.4 Cardiac remodelling and fibrosis

1.8.4.1 Cardiac remodelling

Cardiac remodelling describes the changes that occur in response to disorders such as MI, pressure or volume overload, inflammatory heart muscle disease and idiopathic dilated cardiomyopathy (Cohn et al., 2000). Histopathologically, the remodelling process is characterised by structural rearrangement of components of the normal chamber wall that involves CM disarray and hypertrophy, cardiac fibroblast proliferation and accelerated and aberrant accumulation of extracellular matrix (ECM), referred to as cardiac fibrosis (Massie & Shah, 1996; Sun & Weber, 2000; Swynghedauw, 1999). These alterations at the cellular level eventually lead to changes in heart shape, size and most importantly function. Although these remodelling processes initially play an important role in wound healing and augment performance, over the longer term they often develop to a maladaptive response and progressively impair cardiac function resulting in heart failure. Maladaptive remodelling has been associated with an impaired prognosis in patients after MI and patients with CHF and therefore represents an important therapeutic target for medical therapy (Cohn et al., 2000). Neurohormonal activation including that of the RAAS and the sympathetic nervous system is an important driver of cardiac

remodelling. Consequently drugs interfering with these pathways such as ACEi, angiotensin receptor blockers (ARBs) and MR antagonists ameliorate the remodelling process and thereby slow down disease progression, and improve symptoms, co-morbidities and mortality (1997; Garg & Yusuf, 1995; Packer et al., 1996; Pitt et al., 1999).

1.8.4.2 Cardiac fibrosis

Cardiac fibrosis is an integral feature of cardiac remodelling and is characterised by disproportionate accumulation of ECM. The ECM forms a three dimensional scaffold around individual and bundles of CM to generate a stress-tolerant network. Collagen type I and III are the major stress-bearing elements within the ECM in the heart accounting for approximately 80% and 10%, respectively, of total cardiac collagen content (Jugdutt, 2003). Cardiac fibrosis results from a disproportionate increase in the synthesis and/or inhibition of degradation of ECM proteins and has important functional consequences for the heart. Firstly, collagen accumulation leads to ventricular stiffening, which initially impairs ventricular relaxation resulting in predominantly diastolic dysfunction but if severe can compromise cardiac contraction and result in systolic dysfunction. Secondly, cardiac fibrosis disrupts electrophysiological connections between cardiac myocytes and provides an electrical substrate for arrhythmogenesis. Thirdly, capillary density is reduced by fibrosis and thereby oxygen diffusion distance increases leading to CM hypoxia (Sabbah et al., 1995). Cardiac fibrosis is not uniform and displays characteristic differences depending on the underlying aetiology. While reparative (replacement) fibrosis takes place at sites of myocyte death to replace areas of dead myocytes by a structural scar, reactive fibrosis is characterised by interstitial (collagen appears in the intermuscular space) and/or perivascular (collagen accumulates around coronary

arteries and arterioles) collagen deposition and is not directly associated with CM death (Weber et al., 1989).

CFb are the predominant secretory cells of ECM proteins in the heart and are thus key in normal and pathological cardiac remodeling. Following cardiac injury, fibroblasts undergo a phenotypic transformation into myofibroblasts (MyoFb) which are characterised by their ability to contract and actively secret ECM proteins and thereby facilitate wound closure (Sun & Weber, 2000). MyoFb are characterised by the expression of the pro-contractile protein α smooth muscle actin (α-SMA) and are exclusively found at sites of remodelling but not in the normal heart (with the exception of valve leaflets). Following activation, MyoFb become particularly responsive to pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), transforming growth factor-beta (TGF-β) and vasoactive peptides (including Ang II, endothelin-1) (Porter & Turner, 2009). These stimuli in turn lead to alterations in the proliferative and migratory properties of MyoFb, modulate ECM turnover and regulate the secretion of cytokines and growth factors including TNFα, Il-6 and Il-1β (Porter & Turner, 2009). While apoptosis of the MyoFb has been shown to be associated with the advancement of granulomatous tissue to become a mature scar, persistence of MyoFb has been suggested to be an important driver of maladaptive cardiac fibrosis.

Cardiac ECM homeostasis is a continuous process and is determined by the balance between formation and degradation (Brown et al., 2005; Camelliti et al., 2005). While the aforementioned formation of ECM is directly related to the transcriptional activity of genes encoding for collagens, degradation of collagens requires the expression and activity of matrix metalloproteinases (MMPs). MMPs constitute a proteolytic enzyme family with more than 20 members capable of digesting ECM proteins (Visse & Nagase, 2003). MMP activity is regulated through transcriptional regulation,

activation of latent pro-enzymes and by tissue inhibitors of metalloproteinases (TIMPs), which are naturally-occurring inhibitors of MMP activity (Nagase & Woessner, 1999). MMPs are not exclusively expressed by fibroblasts, but also by CM, endothelial and inflammatory cells and in addition to ECM degradation are also involved in regulating of cell growth and migration, cell survival/death and angiogenesis.

1.9 Corticosteroids and the heart

1.9.1 Clinical studies

1.9.1.1 Corticosteroids and cardiovascular risk

1.9.1.1.1 Aldosterone and cardiovascular risk

Besides its classical physiological role and function in regulating salt and fluid homeostasis, Aldo has emerged as a cardiovascular risk factor in patients with underlying heart disease. The first evidence came from early ACE inhibitor trials such as the cooperative north scandinavian enalapril survival (CONSENSUS) study, which showed a positive relationship between plasma Aldo levels and mortality in patients with CHF (Swedberg et al., 1990), whereas the survival and ventricular enlargement (SAVE) study showed that Aldo levels in patients post MI predict development of heart failure (Vantrimpont et al., 1998). More recent studies confirmed these initial findings and showed that Aldo levels in patients with MI predicted mortality (Beygui et al., 2006; Palmer et al., 2008). Similar findings have been observed in patients with CHF in which high Aldo levels were associated with increased mortality (Guder et al., 2007).

More recent evidence for a direct, blood pressure independent effect of Aldo on the CV system comes from studies in patients with primary hyperaldosteronism (described in section 1.6.3). Echocardiographic studies in these patients have demonstrated that in comparison to patients with essential hypertension matched for blood pressure, cardiac hypertrophy is augmented (Matsumura et al., 2006; Muiesan et al., 2008; Rossi et al., 1996) and cardiac texture suggests increased collagen deposition (Rossi et al., 2002). In addition, primary hyperaldosteronism patients also have a significantly increased risk for MI, stroke and atrial fibrillation in comparison to blood pressure matched patients with essential hypertension (odds ratio: 4.2, 6.5 and 12.1, respectively) (Milliez et al., 2005), again, suggesting a direct detrimental effect of Aldo on the CVS. Similar, blood pressure independent effects of Aldo on the heart have also been described in patients with essential hypertension in whom Aldo levels were positively correlated with left ventricular hypertrophy even after adjusting for mean 24 blood pressure (Duprez et al., 1993). Finally, in normotensive subjects higher Aldo levels predispose to the development of hypertension in the future (Vasan et al., 2004).

1.9.1.1.2 Glucocorticoids and cardiovascular risk

Long term treatment of patients with pharmacological doses of glucocorticoids or chronic endogenous cortisol excess (Cushing's syndrome) causes central obesity, hypertension, insulin resistance and dyslipidaemia, all of which comprise risk factors for cardiovascular disease. As a consequence, mortality due to cardiovascular complications (including early atherosclerosis, coronary artery disease, heart failure and stroke) is 4 times higher in patients with Cushing's syndrome than in the normal population (matched for sex and age) (Etxabe & Vazquez, 1994) resulting in a 5 year all cause mortality of 50% (Plotz et al., 1952). Furthermore, pharmacological

glucocorticoid treatment has been shown to be associated with an increased risk of heart failure (Souverein et al., 2004) and cardiovascular disease (Wei et al., 2004). Cortisol excess in Cushing's syndrome causes hypertension in more than 90% of patients through a number of different mechanisms. In the kidney, excessive cortisol levels swamp the enzyme 11β-HSD2 which in turn is no longer able to protect the MR from illicit cortisol binding. This results in a glucocorticoid induced form of mineralocorticoid hypertension similar to apparent mineralocorticoid excess (AME) (described in section 1.7.2.3). In the vasculature, cortisol increases peripheral resistance by increasing vascular reactivity to vasconstricting stimuli including Ang II, and catecholamines, and by inhibiting mediators of vasodilation including nitric oxide and prostacyclin. In the liver, cortisol excess increases synthesis of angiotensinogen which results in increased levels of Ang II (Arnaldi et al., 2004). Besides its effects on blood pressure, cortisol excess induces adverse metabolic changes including central obesity, insulin resistance and dyslipidaemia which are all well established cardiovascular risk factors associated with macrovascular morbidity and mortality. Clinical studies in patients with Cushing's syndrome suggest a high prevalence of cardiac hypertrophy and impaired cardiac function suggesting direct detrimental effects of glucocorticoids on the heart (Baykan et al., 2008; Muiesan et al., 2003). Interestingly, high normal cortisol levels have recently also been associated with adverse outcome in patients with CHF but otherwise normal HPA function suggesting that cortisol levels even when within the normal reference range may constitute a novel risk factor in CHF (Guder et al., 2007).

1.9.1.2 Mineralocorticoid receptor antagonists

The significance of these observational studies has subsequently been underpinned by randomised double blind placebo controlled intervention studies in which low

doses of MR receptor antagonists were added to standard treatment regimes in heart failure patients. In the randomised aldactone evaluation study (RALES) low doses of Spiro showed a substantial (30%) improvement in cardiovascular morbidity and mortality in patients with CHF compared with those given placebo (Pitt et al., 1999). Similar results were found in the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS). In this study, patients with acute left ventricular failure post MI treated with the selective aldosterone antagonist eplerenone (EPL) showed a 15% reduction in mortality in comparison to placebo (Pitt et al., 2003b). Animal models of extensive MI resulting in acute LV dysfunction underpin these results as they show that double RAAS blockade with EPL and an ACEi or ARB is more effective than EPL alone in improving remodelling (Fraccarollo et al., 2003; Fraccarollo et al., 2005).

In the 4E (Eplerenone, Enalapril and Eplerenone/Enalapril) study, patients with essential hypertension and left ventricular hypertrophy were assigned to treatment with enalapril, EPL or a combination of both. Both drugs by themselves proved to be equally effective in reducing blood pressure and LVH, whereas the combination of both drugs was more effective than EPL alone (Pitt et al., 2003a). Another study assessed cardiac function in hypertensive patients with suspected diastolic dysfunction and found that addition Spiro treatment resulted in echocardiographic improvement in cardiac function in comparison to placebo irrespective of blood pressure (Mottram et al., 2004). Surprisingly however, addition of EPL in asymptomatic patients with moderate to severe aortic stenosis, did not slow down the onset of LV dysfunction, decrease LV mass or reduce progression of valve stenosis compared to placebo (Stewart et al., 2008). The lack of clinical benefit of EPL in this clinical trial contrasts results from a mouse model of aortic stenosis inflicted by aortic

banding in which the administration of EPL slowed down disease progression and improved survival (Kuster et al., 2005).

Treatment with Spiro in patients with CHF has been shown to reduce natriuretic peptides, collagen markers and improve vascular function (endothelial function, vascular ACE activity) which reflect the favourable effects of Spiro in CHF patients (Macdonald et al., 2004; Rousseau et al., 2002). However, the underlying molecular mechanisms accounting for the improvements in remodelling and beneficial outcomes remain largely unknown.

1.9.1.3 Aldosterone escape

Aldo escape or Aldo breakthrough describes a phenomenon in which despite an acute fall in Aldo levels in response to administration of an ACEi, Aldo levels subsequently start to rise, and in some patients return back to baseline levels. Insufficiently suppressed Aldo levels following ACEi treatment have been described in different patient cohorts including hypertensive patients with LVH (Sato & Saruta, 2001), post MI (Borghi et al., 1993) and CHF patients (MacFadyen et al., 1999; Swedberg et al., 1990).

The mechanism by which Aldo levels during ACEi treatment after an initial fall rise again despite ongoing treatment is unclear. Although Ang II levels during ACEi therapy exhibit a similar course (MacFadyen et al., 1999; Roig et al., 2000), however dissociation of Ang II and Aldo has been described (Lee et al., 1999) suggesting an Ang II independent mechanism. This is consistent with findings from the Randomised Evaluation of Strategies for Left Ventricular Dysfunction (RESOLVD) pilot study in which mean Aldo levels after 43 weeks of treatment with maximum doses of enalapril and candesartan had returned back to baseline (McKelvie et al., 1999).

Given the strong association of Aldo levels with poor outcome in patients with heart disease, the phenomenon of Aldo escape may in part explain the beneficial effects observed in the RALES and EPHESUS study, where addition of an MR antagonist on top of standard ACEi or ARB treatment regime dramatically improved morbidity and mortality compared to placebo.

1.9.2 Animal studies

Animal studies have been instrumental in elucidating the functional, cellular and molecular consequences of corticosteroid hormone action, particularly that of Aldo, on the CVS. Insights have been gained from several animal paradigms as well as genetically engineered mice.

1.9.2.1 Experimental studies of corticosteroid hormone action

One of the first reports on the detrimental effects of Aldo on the CVS came from a study by Brilla and Weber (Brilla et al., 1990). In this study an animal paradigm of aortic banding, which induces high blood pressure proximal to the site of banding, was explored. The key observation from this study was that only suprarenal aortic banding lead to activation of the RAAS secondary to renal hypoperfusion and resulted in the development of severe cardiac fibrosis whereas infrarenal banding in which renal perfusion was not compromised and RAAS not activated, did not. Similar changes including high blood pressure levels, cardiac hypertrophy and fibrosis were also observed in uninephrectomised animals treated with Aldo and given high salt solutions to drink. In these animals Aldo levels are high but Ang II levels are suppressed, suggesting that the detrimental fibrotic changes are a direct effect of Aldo and not Ang II (Brilla & Weber, 1992). Importantly, the detrimental cardiovascular effects elicited in this paradigm critically relied on concomitant high

salt intake in addition to treatment with Aldo, as Aldo excess in the presence of a low salt diet is insufficient for the development of hypertension, cardiac hypertrophy and fibrosis. Moreover, it was noted that cardiac fibrosis in this animal model developed similarly in both the right and left ventricle, strongly suggesting that fibrosis was a humoral rather than a haemodynamic consequence of Aldo. Several subsequent studies have now established that the detrimental cardiac effects of Aldo or that of its precursor 11-deoxycorticosterone acetate (DOC) which is also a mineralocorticoid but less potent than Aldo, are blood pressure independent. Administration of low doses of MR antagonists have been shown to prevent or ameliorate cardiac fibrosis without affecting blood pressure (Brilla et al., 1993). Furthermore, infusion of Aldo peripherally or centrally (at 1/100 the dose) results in similar increases in blood pressure but hypertrophy only develops in animals infused peripherally, suggesting a direct blood pressure independent effect (Gomez Sanchez, 1995). Moreover, concomitant intracerebroventricular application of the MR antagonist RU28318 in animals infused with Aldo blunts increases in blood pressure yet does not affect cardiac hypertrophy (Young et al., 1995). Lastly, in Ang II infused rats adrenalectomy (Rocha et al., 2000) as well as treatment with low doses of EPL (Rocha et al., 2002a) prevents cardiac fibrosis without affecting blood pressure.

In sharp contrast to the MR which plays a central role in mediating mineralocorticoid effects, the GR appears to be inert in the pathophysiology of cardiac inflammation and fibrosis in these models as administration of the GR antagonist RU486 does not appear to impact on fibrosis (Rickard et al., 2006; Rickard et al., 2007).

Based on the potent effects of MR antagonist in preventing cardiac inflammation and fibrosis in mineralocorticoid/salt excess, MR antagonists have been studied in other animal paradigms of heart disease which in general are characterised by neurohormonal activation including elevated Aldo levels. In an animal model of aortic

stenosis, administration of EPL has been shown to reduce myocardial fibrosis, myocyte apoptosis, oxidative stress, macrophage infiltration resulting in improved cardiac function and survival (Kuster et al., 2005). Furthermore, in an animal model of MI, administration of EPL 10 days post MI has been shown to improve LV remodelling (Fraccarollo et al., 2005). Addition of an ACEi substantially potentiated this effect by complementary prevention of LV fibrosis and cardiac hypertrophy. The optimal time point to start treatment with MR antagonists after MI is not clear, but a recent report showed that selective MR blockade immediately after MI accelerated macrophage infiltration and transiently increased the expression of healing promoting cytokines and factor XIIIa resulting in enhanced neovascularisation and reduced early LV dilation and dysfunction (Fraccarollo et al., 2008).

MR antagonists have recently been shown to elicit similar beneficial effects even when Aldo levels are low. Dahl salt sensitive rats when fed a high salt diet develop hypertension and cardiac hypertrophy but consistent with this salt excess state the RAAS is suppressed. Nevertheless, Nagata and colleagues have shown that administration of sub-pressor doses of the MR antagonist EPL reduced oxidative stress and vascular inflammation resulting in attenuated hypertrophy and heart failure independent of blood pressure (Nagata et al., 2006). Beneficial cardiac effects of MR antagonists have also been described in a rat model of chronic renal failure, in which administration of sub-pressor doses of EPL attenuated cardiac hypertrophy and cardiac oxidative despite absence of increased Aldo levels compared to control animals (Michea et al., 2008).

Collectively, these animal studies show that a) inappropriate Aldo for salt status leads to cardiac inflammation and fibrosis independent of blood pressure, b) administration of MR antagonists attenuates cardiac sequelae c) cardiac hypertrophy at least

partially results from direct Aldo actions independent of blood pressure, and lastly d) MR antagonists are beneficial even when Aldo levels are not elevated.

1.9.2.2 Genetically engineered mice

Further important insights into the pathophysiological role of corticosteroid hormones have been gained from studies with genetically engineered mice. Given the pivotal role of nuclear steroid hormone receptors in mediating steroid hormone action, global MR and GR knock out mice have been created. However, GR null mice die a few hours after delivery from respiratory failure due to a lack of lung maturation (Cole et al., 1995), whereas global MR null mice die around post natal day 10 from severe salt wasting and dehydration (Berger et al., 1998). To date, no results for cardiac specific MR or GR knock out mice have been published.

Nevertheless, the consequences of reduced levels of both receptors have been studied in knock down models in which antisense RNA to the respective mRNA of the GR or MR is overexpressed in CM leading to formation of double stranded RNA which no longer can be translated into protein and subsequently is degraded. The results from these studies have been puzzling. MR knock down mice show a 50% reduction in cardiac MR mRNA levels and as a consequence surprisingly develop severe cardiomegaly, heart failure and cardiac fibrosis at 3 months (Beggah et al., 2002). In this model, reduced expression levels of the MR could be reverted to normal levels by turning off antisense RNA expression, which resulted in complete reversal of the phenotype.

Using a similar model, the same group also studied consequences of CM specific overexpression of the MR (Ouvrard-Pascaud et al., 2005). These mice show increased mortality as a result of severe ventricular arrhythmias which could be prevented by the administration of Spiro. However, no structural abnormalities of the

heart were reported in this model. The consequences of CM specific GR overexpression have also been addressed (Sainte-Marie et al., 2007). These mice exhibit electrocardiogram (ECG) abnormalities including a prolonged PQ interval, increased QRS and QTc duration as well as chronic atrio-ventricular block, in the absence of any structural cardiac abnormalities such as hypertrophy or fibrosis.

In addition to these studies focusing on the consequences of altered steroid hormone receptor levels, the effects of changes in cardiac steroid hormone levels have been addressed by creating transgenic which overexpress enzymes involved in steroid synthesis and metabolism in CM. Given that 11β-HSD2 is not expressed, both glucocorticoids and mineralocorticoids must be considered as physiological MR ligands in the heart. As the concentration of glucocorticoids in the circulation exceeds those of Aldo by at least by 100 fold, glucocorticoid binding may protect the MR from Aldo through tonic inhibition of the receptor. This theory is supported by an animal model in which overexpression of 11β-HSD2 in CM resulted in inactivation of corticosterone to its inert compound 11-dehydrocorticosterone. These mice develop severe cardiac hypertrophy, CHF and cardiac fibrosis and die prematurely by the age of 4-6 months (Qin et al., 2003). Consistent with an MR mediated effect, administration of the MR antagonist EPL in this model ameliorated the phenotype. Garnier *et al* assessed the consequences of overexpression of aldosterone synthase, the terminal enzyme involved in Aldo synthesis (1.3.2.2) (Garnier et al., 2004). These mice merely exhibit coronary dysfunction but otherwise have no alterations in cardiac structure or function despite 1.7 fold elevated cardiac Aldo levels compared to WT mice.

The downstream effects of steroid hormone action in the heart are not well understood. However, animal models with deletions of genes involved in steroid hormone signalling and action have shed light on some of the underlying

mechanisms. SgK1 is an important target of Aldo action in epithelial cells of the distal nephron in the kidney, and elevated SgK1 levels have been reported in a range of inflammatory/fibrotic diseases (Lang et al., 2000; Waerntges et al., 2002). Compared to WT mice, DOCA/salt treated SgK1 knock out mice are protected from cardiac fibrosis despite similar blood pressure levels (Vallon et al., 2006), suggesting a pivotal role for SgK1 in mediating the fibrotic response in this model. One of the molecular effects of Aldo action *in vitro* is induction of reactive oxygen species (ROS) by activation of the NADPH dependent oxidase (NOX) (Rude et al., 2005). These findings are supported by mice harboring a deletion of the NOX2 gene which encodes for a subunit of the multimeric NADPH dependent oxidase complex. Compared to WT mice, NOX2 knock out mice treated with Aldo in the presence of high salt in the drinking water are protected from cardiac fibrosis (Johar et al., 2006), consistent with a pivotal role of ROS in mediating cardiac fibrosis.

Although some of these animal models show unexpected and unexplained phenotypes which might be partly caused by technical issues related to the transgene expression, they do provide important insights into the pathophysiological function of corticosteroid hormone action in the heart.

1.9.3 Mechanisms of corticosteroid hormone action in the heart

The heart consists of four main cell types: cFb, CM, endothelial cells (ECs) and, vascular smooth muscle cells (VSMCs) (Figure 1-20). Corticosteroid hormone action in the heart is determined by several factors. First of all, action of steroid hormones critically relies on the presence of steroid hormone receptors and thus, only cells which express these receptors can be considered as steroid target cells. The magnitude of steroid effects is directly related to their concentration. With regard to steroid hormones that are exclusively produced in endocrine glands, circulating levels

are decisive for the effects in peripheral tissues. However, steroids may also be synthesised locally in the heart, either *de novo* from cholesterol or from other pre-cursor steroids such as DOC. Steroid hormones may also be modulated by steroid modifying enzymes within target cells. Through this mechanism, also referred to as pre-receptor modulation, steroids may be activated or inactivated within target cells prior to exhibiting their effects via their cognate receptors.

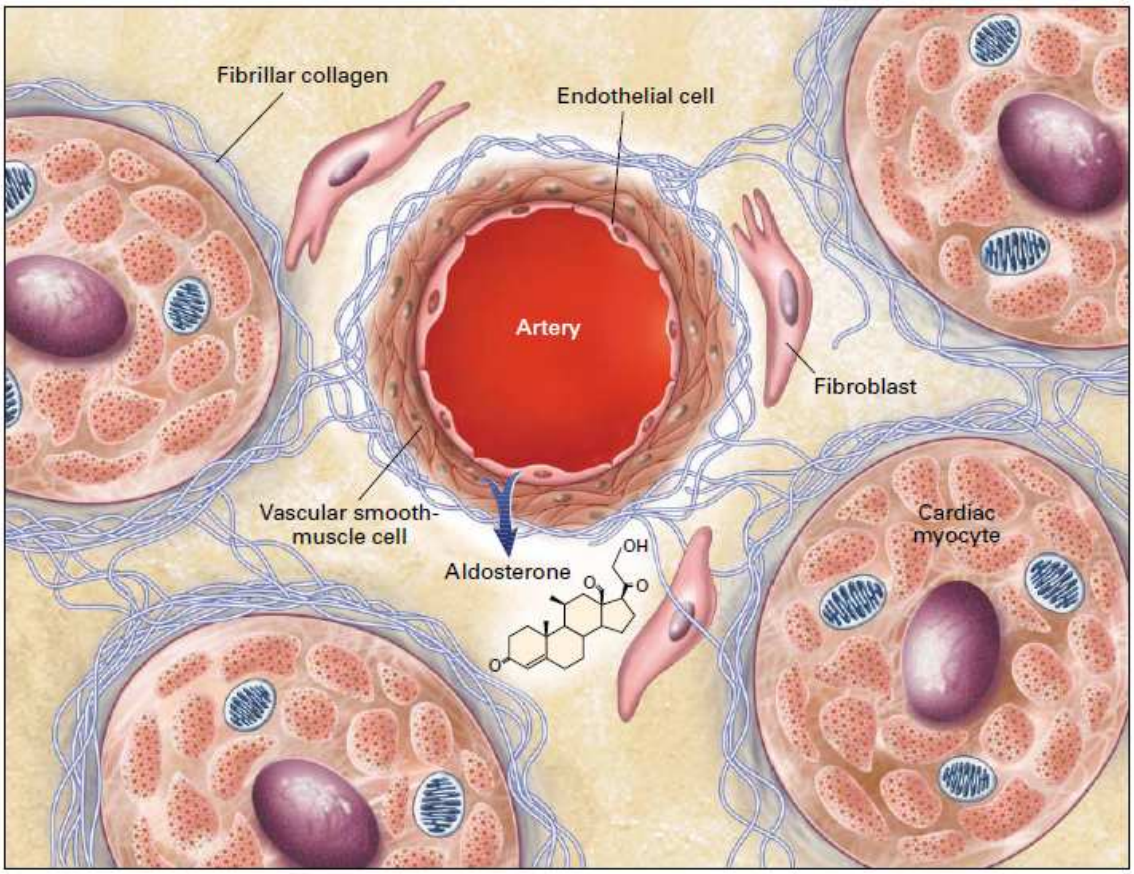

Figure 1-20 Schematic illustration of cellular architecture in the heart (Weber, 2001).

1.9.3.1 Steroid hormone receptor expression

The GR is ubiquitously expressed, and thus, virtually all cells are considered as potential glucocorticoid target cells. Presence of the GR in the heart has been

detected by various techniques such as binding studies (Turner & Moses, 1986), northern blot analysis (Kalinyak et al., 1987) and by Western blotting (Pujols et al., 2001). The predominant isoform expressed in the human heart is GRα whereas GRβ expression levels are much lower (Pujols et al., 2001). In contrast to GRα, GRβ is unable to bind glucocorticoids. It functions as a dominant negative isoform of GRα-induced transactivation of GRE-containing, glucocorticoid-responsive promoters and therefore is a natural inhibitor of glucocorticoid actions.

Expression levels of the GR are regulated by circulating glucocorticoids by negative feedback inhibition as adrenalectomy results in up-regulation, and conversely, treatment with pharmacological doses of dexamethasone (DEX) causes down-regulation of the GR in the heart as well as in other tissues (Kalinyak et al., 1987; Turner & Moses, 1986).

In contrast to the GR, expression of the MR is much more restricted. More than 20 years ago high affinity binding sites for Aldo (so called type I receptors, which are now referred to as MR) were described in the heart. (Barnett & Pritchett, 1988; Pearce & Funder, 1987). Moreover, expression of the MR in the heart has been confirmed in several species by various techniques including Northern blot analysis (Arriza et al., 1987), *in situ* hybridisation (Brown et al., 1996b; Lombes et al., 1995) and immunohistochemistry (Lombes et al., 1995; Lombes et al., 1992). From these studies, expression of the MR appears to be restricted to CM and ECs whereas expression in cFb is undetectable. These findings are underlined by RNAse protection assays in isolated cells from neonatal rat hearts, which show expression of the MR only in CM but not in cFb (Sheppard & Autelitano, 2002).

1.9.3.2 Cardiac steroidogenesis

Glucocorticoids and mineralocorticoids are synthesised in the cortex of the adrenal gland (1.3.2). However, given their effects in modulating cardiovascular disease, a number of studies have addressed whether the heart is capable of synthesising corticosteroids which may then directly impact on the development and progression of cardiac disease. A pre-requisite for *de novo* synthesis of corticosteroids is the presence of steroidogenic enzymes such as P450scc, 3β-HSD, P450c21, P450c11 and P450c11AS whereas Aldo synthesis from precursor steroids such as DOC from the circulation only requires the presence of aldosterone synthase.

Slivestre and colleagues assessed steroidogenic enzymes in isolated rat hearts and were able to detect both expression and enzymatic activity of P450c11 and P450c11AS. Moreover, enzymatic activities in the heart were further induced by ACTH and Ang II treatment (Silvestre et al., 1998). In addition, following MI, expression of aldosterone synthase was increased in rat hearts whereas expression in the adrenal glands remained unchanged, suggesting that locally synthesised Aldo might play a modulatory role post MI (Silvestre et al., 1999). These results are underlined by a study from Takeda and colleagues who report increased P450c11AS expression and activity in spontaneously hypertensive rats (SHRSP) compared to Wistar-Kyoto rats, which suggests a role for locally produced Aldo in cardiac hypertrophy (Takeda et al., 2000). However, expression levels were found to be 1000 fold lower compared to the adrenal gland raising the question of the biological significance of these findings. A subsequent study has questioned these findings as cardiac levels of Aldo following adrenalectomy (ADX) were either undetectable or present only at extremely low concentrations suggesting that cardiac Aldo levels only reflect those in the circulation (Gomez-Sanchez et al., 2004). Recently, cardiac

glucocorticoid production was assessed in two animal models of cardiac hypertrophy. In both Dahl salt sensitive (DS) rats on a high salt diet and mice subjected to transthoracic aortic banding (TAC) there was increased tissue concentrations of corticosterone compared to control animals (Ohtani et al., 2009). Moreover, expression levels of P450scc, P450c11 and StAR but not those of P450c11AS were found to be significantly up-regulated compared to control animals, suggesting that cardiac glucocorticoid synthesis may contribute to the development of cardiac hypertrophy.

Expression of steroidogenic enzymes has also been studied in human heart tissue. In both healthy and failing hearts, P450scc, 3β-HSD and P450c21 mRNA have been found to be expressed by reverse transcriptase polymerase chain reaction (RT-PCR), albeit at very low levels, whereas P450c11 and P450c11AS could not be detected suggesting that human hearts are incapable of *de novo* glucocorticoid and mineralocorticoid synthesis (Kayes-Wandover & White, 2000; Young et al., 2001). However by contrast, Satoh and colleagues found P450c11AS to be expressed in biopsies of patients with CHF (Satoh et al., 2002). Expression levels were higher in patients with more severe heart failure defined by an ejection fraction of less than 30% and were positively correlated with collagen content. In agreement with these findings, P450c11AS expression levels were increased in hearts of deceased CHF patients compared to patients without cardiovascular disease (Yoshimura et al., 2002). Cardiac steroidogenic activity has also been assessed *in vivo* by subtracting steroid hormone concentrations in arterial blood from that of the coronary sinus, which drains blood from the heart. The difference between these two concentrations is thought to reflect locally produced steroids. Using this method, Aldo concentrations were found to be increased in patients with systolic and diastolic left ventricular dysfunction (Mizuno et al., 2001) as well as in hypertensive patients (Yamamoto et

al., 2002) compared to healthy controls suggesting that Aldo is synthesised in the heart in patients with heart failure or left ventricular hypertrophy, respectively.

In conclusion, these studies suggest that the heart expresses some but not all steroidogenic enzymes necessary for *de novo* steroid synthesis, albeit at very low levels. In patients with heart failure and hypertension Aldo appears to be produced by the heart but it is likely that its synthesis relies on adrenal precursor steroids such as DOC. However, the biological significance of these findings in the development and progression of heart disease is likely to be minor.

1.9.3.3 11β-HSD1 and the cardiovascular system

11β-HSD1 facilitates GR mediated action by converting cortisone (11-dehydro-corticosterone in rodents) to cortisol (corticosterone in rodents). A few studies have addressed expression levels, cellular localisation and activity of 11β-HSD type1 in the heart. Compared to liver, where 11β-HSD1 is highly expressed, both enzymatic activity and expression levels of 11β-HSD1 were much lower in the heart (Brereton et al., 2001; Walker et al., 1991). Moreover, expression of 11β-HSD1 within the heart appeard to be restricted to interstitial fibroblasts of the endocardium and isolated cells surrounding cardiac vessels and could not be detected in CM (Brereton et al., 2001). Consistent with this restricted pattern of expression, enzymatic activity observed in cardiac homogenates were found to be low (Walker et al., 1991). However by contrast, high levels of 11β-HSD1 oxoreductase activity and expression have been reported in isolated CM as well as fibroblasts from neonatal rat hearts (Sheppard & Autelitano, 2002).

Regulation of 11β-HSD1 has been studied in hearts from Dahl salt sensitive (DS) and Dahl salt resistant (DR) rats which were either fed a low or high salt diet. Both activity and expression levels of 11β-HSD1 were higher in DR compared to DS rats.

However, no differences in 11β-HSD1 expression and activity were observed in DS on a high compared to low salt diet despite markedly increased blood pressure levels and cardiac hypertrophy (Mazancova et al., 2005).

To date, the biological role of 11β-HSD1 in the heart remains unclear. Mice with a deletion of the 11β-HSD1 gene (11β-HSD1$^{-/-}$) do not seem to exhibit an obvious cardiac phenotype at rest (Kotelevtsev et al., 1997). However, in a mouse model of MI 11β-HSD1$^{-/-}$ compared to WT mice exhibited increased cardiac angiogenesis and improved cardiac function suggesting that 11β-HSD1 by modulating local glucocorticoid levels plays a role in post MI remodelling (McSweeney et al.; Small et al., 2005).

1.9.3.4 11β-HSD2 and the cardiovascular system

In the kidney, 11β-HSD2 plays a pivotal role in protecting the MR from glucocorticoid binding and thereby ensuring Aldo specificity. Hence, there has been great interest in the presence and function of 11β-HSD2 in the heart as Aldo has emerged as a CV risk factor and pharmacological blockade of the MR in heart failure patients has been shown to greatly reduce mortality.

At the time 11β-HSD2 was cloned, no expression was reported in human heart tissue (Albiston et al., 1994; Funder et al., 1988). However, in subsequent studies an NAD^+ dependent enzyme with a high affinity for corticosterone was detected in heart tissue from human transplant donors, suggesting presence of 11β-HSD2 which was confirmed by RT-PCR analysis (Slight et al., 1996). In contrast, in rat hearts, NAD^+ dependent enzymatic activity was absent (Walker et al., 1992) and, using *in situ* hybridisation, expression could not be detected (Roland & Funder, 1996). During early embryological development, high expression levels of 11β-HSD2 have been detected in the murine heart by *in situ* hybridisation (Brown et al., 1996b). However,

beyond embryonic day 13, 11β-HSD2 expression ceases and is not detectable in adult life. The phenotype of mice with deletion of the 11β-HSD2 gene (11β-HSD2$^{-/-}$) resembles that of humans with inactivating mutations in HSD11B2 (Kotelevtsev et al., 1999). 11β-HSD2$^{-/-}$ mice develop high blood pressure and exhibit an increased heart / body weight ratio which develops as a consequence of hypertension but otherwise no cardiac abnormalities have been reported.

In conclusion, expression of 11β-HSD2 in the heart remains controversial. If detectable, levels of 11β-HSD2 appear to be extremely low, and thus, its biological relevance in the heart is more than questionable. Therefore, unlike classical Aldo target cells, the MR in the heart is considered not to be protected by 11β-HSD2 and therefore is a target for both glucocorticoids and mineralocorticoids.

1.9.3.5 Molecular mechanisms of steroid hormone action in the heart

1.9.3.5.1 Corticosteroid effects on cardiac fibroblasts

Given that Aldo excess results in severe interstitial and perivascular fibrosis while MR blockade protects the heart from these fibrotic changes, cFb were believed to be the primary target of Aldo action. Initial studies showed that Aldo and Ang II were indeed capable of inducing collagen production in isolated rat cFb *in vitro,* determined by ^{3}H-proline incorporation (Brilla et al., 1994). These effects could be blocked by co-incubation with the MR antagonist Spiro and the AT2-receptor antagonist PD12377, respectively. A subsequent report by the same group confirmed these results and, in addition, showed that Aldo and Ang II were capable of inducing expression of type I collagen mRNA expression (Zhou et al., 1996). However, in contrast, a different group employing the same technique was unable to detect a direct effect of Aldo on collagen production in isolated neonatal and adult cFb from various rat strains

(Sprague-Dawley, WKY, SHR), suggesting that Aldo does not directly affect collagen production in cFb (Fullerton & Funder, 1994).

Stockand and Meszaros found that near-physiological concentrations of Aldo (10 nmol/l) promote proliferation of cFb via activation of the k-Ras and MAPK 1/2 signalling pathways. These effects could be blocked by Spiro but not the GR antagonist RU486, confirming that Aldo was acting through the MR (Stockand & Meszaros, 2003). Although these studies suggest that Aldo has direct effects on cFb, the significance of these findings *in vivo*, particularly with regard to the direct induction of collagen synthesis by Aldo, remains unclear.

1.9.3.5.2 Direct effects of aldosterone on cardiomyocytes

In order to better understand mechanisms of Aldo action in the heart, a number of studies have addressed direct effects of Aldo on cell viability, hypertrophy and gene expression in isolated CM *in vitro*.

Administration of Aldo to rats resulted in increased apoptosis of cardiomyocoytes and skeletal myocytes after 48h *in vivo* (Burniston et al., 2005). Both CM and skeletal myocyte apoptosis in this model could largely be prevented by prior application of the MR antagonist Spiro, confirming that apoptosis following Aldo administration was an MR mediated effect. Subsequent *in vitro* studies in isolated neonatal rat cardiomyocytes (nrCM) by Mano and colleagues confirmed these findings. Treatment with high doses of Aldo (1-10 µM) resulted in a rapid calcium influx in nrCM via an Aldo membrane receptor which accelerates the mitochondrial apoptotic pathway through calcineurin activation and dephosphorylation of the pro-apoptotic factor Bad (Mano et al., 2004).

The increased mortality in patients with CHF is in part due to an increased incidence of ventricular arrhythmias and treatment of CHF patients with Spiro drastically

reduced arrhythmic events suggesting that MR signalling contributes to arrythmogenicity (Pitt et al., 1999). A number of electrophysiological studies have investigated Aldo effects on myocyte ionic homeostasis. Aldo has been shown to increase L-type Ca^{2+} current and decrease transient outward K^+ current (Ito) in rat CM (Benitah et al., 2001; Benitah & Vassort, 1999). Moreover, Aldo increases the fast Na^+ current in adult mouse ventricular myocytes (Boixel et al., 2006) and has been shown to induce expression of the Na^+/K^+-ATPase in nrCM (Ikeda et al., 1991). Lalevee and colleagues reported that Aldo increases the beating frequency of nrCM by enhanced T-type and L-type Ca^{2+} currents following stimulation of gene expression (Lalevee et al., 2005). Increased spontaneous beating in response to Aldo was also reported in nrCM which was found to be mediated by increased I_f currents resulting from increased expression of the I_f channels HCN2 and HCN4 (Muto et al., 2007). These electrophysiological studies demonstrate that Aldo exhibits complex electrophysiological effects on Na^+, K^+ and Ca^{2+} currents in CM which are likely to be involved in the increased risk of ventricular arrhythmias in patients with CHF and cardiac hypertrophy.

Clinical studies also suggest that Aldo is directly involved in cardiac hypertrophy independent of blood pressure levels (Rossi et al., 1996). *In vitro* studies have underpinned these findings, as Aldo treatment augmented protein incorporation and increased myocyte surface in nrCM which are surrogate markers of CM hypertrophy (Okoshi et al., 2004). Aldo treatment has also been shown to lead to cell enlargement of murine atrial HL-1 cells via induction of corticotrophin-1 (CT-1) as this effect could be blocked by co-administration of antibodies against CT-1 or its receptors (Lopez-Andres et al., 2008). CT-1 induction in this study could be blunted by both MR and GR antagonists suggesting that both receptors are involved in CT-1 up-regulation by Aldo. *In vivo* studies underpin these findings as only WT but not CT-1 knock out mice

exhibited increased expression of cardiac markers of hypertrophy in response to Aldo. (Lopez-Andres et al., 2008).

Besides its apoptotic and hypertrophic effects, Aldo has been shown to directly regulate gene expression in CM. In the circulation Ang I is converted to Ang II by ACE. However, the ACE gene is also expressed in the heart where it is believed to facilitate local Ang II production. In failing human hearts ACE expression is increased, suggesting a contributory role in cardiac remodelling and progression of heart failure (Studer et al., 1994). *In vitro* studies show that Aldo by acting through the MR directly induces ACE expression in nrCM and by this mechanism may contribute to cardiac Ang II production (Harada et al., 2001). More recently, an ACE isoenzyme (ACE2) has been identified which converts Ang I to a shorter fragment which is biologically inactive. In nrCM Aldo represses expression levels of the ACE2 gene through an MR dependant mechanism and thereby further facilitates local Ang II production by inhibiting Ang I inactivation (Yamamuro et al., 2008). In summary, Aldo appears to regulate cardiac Ang II production by simultaneous up-regulation of the ACE1 and down-regulation of the ACE2 gene.

Cardiac remodelling is characterised by an inflammatory reaction and increased myocardial expression of pro-inflammatory markers including osteopontin (OPN), cyclooxygenase-2 (COX-2), plasminogen activator inhibitor (PAI-1), interleukin-6 (Il-6) and macrophage chemo attractant protein-2 (MCP-1) (Rocha et al., 2002b). Aldo plays an important role in inducing and mediating these inflammatory responses suggesting that Aldo may directly regulated these genes (Rocha et al., 2002a; Rocha et al., 2002b). Following MI cardiac expression levels of the pro-inflammatory markers COX-2 and Il-6 are increased. Administration of the MR antagonist RU28318 completely prevents COX-2 induction and partially blocks that of Il-6 suggesting that Aldo via MR is implicated in the regulation of these genes. *In vitro* studies have

confirmed that Aldo is capable of inducing COX-2 and Il-6 expression in nrCM but not in cFb (Rebsamen et al., 2004).

Connective tissue growth factor (CTGF) promotes fibrosis by inducing collagen type I and fibronectin expression. CTGF expression is up-regulated in ischaemic hearts in CM and mesenchymal cells adjacent to the infarct zone suggesting that CTGF might be regulated by Aldo. Indeed, subsequent *in vitro* studies show that Aldo induces CTGF expression via the p38 MAPK pathway in embryonic ventricular CM (Lee et al., 2004). Interleukin-18 (Il-18) is another pro-inflammatory cytokine that belongs to the Il-1 family. It plays a critical role in myocardial inflammation and CM hypertrophy and is up-regulated on a transcriptional level by Aldo in nrCM (Doi et al., 2008). However, this effect appears to be indirect as it is dependent on endothelin-1 and Ang II.

In addition to these transcriptional effects, Aldo also exhibits posttranslational effects on CM independent of transcription. In one report Aldo diminished nitric oxide production by directly inhibiting inducible nitric oxide synthase (iNOS) activity (Chun et al., 2003) whereas another study reported activation of MMP-2 and MMP-9 by Aldo which was mediated via production of NADPH-oxidase dependent reactive oxygen species (Rude et al., 2005).

Collectively, these studies provide important mechanistic insights into the role of Aldo as a regulator of genes involved in cardiac remodelling and fibrosis. However, the pathophysiological relevance of these studies is hampered by two facts. Firstly, significant effects of Aldo in these studies are by and large only observed at supraphysiological Aldo concentrations, equal to or greater than 10nM. Secondly, given that the MR has equal affinities for glucocorticoids and Aldo and that in the absence of 11β-HSD2 both steroids must be considered as MR ligands, the specificity for Aldo in mediating these effects is questionable as glucocorticoids through the MR or possibly even the GR may exhibit similar effects on the

transcriptional regulation of these genes. For this reason, identification of genes that are regulated exclusively by Aldo but not glucocorticoids might be key for our understanding of Aldo pathophysiology.

1.10 Hypothesis and aims

1.10.1 Characterisation of steroid hormone action in the heart

It is now clear that Aldo and glucocorticoids have detrimental effects on the CVS (described in detail in section 1.9). However, so far it has not been clarified which cell type in the heart constitutes the primary target of steroid hormone action. So far, expression levels of key components involved in corticosteroid hormone signalling including the GR, MR, 11β-HSD1 and 2 have only been studied in nrCM.

In chapter 3 we will characterise expression of key components involved in steroid hormone signalling in primary cultures of adult rat CM and cFb in an attempt to better understand corticosteroid hormone action in the adult heart. Furthermore, we will investigate glucocorticoid hormone modulation by 11β-HSDs and the effects on gene regulation in primary cultures of rat CM and cFb.

1.10.2 Differential gene regulation by corticosteroids in cardiomyocytes

There is a large body of evidence from animal and clinical studies suggesting that Aldo has detrimental effects on the CVS and that blockade of the MR is cardioprotective. However, on a molecular basis this phenomenon remains less well defined. The MR has equal affinities for corticosterone and Aldo. In the kidney specificity for Aldo is achieved by the enzyme 11β-HSD2 which inactivates corticosterone to 11-dehydrocorticosterone which is no longer able to bind and activate the MR. In non-epithelial cells such as CM, 11β-HSD2 does not seem to be

expressed leaving the MR accessible for both gluco- and mineralocorticoids which raises the question of ligand specific effects.

In chapter 4 we will therefore aim to identify genes differentially regulated by corticosteroid hormones in CM employing microarray technology. We hypothesise that the adrenal steroids Aldo and corticosterone induce a differential transcriptome via their cognate steroid hormone receptors. These differentially regulated genes may give important mechanistic insights in our understanding of the detrimental effects of Aldo on the CVS in the absence of 11β-HSD2.

1.10.3 Steroid hormones in essential hypertension

Blood pressure is influenced by steroid hormones. This is exemplified in conditions of glucocorticoid and mineralocorticoid excess such as Cushing's syndrome and primary hyperaldosteronism, respectively, which invariably result in high blood pressure. However, the role of steroids on blood pressure in the absence of overt excess states is less clear. Some but not all studies have suggested that polymorphisms in the HSD11B2 gene encoding for 11β-HSD 2 and the CYP11B2 gene encoding for aldosterone synthase are associated with hypertension.

In chapter 5 we set out to characterise corticosteroid hormone secretion and metabolism in essential hypertension employing urinary steroid hormone metabolite analysis by gas chromatography / mass spectrometry.

1.10.4 Effects of spironolactone on corticosteroid hormone secretion and metabolism in chronic kidney disease

MR antagonists such as Spiro and EPL are now widely used in patients with primary hyperaldosteronism, resistant hypertension and heart failure. By reducing blood pressure and improving cardiac remodelling, respectively, the aim is to lower

cardiovascular morbidity and mortality. It is unknown if MR inhibition may also alter corticosteroid hormone secretion and metabolism which may further contribute to the protective effects of MR antagonists. We hypothesise two different mechanisms by which Spiro may impact on glucocorticoid hormone secretion. Firstly, that MR in the central nervous system are involved in the regulation of HPA drive and secondly that there is direct inhibition of the 11β-hydroxylase enzyme by Spiro or one of its metabolites.

In chapter 6 we will characterise the effects of Spiro on steroid hormone secretion and metabolism in patients with mild to moderate chronic kidney disease. We hypothesise that MR blockade results in altered steroid hormone secretion and metabolism.

Chapter 2 - General Methods

2.1 In vitro techniques

Unless otherwise stated, reagents were purchased from Sigma (Sigma, UK). The following reagents were purchased from VWR: NaCl, KCl, $MgSO_4$, $NaHCO_3$, $CaCl_2$, Pyruvate, D-Glucose, KH_2PO_4, H_20 (VWR International, Pool, UK)). Cell culture plastic ware was purchased from Corning (Artington, Surrey, UK).

2.1.1 Cell culture

2.1.1.1 Isolation of rat cardiomyocytes and cardiac fibroblasts

2.1.1.1.1 Principle

CM are firmly connected to one other by intercalated discs consisting of tight junctions and gap junctions and so are difficult to cleave without damaging the cells. In order to obtain and isolate intact rod-shaped CM, freshly isolated hearts need to be applied to a Langendorff apparatus by cannulation of the ascending aorta (Figure 2-1). This then allows retrograde perfusion of the heart through the coronary arteries. In order to keep the heart at a physiological temperature during the isolation procedure, the cannulated heart is placed into the inner chamber of an organ bath which is perfused through an outer chamber with water at 37ºC. First the heart is perfused via the aortic cannula with a solution with a low concentration of calcium ions. Subsequently the low calcium solution is switched to solution containing collagenase to digest and break up the ECM freeing individual cells. Following digestion, CM can be separated from all other cardiac cell types including fibroblasts and endothelial cells by gravity centrifugation. CFb are then separated from endothelial cells by making use of their ability to quickly adhere to non-coated culture dishes within a few hours whereas endothelial cells remain in the supernatant.

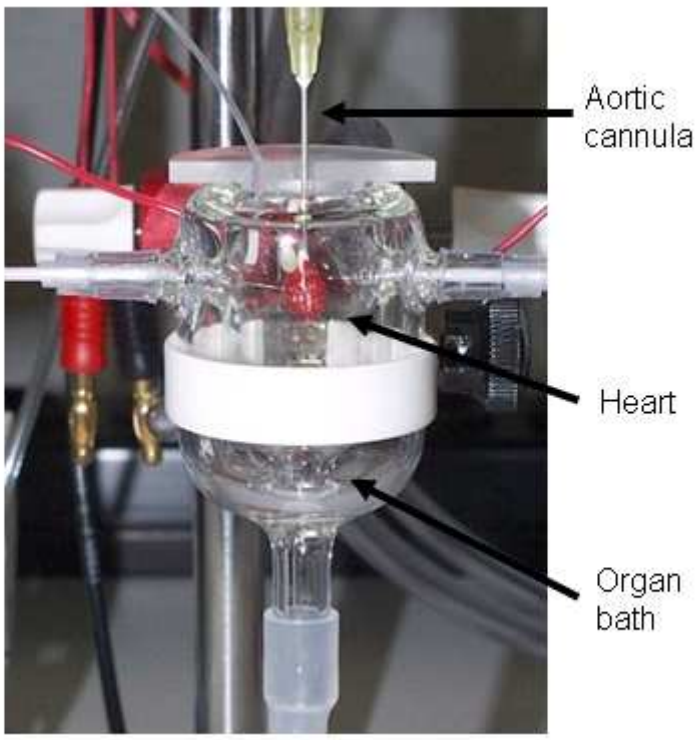

Figure 2-1 Photograph of a cannulated heart in a Langendorff apparatus (http://www.fmigmbh.de).

2.1.1.1.2 Methods

CM from adult rats were isolated by digestion of intact perfused ventricle as previously described (Lewis et al., 2004). Male Sprague–Dawley (300-340 g) rats were killed by cervical dislocation and the heart was rapidly excised and placed into an ice-cold low calcium solution ($CaCl_2$ 10µM, NaCl 120mM, KCl 5.4mM, $MgSO_4$ 5mM, pyruvate 5mM, glucose 20mM, taurine 20mM, HEPES 10mM, nitrilotriacetic acid (NTA)). The aorta was cannulated and the heart was retrogradely perfused for 8 minutes with low calcium solution using a Langendorff apparatus at a constant flow of 5ml/min. Thereafter the heart was perfused for 20 minutes with enzyme solution ($CaCl_2$ 200µM, NaCl 120mM, KCl 5.4mM, $MgSO_4$ 5mM, pyruvate 5mM, glucose 20mM, taurine 20mM, HEPES 10mM, pH 7.4) supplemented with, collagenase (1mg/ml) (Worthington, Lakewood, USA) and hyaluronidase (0.6mg/ml) (Sigma,

York, UK) equilibrated with 100% O2. Following perfusion, the atria were separated from the ventricles and the pre-digested heart was chopped into small pieces which were incubated for another 30 minutes at 37℃ in a shaking water bath. Single CM and cFb were separated from undigested tissue by filtering the cell suspension through a gauze of mesh (300µm) (Cadisch, London, UK). CM were pelleted for 3 min at 400xg and the supernatant containing cFb and endothelial cells was transferred to a 50ml Falcon tube. The CM pellet was resuspended in 50ml enzyme solution (without any enzymes added) and allowed to settle by gravity for 15 minutes. CM were then washed twice with media M199 (Invitrogen Ltd, Paisley, UK) and the supernatant containing cFb was transferred to 50ml Falcon tubes.

2.1.1.1.3 Culturing of primary cardiomyocytes

CM were resuspended in CM culture media and viability and purity of CM was assessed by light microsopy. Healthy and viable CM have a rod shape appearance and spontaneously contract in culture (Figure 2-2). During preparation and isolation of CM a proportion of CM die as a result of membrane disruption through mechanical force or from calcium influx through gap junctions. Non viable cells have a round shape appearance and following staining with trypan blue take up dye and stain blue (described in section 2.1.2). The percentage of dead cells varied from preparation to preparation from 10% to 40% (Figure 2-3). CM were counted in a haemocytometer and approximately 200,000 rod shaped CM were incubated in CM culture media (M199 media (Invitrogen, UK), 1% penicillin/streptomycin, 0.1mM ascorbic acid, 5mM creatine monohydrate, 2mM carnitine, 5mM taurine) in the presence of 10% fetal calf serum (FCS) for 1h to allow CM to attach and adhere to the culture plate. Non-adherent CM were aspirated and the adherent CM were washed once with M199 media and subsequently incubated in CM culture media.

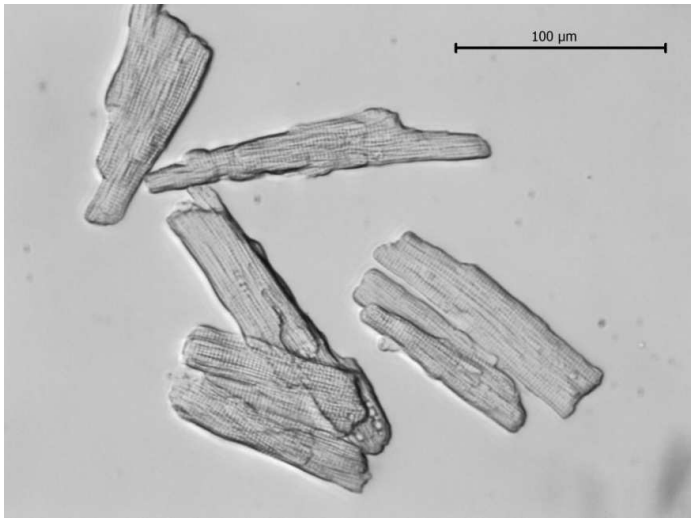

Figure 2-2 Isolated and purified rat cardiomyocytes in culture.

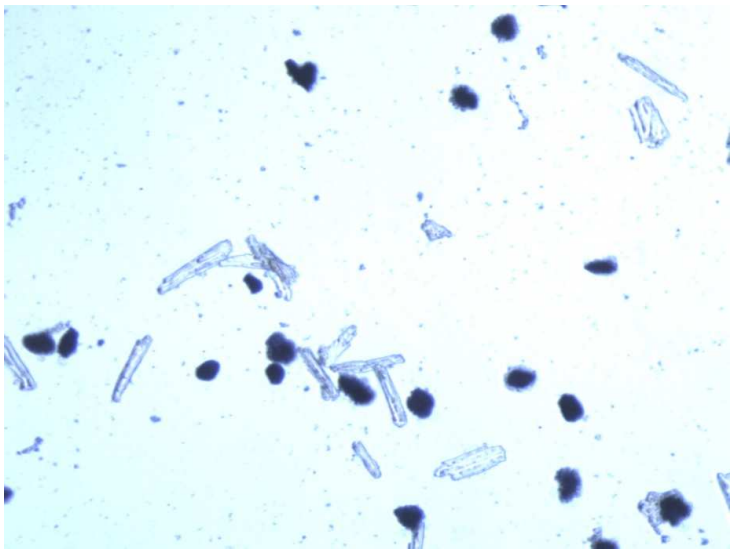

Figure 2-3 Trypan blue staining of isolated cardiomyocytes in culture. Dead cells have a round shape appearance from spontaneous contracture resulting from calcium influx and take up trypan blue stain.

2.1.1.1.4 Culturing of cardiac fibroblasts

Falcon tubes containing cFb in suspension were centrifuged for 10min at 250 x g. The cell pellet was resuspended in cardiac fibroblast media (DMEM (PAA, UK), containing 10% FCS and 1% penicillin/streptomycin) and incubated in a 75cm^2 culture flask for 2h to let cFb adhere to the flask. Non attached cells were removed

and cFb were washed three times with cFb culture media. After 3-4 days cFb had grown to approximately 80% confluence and were used for further experiments.

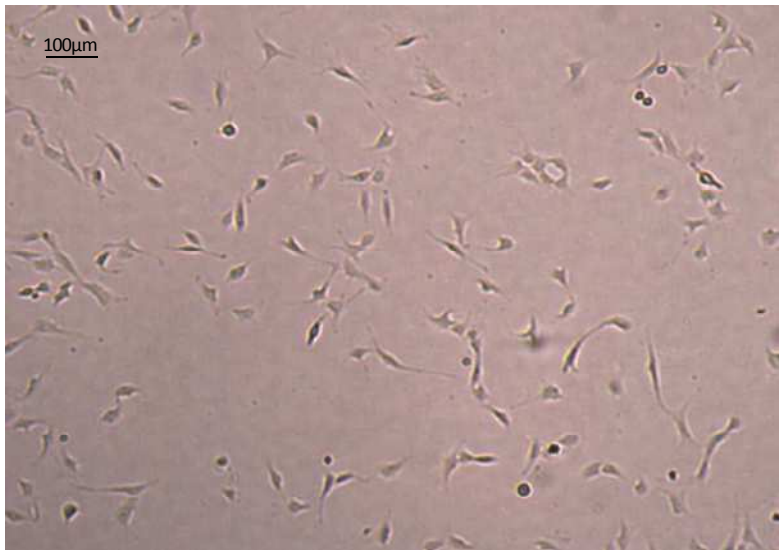

Figure 2-4 Isolated cardiac fibroblasts (cFb) in culture.

2.1.2 Trypan blue staining

2.1.2.1 Principle

Trypan blue is a diazo dye and used to selectively colour dead cells blue. Viable cells with intact cell membranes are not coloured, whereas in a viable cell trypan blue is not absorbed and cells are excluded from staining.

2.1.2.2 Method

100 µl Trypan blue 0.4% is added and gently mixed with 100 µl cell suspension and incubated at room temperature for 5 minutes. Subsequently, the percentage of dead cells is determined by counting of stained (dead) and unstained (viable) cells using a hemocytometer.

2.1.3 Proliferation assay

2.1.3.1 Principle

The effect of cytokines, hormones and drugs on cellular proliferation can be assessed by measuring the incorporation of ^{3}H-thymidine into newly synthesised DNA. The amount of radioactivity as measured by liquid scintillation counting as a marker of DNA synthesis and reflects cellular proliferation.

2.1.3.2 Method

Primary cFb were split and seeded at a density of 12,500 cells per well in 6 well plates in the presence of culture media (DMEM supplemented with 10% FCS and Penicillin/Streptomycin (1x)). The following day, cells were treated with steroids and incubated for 24h. Subsequently, 10µl ^{3}H-thymidine (1:50) (Perkin Elmer, Bucks, UK) was added to the cell culture media and cells were incubated for 4h. Media was removed and cells were washed with PBS and ice cold 5% trichloroacetic acid was added for 30 min to precipitate DNA. Subsequently, trichloroacetic acid was removed, cells washed with PBS and detached from the culture dish by adding 300µl 100mM NaOH. Cells were scraped off the culture dish using a rubber policeman and 100µl cell suspension was added to scintillation vials containing 5ml of scintillation fluid. Radioactivity was measured on a liquid scintillation counter (Model 2500 TR, Packard Instruments) for 5 minutes per sample.

2.1.4 RNA extraction using TriReagent

2.1.4.1 Principle

For isolation of total RNA from cultured primary rat CM and from human left ventricular biopsies, Tri Reagent® (Ambion, Huntingdon, UK) was used. The

principle of this extraction is based in the one-step acid guanidinium method first described by Chomczynski and Sacchi (1987) (Chomczynski & Sacchi, 1987).

2.1.4.2 Method

Total RNA was extracted from heart biopsies and monolayer cells using TriReagent. For monolayer cells, 1ml of TriReagent was added to cells and the cell lysate was transferred to an Eppendorf tube. Heart biopsies were homogenised in 1ml TriReagent in round bottom silicate tubes using a homogeniser and minced tissue was transferred to an Eppendorf tube.

Per 1ml of TriReagent® containing the cell/tissue lysate 0.2ml of Chloroform was added, mixed and incubated at RT for 15 min. The solution was then centrifuged at 12,000xg for 15 min at 4ºC to separate the RNA containing aqueous phase from the protein containing organic phase. The aqueous phase was carefully transferred to a new Eppendorf tube and 0.5 ml of isopropanol was added to precipitate the RNA. For small amounts of RNA, 1µl GlycoBlue® (Ambion, Huntingdon, UK) was added for RNA precipitation and to stain the RNA pellet with a blue color which is easily visible. The RNA was precipitated by centrifugation at 12,000xg for 15 min at 4ºC. Subsequently, the supernatant was aspirated, the pellet washed with 75% ethanol and centrifuged for at 8,000xg for 5 min at 4ºC. The supernatant was removed and the pellet air dried for 5 min and subsequently resuspended in an appropriate volume of nuclease free water and stored at -80ºC.

The concentration and purity of the extracted RNA was checked by a Nanodrop (ND-1000) spectrophotometer (Thermo Scientific, Cheshire, UK). Protein contamination of RNA was assessed by the $OD_{260/280}$ with a ratio of 1.85-2.1 indicating minimal contamination. RNA concentration was quantified by OD_{260} with one unit being equivalent to 40µg of RNA. The integrity of the RNA was checked on 1% agarose gel

containing 0.05 µl/ml ethidium bromide and visualised under ultra violet light. Intact, non degraded RNA exhibits a clear 28s and 18s rRNA bands of which the 28s band is about twice as intense as the 18s band.

2.1.5 RNA extraction using RNeasy® spin columns

2.1.5.1 Principle

For microarray studies (Chapter 2.1.13) RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol. This method is based on an affinity column which contains a matrix that selectively binds RNA yielding pure RNA free of genomic DNA.

2.1.5.2 Method

CM were homogenised in 350µl RLT buffer (provided by Qiagen) by gentle pipetting and transferred to an Eppendorf tube. After addition of 350µl 70% ethanol homogenates were transferred to a Qiagen RNA column and centrifuged for 30 seconds at 13,000 rpm in order to bind the RNA to the matrix of the column. The RNA was then washed twice with 500µl RPE buffer (provided by Qiagen) and eluted with 15µl ddH_20 by centrifugation at 13,000 rpm for 1min.

2.1.6 Reverse transcription and polymerase chain reaction

2.1.6.1 Principles

2.1.6.1.1 RT-PCR

Reverse transcription (RT) is a technique by which RNA is converted into single stranded complementary DNA (cDNA). After extraction, total RNA is incubated with random hexamers, deoxy-nucleotides, reverse transcriptase in the presence of

RNAse inhibitor for cDNA synthesis. Following the RT reaction, single stranded DNA can be used for amplification by means of polymerase chain reaction.

2.1.6.1.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique which allows amplification of DNA by using oligonucleotides which are complementary to the 5' and 3' ends of the DNA region to be amplified. After annealing of the oligonucleotides to the corresponding DNA strand, they are extended by Taq polymerase which elongates the new DNA strand by incorporation of nucleotides. The newly synthesised DNA strands then serve as templates for subsequent cycles resulting in exponential DNA amplification until production plateaus due to limitation of nucleotides and/or polymerase.

2.1.6.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis separates a mixed population of DNA and RNA fragments by length. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. As shorter molecules migrate more easily through the pores of the gel they move faster and migrate farther than longer molecules. Size was assessed using ladders consisting of nucleic acid standards of defined sizes.

2.1.6.2 Methods

2.1.6.2.1 Reverse transcription polymerase chain reaction

For reverse transcription polymerase chain reaction (RT-PCR) a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) was used according to the manufacturer's manual.

Up to 1 µg of total RNA was used in a 20µl reaction: reaction buffer (1x), dNTP (1x), random primers (1x), RT buffer (1x), RNAse inhibitor (1x) and MuLV reverse transcriptase (1x). In a thermal cycler, samples were incubated at 26º for 10min, 37º for 2h followed by a 5 min incubation at 95º to inactivate the reverse transcriptase. cDNA was then diluted to a final concentration of 1.25-5 ng cDNA / µl using nuclease free water. cDNA was stored at -20ºC until further use. Efficiency of the RT reaction was assessed by amplification of the housekeeping gene 18s.

2.1.6.2.2 PCR

PCR was carried out in 20 µl reactions containing the following components (final concentration 1x): reaction buffer (1x), MgCl2 2mM, dNTPs (200 µM), Taq polymerase (0.05 U/µl) (Bioline, London, UK), forward and reverse primer (0.4 µM) and 5-10ng of cDNA. Samples were incubated in a thermal cycler with an initial DNA denaturing step of 95ºC for 5 min followed by 32-34 cycles of 95ºC (30 sec), 60ºC (30sec), 72ºC (1min) and for final extension samples were incubated at 72ºC for 7 min.

2.1.6.2.3 Agarose gel electrophoresis

PCR products were analysed by agarose gel electrophoresis. 5µl of the PCR reaction was mixed with 1µl of loading dye (Bioline, London, UK), loaded onto a 2% agarose gel containing 0.05µl of ethidium bromide and electrophoresed at 100V in Tris borate EDTA buffer (TBE) buffer (89mM Tris, 89mM boric acid, 2mM EDTA) for 20 minutes. DNA was visualised under ultra violet light. Size of PCR products was assessed using HyperLadder IV (Bioline, London, UK).

2.1.7 Relative quantitative (real-time) polymerase chain reaction

2.1.7.1 Principle

Quantitative real-time PCR (qPCR) is a technique based on the polymerase chain reaction, which enables quantification of the amplified DNA product in real time. The set up of the reaction is similar to conventional PCR, but is carried out in a real-time thermal cycler that allows measurement of fluorescence of the samples. Similar to conventional PCR a 5' and 3' oligonucleotide primers flanking the DNA region that is to be amplified are used but in addition, in TaqMan real-time PCR a probe is added to the reaction which consists of a single-stranded oligonucleotide complementary to a segment of 20-60 nucleotides within the DNA template located between the two primers and contains a fluorescent reporter (fluorophore) and a quencher (Figure 2-5). The proximity of the quencher dye inhibits the fluorescence emitted by the reporter through fluorescence resonance energy transfer (FRET). During polymerisation, the probe gets degraded by the 5' exonuclease activity of the polymerase and the fluorophore gets released and breaks the close proximity to the quencher, allowing fluorescence of the fluorophore. Therefore fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. The exponential increase of the fluorescent signal is used to determine the threshold cycle (CT) which refers to the PCR cycle number at which a significant exponential increase in fluorescence is detected, and which is directly correlated with the number of copies of DNA template present in the reaction. The higher the copy number of the target DNA in the sample the lower the cycle at which fluorescence is detected and, hence, the lower the Ct value. In order to determine relative expression levels, the Ct values of the target

gene is subtracted from the Ct value of a housekeeping gene (ie the 18s ribosomal RNA subunit) of which expression levels are constant.

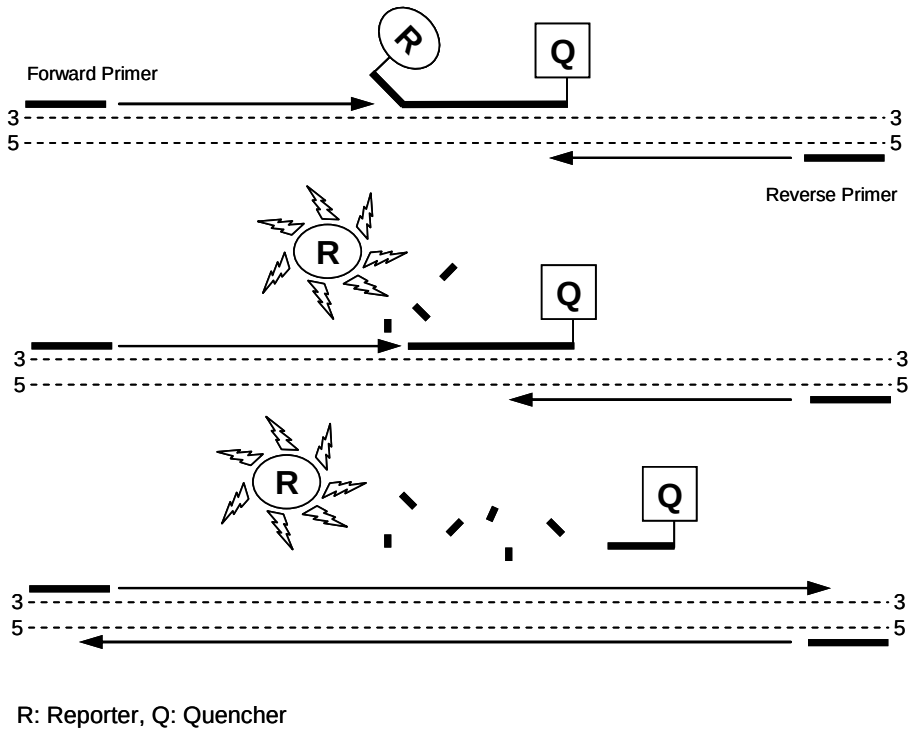

Figure 2-5 Schematic depiction of the TaqMan real time PCR principle. R: reporter; Q: quencher.

2.1.7.2 Methods

Quantitative PCR (qPCR) was carried out employing reagents and expression assays from Applied Biosystems (Applied Biosystems, Qarrington, UK). Reactions for genes of interest and the housekeeping gene 18s were always carried out in duplicated and either as singleplex (i.e. separate reactions for gene of interest and 18s) or multiplex (i.e. amplification of gene of interest and 18s in the same reaction) reactions. The fluorophore for probes of genes of interest was TAMRA whereas 18s probes were labeled with VIC (a pre-requisite for multiplexing). For multiplexing reactions, independent amplification of the housekeeping gene 18s and the gene of

interest was ensured through a dilution series to rule out amplification and/or detection interference of the two probes. The total reaction volume was 10µl consisting of 5µl (2x) master mix, 4µl (1.25-5ng) of cDNA and for amplification and detection of target genes 0.5 µl (20x) expression assay (containing primers and probes) or for amplification of the housekeeping gene 18s 0.3 µl of 18s expression assay and water. For multiplexing reactions both primers and probes of genes of interest and 18s were added to the same reaction. Amplification and detection was carried out on an ABI 7500 real time PCR machine. ΔCt values were calculated by subtracting Ct values for 18s from Ct values for the gene of interest. Data were expressed as arbitrary units which were calculated from ΔCt values employing the formula: expression = $1000 * 2^{-\Delta Ct}$.

2.1.8 In-house production of ^{3}H-11-dehydrocorticosterone

In contrast to tritiated corticosterone (B), 11-dehydrocorticosterone (A) is not commercially available. Therefore a protocol was developed to convert ^{3}H-B into ^{3}H-A. 20µl of ^{3}H-B (Perkin Elmer, Bucks, UK) was converted into ^{3}H-A in the presence of 250 µl of homogenised human placenta, NAD^{+} (500µM) in a total volume of 500 µl of 0.1 M phosphate buffer, pH 7.4. The mixture was incubated for 3h in a shaking water bath at 37ºC. Steroids were extracted by adding 7ml of dichloromethane and vigorous shaking and vortexing for 1 min. The organic phase containing the steroids was separated from the aqueous phase by centrifugation at 80xg for 15 min. Subsequently the top aqueous phase was aspirated and the organic phase was evaporated at 55ºC und constant air flow using a sample concentrator (Techne, UK). Steroids were then resuspended in 100µl of dichloromethane and spotted on a silica plate (Fluka, Sigma-Aldrich, UK) and separated using a thin layer chromatography (TLC) chamber with chloroform:ethanol (92:8) as a mobile phase for 2 h. Dried silica

plates were scanned using an imaging scanner (Bioscan 200, Lablogic, Sheffield, UK) to localise tritiated steroids. Following detection of tritiated A, the corresponding spot of the silica plate was cut out, silica was scraped off into a glass tube and the ^{3}H-A was eluted overnight in 500µl ethanol. The next day the silica ethanol suspension was centrifuged for 15 min at 100xg and the ethanol was transferred to a new glass tube. Purity and activity of the extracted ^{3}H-A was assessed by running one microliter on a TLC plate as described above. ^{3}H-A was considered pure and suitable for further experiments if a single peak was detected representing the ^{3}H-A with an activity of 1000 counts per minute. In case the activity was lower, the ^{3}H-A stock was concentrated until the desired activity was achieved. The ^{3}H-A was stored -20ºC until use.

2.1.9 11β-hydroxysteroid dehydrogenase enzyme assays

2.1.9.1 Principle

The activity of the 11β hydroxysteroid dehydrogenase (11β-HSD) enzyme was analysed by measuring the interconversion of corticosterone and 11-dehydrocorticosterone. A known concentration of substrate (A for oxoreductase activity (conversion of A to B), B for the dehydrogenase activity (conversion of B to A)) and a trace amount of radioactively labeled steroid was added to either isolated cells or tissue and the reaction mixture was incubated at 37 ºC for a previously optimised period. Steroids were then extracted with organic solvent and steroids separated by TLC (Figure 2-6). Fractional conversion was calculated as pmols of product per mg protein (for cells grown in a monolayer), 1 Mio cells (CM grown in suspension) or g wet tissue (for explanted heart tissue).

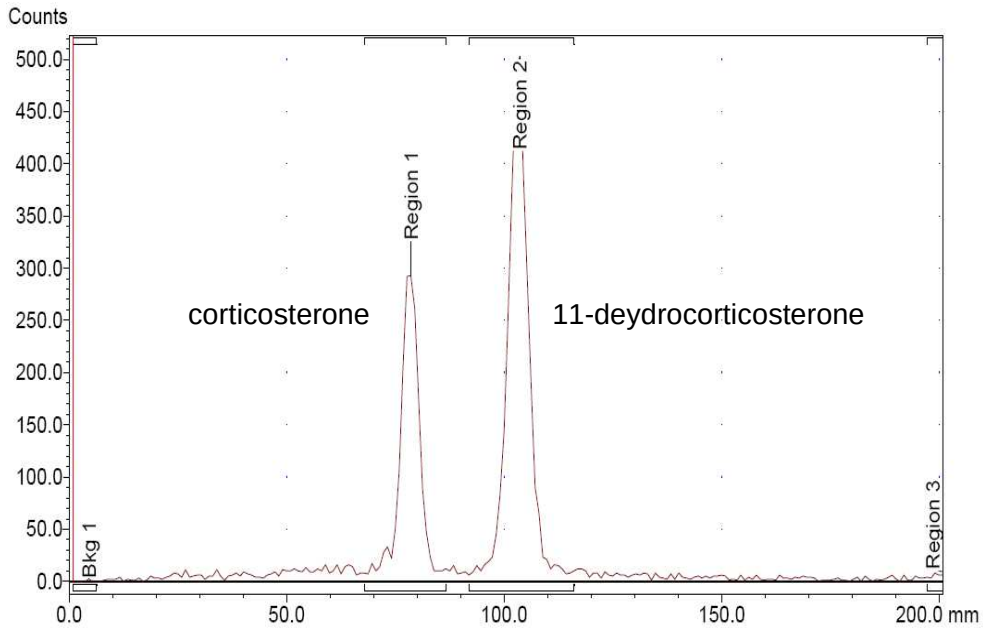

Figure 2-6 Typical Bioscan trace reflecting tritiated steroids separated by thin layer chromatography. Peaks show corticosterone and 11-dehydrocorticosterone.

2.1.9.2 Solutions

Stock solutions of corticosterone and 11-dehydrocorticosterone were prepared as follows: 3.464mg B / 3.444mg A were diluted in 1ml of absolute ethanol and stored at -20 ºC for up to 6 months. On the day of experiment, steroids were diluted to the desired concentration in cell culture media, resulting in a final ethanol concentration of 0.1%

^{3}H-B working solution was prepared from ^{3}H-B stock (1mCi/ml, Perkin Elmer, Bucks, UK) diluted with ethanol 1:10, ^{3}H-A working solution was prepared from ^{3}H-B as described in section 2.1.8.

2.1.9.3 Protocol

CM and cFb were isolated, purified and cultured as described in section 2.1.1.1. For murine heart explants, ventricles were separated from the atria and cut in

approximately 20-30 tissue pieces. 11β-HSD dehydrogenase and oxoreductase assays were either carried out by incubating cultured CM, cFb or murine heart explants in the presence of 100 nM cold substrate and a trace amount of tritiated steroid substrate at 37 ºC degrees in air/5%CO_2 for a suitable time period which was established in preliminary experiments. The reaction was stopped by transferring culture media to 10ml glass tubes and addition of 7ml of dichloromethane. Steroids were extracted by vigorous shaking and vortexing for 10 seconds and the aqueous phase was separated from the steroid containing organic phase by centrifugation for 10 min at 1000xg at RT. The aqueous phase and interphase were aspirated and the organic phase was evaporated at 55ºC under constant air flow. Steroids were then resuspended in 60 µl dichloromethane and spotted onto a TLC silica plate together with 1 µl of a standard containing a mixture of A and B (10^2 M). Steroids were separated using 200ml of a mobile phase consisting of chloroform:ethanol (92:8). Cold standards were visualised under ultra violet light to determine steroid migration. Subsequently, TLC plates were scanned on a Bioscan System 200 and fractional enzymatic activity was determined by calculating the percentage conversion ($counts_{product}$/($counts_{substrate}$ + $counts_{product}$)) per mg protein (monolayer cells), 100,000 CM (suspension cells) or per g wet tissue (cardiac explants) per h and expressed as pmol/10^6 cells/h (pmol/g wet tissue/h).

2.1.9.4 11β-hydroxysteroid kinetics

2.1.9.5 Principle

In order to characterise the properties of any enzyme, the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) for each substrate needs to be determined experimentally (Figure 2-7). Determination of V_{max} and K_m is useful for various

biochemical purposes such as estimation of reaction rates, isoform comparison and definition of the potency of inhibitors.

2.1.9.6 Methods

To determine kinetic properties of 11β-HSD oxoreductase activity, CM were isolated and 100,000 rod-shaped cells were cultured as described in section 2.1.1.1 in 6 well plates. Cells were then incubated in the presence of increasing concentrations of 11-dehydrocorticosterone and traces of radioactively labeled ^{3}H-11-dehydrocorticosterone. Following a 2h incubation period, steroids were extracted using dichloromethane, spotted onto TLC silica plates and separated by TLC (described in section 2.1.9.3). Subsequently, TLC plates were scanned and fractional enzymatic activity was determined by calculating the percentage conversion. Concentration dependant enzymatic activities were calculated and expressed as pmol/mg protein/hour or pmol/10^{6} cells/h. Following Lineweaver-Burk transformation, the Michaelis-Menten constant (K_m) and V_{max} was calculated for 11β-HSD oxoreductase activity in CM.

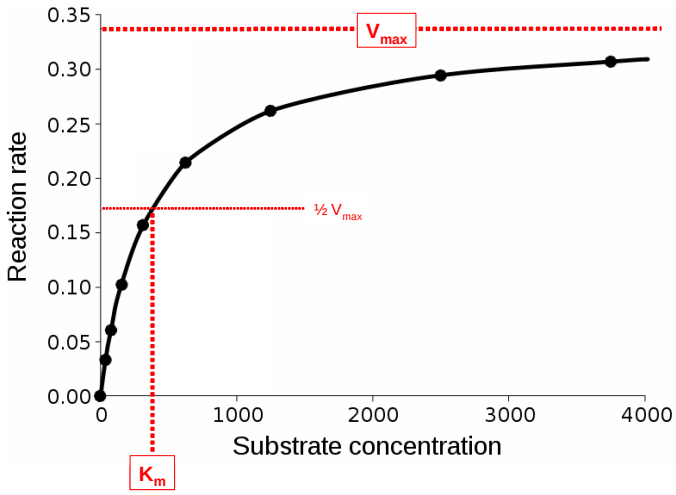

Figure 2-7 Illustration of the reaction rate (velocity) of an enzyme in relation to substrate concentration. K_m denotes the substrate concentration at half of the maximum velocity.

2.1.10 Protein extraction

2.1.10.1 Principle

Monolayer cells are lysed in a buffer containing detergent to break open cell membranes. In addition, cells are freeze thawed once to further disrupt cell membranes

2.1.10.2 Methods

Culture media was removed from cells grown in monolayer and cells were briefly rinsed with PBS. RIPA buffer (50mM Tris pH 7.4, 1% NP40, 0.25% SDS, 150mM NaCl, 1mM EDTA pH 8) (if cell lysate were used for Western blot analysis, protease inhibitors were added to the RIPA buffer in addition) was added to the cells (50 µl/well (12 well plate), 200 µl/well (6 well plate)) and cells were frozen at -80ºC until further use. After thawing, cells were scraped with a rubber policeman and transferred to an Eppendorf tube and centrifuged at 12,000xg for 15 min at -4ºC to spin down insoluble cell debris. The supernatant was transferred to a new Eppendorf tube and stored at -80ºC until further use.

2.1.11 Protein quantification

2.1.11.1 Principle

Protein concentrations of cell extracts were measured using the BioRad RC DC protein assay (BioRad, Hemle Hempstead, Herts). This is a colourimetric assay for protein concentration following detergent solubilisation. The assay is based on the Lowry method and the reaction of protein with an alkaline copper tartrate solution and then reduction of Folin reagent. Folin reagent is reduced by the loss of 1, 2 or 3 oxygen atoms, primarily due to the amino acids tyrosine, tryptophan and to a lesser

extent, cystine, cysteine and histidine. The reduction of Folin reagent can produce several reduced species which have a characteristic blue colour, with a maximum absorbance at 750nm and a minimum absorbance at 405nm. The protein concentration of a sample was determined employing a standard curve which was generated from a dilution series of a sample with a known protein concentration (Figure 2-8).

2.1.11.2 Methods

Protein assays were carried out in 96 well plates according to the manufacturer's protocol. Per well, 5µl of protein standard (range 0, 0.5, 1, 2, 4, 6, 8 mg/ml BSA) or sample was added. Subsequently, 25µl of solution A (alkaline copper tartrate solution) was added followed by addition of 200µl of solution B (Folin reagent). The plate was incubated for 10 and then read at 690nm on a Victor3 1420 multilabel counter (PerkinElmer, Bucks, UK).

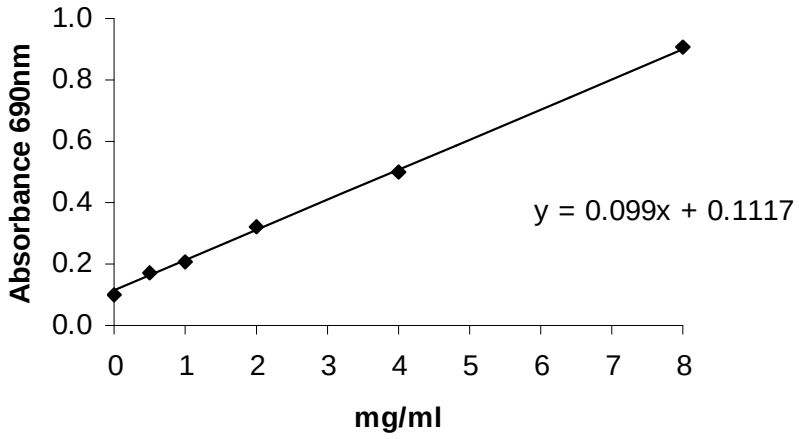

Figure 2-8 Representative protein standard curve from protein standards containing 0, 0.5, 1, 2, 4, 8 mg of protein/ml.

2.1.12 Western Blotting

2.1.12.1 Principle

Western blotting allows detection and measurement of relative amounts of a specific protein in a mixed protein sample (Towbin et al., 1992). The proteins are separated by size by SDS-PAGE and subsequently transferred to a membrane, usually nitrocellulose or polyvinylidene difloride (PVDF). The membrane is blocked with a generic protein, such as cow milk proteins or BSA, to prevent unspecific binding of antibodies to the membrane. Thereafter, the membrane is incubated with the primary antibody that binds specifically to the protein of interest. The membrane is then incubated with an enzyme conjugated secondary antibody that is directed against the primary antibody. The secondary antibody is typically conjugated to horseradish peroxidise (HRP) which catalyses the oxidation of a chemiluminescent substrate producing light. The reaction is captured onto photographic film.

2.1.12.2 Method

SDS-PAGE and protein transfer were performed in a BioRad mini protein 3 apparatus using a modified protocol provided with the kit. Between 10-40µg of protein was mixed with the appropriate amount of 4 X loading buffer and boiled for 5 mins. Samples were loaded on an 8-12% SDS-PAGE gel and run at 200V for 1hr to 1 hr 30 min. Proteins were transferred onto nitrocellulose membrane at 140V for 1-1.5 hr depending on the molecular weight of the protein to detect. To check for efficient transfer of proteins, membranes were incubated in Ponceau staininig solution with slight agitation for 30 sec, and then rinsed with water until protein bands appeared. Bands should be distinct and well spread out. Membranes were then blocked in 10mL of blocking buffer for 1hr with constant agitation, rinsed twice with washing

buffer, incubated in primary antibody overnight at 4ºC with constant agitation. Membranes were then washed 3 times for 15 min in washing buffer (PBS, Tween 0.1%). Secondary antibody incubation was for 1 hr at room temperature followed by washing 3 times for 15 min in 50mL of washing buffer. Chemiluminesence was used for antibody detection; substrate A and B were mixed in equal quantities (0.5 mL each per membrane) and allowed to equilibrate for 5 min. 1mL of substrate was put on each membrane and incubated for 2 min. Membranes were placed between two sheets of clear plastic and in the dark exposed to photographic film for 2 sec to 1 hr. Photographic film was developed on Compact X4 (Xograph Imaging Systems, Gloustershire, UK)

2.1.13 Microarray studies

2.1.13.1 Principle

Whole genome DNA microarrays are used to measure changes in expression levels of multiple genes. Whole genome microarrays cover all genes of a particular genome and allow the study of expression levels at baseline as well as changes in response to a treatment.

A microarray consists of a series of thousands microscopic spots, so called features, which contain picomoles of single stranded DNA oligonucleotides that are immobilised on a solid surface such as glass or silicone. Each spot represents one gene as the DNA sequence of the oligonucleotides is complimentary to that of the messenger RNA of the corresponding gene. In order, to assess gene expression levels, the mRNA is labeled with a fluorochrome and hybridised to the immobilised complementary oligonucleotides on the array at a high stringency. The intensity of

fluorescence of each spot directly reflects expression levels of the corresponding gene (Figure 2-9).

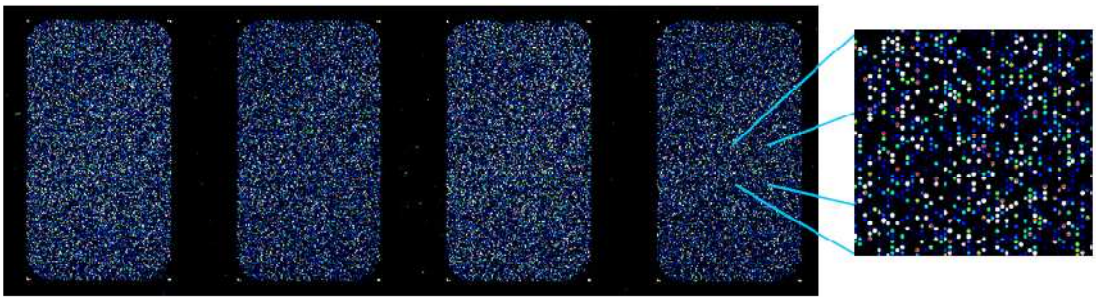

Figure 2-9 Illustration of a scanned microarray slide. One slide contains four individual microarrays containing 43,380 features representing genes. Following hybridisation of the labeled cRNA probe, the microarray slides are scanned. The intensity of each spot reflects the expression level (the brighter the spot the higher the level of expression) of the corresponding gene.

2.1.13.2 Method

Figure 2-10 gives an overview of the multistep protocol involved in the generation of labelled cRNA probes. In brief, extracted RNA is reversely transcribed into 1st and 2nd strand cDNA by reverse transcriptase from the moloney mouse leukemia virus (MMLV RT). The 2nd strand cDNA subsequently serves as a template for the T7 RNA polymerase to finally synthesis labelled complementary RNA (cRNA) by incorporating Cytosins which are linked to the fluorophore Cy3. For details see materials and methods in Chapter 4.

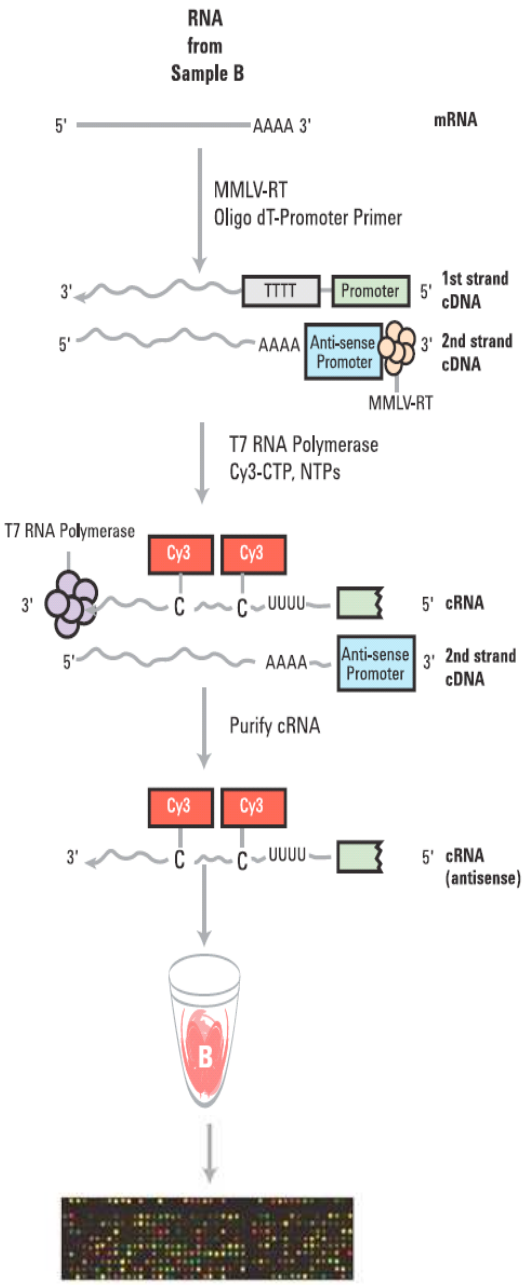

Figure 2-10 Schematic illustration of RNA labeling. RNA is reversely transcribed into cDNA which then serves as a template for the synthesis of complementary RNA (cRNA) in which Cy3-labeled Cytosine is incorporated.

2.1.13.3 Bioinformatic analysis

Assessment and analysis of microarray data was carried out using the software “R: A language and environment for statistical computing” (http://www.r-project.org/) using packages from Bioconductor, open source software for bioinformatics.

(http://www.bioconductor.org/). In order to compare microarray results, raw microarray data was normalised using quantile normalisation. Following quality assurance employing the principal component analysis (PCA) and correspondence analysis (CA), the linear models for microarray analysis (limma) package was used to determine differentially expressed genes. For details see materials and methods in Chapter 4.

2.2 Clinical studies

Several clinical studies are reported in this thesis. All patients were recruited from the University Hospital Birmingham NHS trust. All studies had ethical approval of the Local Research Ethics Committee and all patients gave their informed written consent to participate. Studies were conducted at the Wellcome Trust Clinical Research Facility, Queen Elizabeth Hospital, Birmingham. Study protocols are described in the individual chapters.

2.2.1 Biochemical analysis

2.2.1.1 Gas chromatography/mass spectrometry for urinary steroid hormone metabolites

2.2.1.1.1 Method

The gas chromatography/mass spectrometry (GC/MS) method used is based on the method described by Palermo and Shackelton (Palermo et al., 1996). Steroid extraction was carried out by Seppak C19 cartridges. To this extract, 200ng of sigmasterol and cholesteryl-butyrate were added as external standard and methyloxime-trimethylsilyl ether were prepared according to established procedures. Following derivatisation, the excess reagent was removed by Lipidex

chromatography; the samples were automatically introduced in a Hewlett Packard 5970 mass spectrometer with a 15m DB1 capillary column. Quantification was achieved by monitoring selected ions for the analytes (fragment 531 for cortisone and 605 for cortisol) and internal standards (m/Z 534 and 609, respectively). Relative peak areas were determined and reported as µg/24 h of the individual compound. Validation of the method was performed by comparing the curve of increasing amounts of the analyte to the ratio between the areas of different amounts of the analyte and fixed concentrations of the internal standard (r=0.998 for cortisol and 0.999 for cortisone). Intra and inter-assay coefficients of variation were <10% for both cortisol and cortisone.

2.2.1.1.2 Urinary steroid metabolites and their ratios

The sum of total cortisol metabolites (THF, THE, 5α-THF, α-cortolone, cortisone, cortisol, cortisol, β-cortolone, β-cortol, and α-cortol) provides a reflection of the cortisol secretion rate. The ratio of tetrahydro-metabolites of cortisol (THF + 5α-THF) to those of cortisone (THE) provides a reflection of 11β-HSD1 activity when considered with the ratio of urinary free cortisol (UFF) to cortisone (UFE), which more accurately reflects renal 11β-HSD2 activity (Palermo et al., 1996). The ratios of cortols to cortolones and of 11β-hydroxy-etiocholanolone and 11β-hydroxy-androsterone combined to 11oxo-etiocholanolone also reflect 11β-HSD1 activity (Walker et al., 2007). The activities of 5α- and 5β-reductases can be inferred from measuring the ratio of 5α-THF to THF and androsterone to etiocholanolone. TH-Aldo is the main urinary Aldo metabolite and reflects 24h Aldo production. The activity of 11β-hydroxylase can be inferred from measuring the ratio of total cortisol metabolites (see above) to tetrahydro-11-desoxycortisol (THS) (Freel et al., 2007).

2.2.1.2 Plasma measurements

Urea, creatinine and electrolytes were measured using standard laboratory methods at the Department of Biochemistry, University Hospital of Birmingham NHS trust.

2.2.1.2.1 Steroid secretion and metabolism in essential hypertension

Plasma renin activity (PRA) and Aldo were kindly measured by Dr C Webster, Dept of Clinical Biochemistry, Birmingham Heartlands Hospital, Birmingham, UK. PRA was measured using the Biodata Renin MAIA assay (Serono Diagnostics, Woking, Surrey, UK) and plasma Aldo using a solid phase (coated tube) radioimmunoassay technique, DPC Coat-a-Count assay (DPC, Llanberis, Caernarfon, Gwynedd, UK). The intra-assay correlation coefficients of these measurements were all below 10%. The reference range in the supine position ranged from 0.51 - 2.64 ng/ml/h for PRA and 28 - 445 pmol/l for Aldo.

2.2.1.2.2 Spironolactone in chronic kidney disease patients

PRA, Ang II and Aldo in the circulation were kindly measured by Dr J Morton, Department of Cardiology, Western Infirmary, Glasgow, Scotland, United Kingdom. PRA was measured by an in-house antibody trapping technique in the presence of added excess renin substrate (Millar et al., 1980). The coefficient of variation was 3.4%. An in-house radioimmunoassay was used for plasma Ang II as previously described (Morton & Webb, 1985). Ang II was pre-extracted from plasma before assay. The coefficient of variation was 10%. Aldo was measured with a solid-phase (coated tube) radioimmunoassay kit supplied by Diagnostic Products (UK) Ltd. The coefficient of variation was <8.3%.

2.3 Collection of human left ventricular cardiac biopsies

During cardiac surgery full-thickness free wall LV biopsy samples were obtained using Tru-Cut Biopsy Needles (Allegiance Healthcare Corp, McGaw Park, Ill) immediately before AXC placement (pre-ischemia). Biopsies were immediately snap frozen in liquid nitrogen and stored at -80ºC until further use.

2.4 Blood pressure measurements

Office brachial blood pressure was recorded with the subject lying supine in the non-dominant arm using a validated oscillometric sphygmomanometer (Dinamap® Procare, GE). 24 hour ambulatory blood pressure (24h ABP) measurement was performed using a Meditech® ABPM-04 (Budapest, Hungary).

2.5 Statistical analysis

2.5.1 General statistics

All statistics were performed using SPSS 16.0 software (SPSS inc., Chicago, USA). Parametric data are described as mean ± standard deviation (SD), non-parametric data are described as Mean (interquartile range). Where applicable non-parametric data was log transformed. For comparisons between two groups student's t-test for parametric data, Mann-Whitney U test for non-parametric data and the chi-square test for categorical data was used. When more than two groups were compared, analysis of variation (ANOVA) was used. Correlation coefficients (Pearson for parametric, Spearman for non-parametric data) were obtained when the strength of a relationship between two variables was examined.

Chapter 3 - Characterisation of Glucocorticoid Hormone Action in the Mammalian Heart

3.1 Introduction

The CVS has emerged as an important target of corticosteroid hormone action. The GR and the MR are both expressed in the heart (described in section 1.9.3.1) and a growing body of evidence from both experimental and clinical studies has established an important role for steroid hormones in the development and modulation of cardiovascular disease (described in section 1.9).

Most experimental studies have focused on the modulatory effects of Aldo in heart disease, considerably less is known about the effects of glucocorticoids although chronic glucocorticoid excess similar to Aldo excess has adverse effects on the CVS. This is exemplified in patients with Cushing's syndrome, characterised by excessive endogenous cortisol production, who have a high prevalence of left ventricular hypertrophy (Muiesan et al., 2003; Muiesan et al., 2008). In keeping with these findings, cardiovascular risk in these patients is increased 4 times compared to the normal population (Etxabe & Vazquez, 1994). Glucocorticoid treatment is associated with an increased risk of heart failure (Souverein et al., 2004) as well as cardiovascular disease (Wei et al., 2004). Moreover, high-normal cortisol levels have recently been associated with increased mortality in patients with CHF (Guder et al., 2007).

Glucocorticoid action in target tissues is not exclusively dependent on circulating levels but also determined by the inter-conversion of active and inactive forms of the steroid within target cells. This pre-receptor metabolism is catalysed by two isozymes of 11β-HSDs (described in section 1.7). 11β-HSD2 is expressed mainly in classic Aldo target tissues such as the kidney, colon and salivary glands where it functions as a dehydrogenase converting cortisol to cortisone (in rodents: corticosterone to 11-dehydrocorticosterone). By this mechanism MR specificity for Aldo is maintained as

the MR has equal affinities for glucocorticoids and mineralocorticoids *in vitro* (Funder et al., 1988). In the heart, 11β-HSD2 appears to be expressed only at very low levels (Kayes-Wandover & White, 2000; Slight et al., 1996) or is undetectable (Walker et al., 1992) and thus, is unlikely to sufficiently protect the MR from glucocorticoids (described in section 1.9.3.4). Experimental evidence from a mouse model overexpressing 11β-HSD2 in CM suggests that in fact glucocorticoids may be beneficial by tonically occupying the MR and thereby protecting it from Aldo binding and action (Qin et al., 2003).

In contrast to the type 2 isozyme, 11β-HSD type 1 (11β-HSD1) is widely expressed and predominantly functions as an oxoreductase *in vivo* by converting inactive into biologically active glucocorticoids (described in section 1.7.1). 11β-HSD1 oxoreductase activity critically relies on the presence of NADPH which is generated from $NADP^+$ by the enzyme H6PDH. To date, the role of 11β-HSD1 in cardiac physiology and disease remains unclear.

In order to better understand glucocorticoid hormone action in the heart, we characterised expression and function of key components of glucocorticoid hormone action in isolated adult rat CM, cardiac MyoFb, rodent hearts and human left ventricular biopsies.

3.2 Materials and Methods

3.2.1 Isolation of rat cardiomyocytes

Animal studies were performed in accordance with Home Office Guidance (Animals Scientific Procedures Act 1986). CM from adult male Sprague-Dawley rats (250-300g) were isolated as described in section 2.1.1.1. Following isolation, CM were incubated in CM culture media.

3.2.2 Collection of heart from WT and H6PDH$^{-/-}$ mice

The H6DPH/KO mice were generated by the replacement of exons 2 and 3 of the H6PDH gene with a neomycin resistance cassette as reported (Lavery et al., 2006). Homozygous null mice (H6PDH$^{-/-}$) were generated by heterozygous mating. Mice were housed in pathogen-free conditions and had a 12-h light, 12-h dark cycle and unlimited access to standard mouse chow and water.

3.2.3 Cell treatment

For gene expression studies 200,000 rod shaped CM in suspension were seeded per well in 6 well plates in CM culture medium containing 10% FCS to facilitated attachment of CM. Following a 1h incubation period, medium and non attached cells were discarded and attached CM were gently washed with M199 and incubated in CM culture media with no FCS. Cells were then treated with steroid (1-100nM) in the presence or absence of corticosteroid receptor antagonists (RU486, RU28318) at a concentration of 10μM for 8 hours.

3.2.4 RNA extraction

Total RNA from cultured rat CM, cFb or human left ventricular cardiac biopsies was extracted employing TriReagent (Applied Biosystems, Warrington, Cheshire, UK). For cultured rat CM and cFb 1ml of TriReagent was added to cultured cells; human left ventricular cardiac biopsies were homogenised in 1ml of TriReagent using a tissue homogeniser. Total RNA was extracted as described in section 2.1.4.

3.2.5 cDNA synthesis

Total RNA (0.5 μg) was reversely transcribed in 25 μl total volume using TaqMan Reverse Transcription Reagents (Applied Biosystems) as described in section

2.1.6.2.1. Following the reverse transcription reaction, the cDNA was diluted to a final concentration of 5 ng/µl (human biopsies: 1.25 ng/µl) and stored at −20 ℃ until further use.

3.2.6 Conventional reverse transcription polymerase chain reaction

RT-PCR was carried out in a total volume of 20µl using 20ng of cDNA as described in section 2.1.6.2.2. Primers used for conventional RT-PCR are listed in Table 3-1. For amplification of GR, MR, 11β-HSD1 and 2 in CM and cFb 33 cycles, for characterisation of cFb 31 cycles of PCR amplification were carried out. The temperate profile for amplification was 1 min at 95℃, 30 sec at 95℃, 1 min at 72℃ with a final cycle of 7 min at 72℃.

Table 3-1 Primer pairs used for gene amplification by RT-PCR.

Gene symbol	F/R	Sequence	Product (bp)
GR	F	AAGAGCACTGGAAGGACAGC	474
	R	TCATTAATAATCAGATCAGGAGCAA	
MR	F	GTGGACAGTCCTTTCACTACCG	286
	R	TGACACCCAGAAGCCTCATCTC	
11β-HSD1	F	GGAGCCCATGTGGTATTGAC	577
	R	CTGTGCCTTTGATGATCTCC	
11β-HSD2	F	TGGCAAGGAGACAGCTAAGAA	679
	R	GCAATGCCATTCTGAGTGAA	
PECAM-1	F	CTGAGGTGGGCCTCAGTC	449
	R	TGTCACCTCCTTTTTGTCCA	
Vimentin	F	CCAAGTTTGCTGACCTCTCTG	337
	R	TCCAGCAGCTTCCTGTAGGT	
ANF	F	TCTGATGGATTTCAAGAACCTG	366
	R	TTCGGTACCGGAAGCTGT	
α-SMA	F	CGGGAGAAAATGACCCAGAT	548
	R	ATAGGTGGTTTCGTGGATGC	
18s	F	GTTGGTGGAGCGATTTGTCT	397
	R	GGCCTCACTAAACCATCCAA	

F: forward, R: reverse, bp: base pairs.

3.2.7 Real-time quantitative polymerase chain reaction

Expression levels of genes of interest were measured using ABI 7500 system (Perkin-Elmer, Biosystems). All primers and probes were purchased from Applied Biosystems. Primers and probes for 18s rRNA, GR, MR, 11β-HSD1, VEGFa, ACE1 which were supplied as expression assays (Applied Biosystems, Warrington, Cheshire, UK). All reactions were normalised against the house keeping gene 18S rRNA.

3.2.8 Protein extraction and immunoblotting

Rat heart, liver, kidney and cultured CM were homogenised in RIPA buffer and total protein concentration was determined as described in section 2.1.11. Forty micrograms of protein sample were resolved on an 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane Hybond ECL (GE Healthcare, Chalfont St Giles, UK). Primary antibodies raised against the MR and GR (Santa Cruz, UK) were used at a dilution of 1:1000. Secondary antibodies were used at a dilution of 1/10000 (Abcam plc, Cambridge, UK). Membranes were reprobed for ß-actin at a dilution of 1/5000 (Abcam plc, Cambridge, UK).

3.2.9 11β-HSD enzyme assays

For enzymatic assays 200,000 rod shaped CM in suspension per well were cultured in 6 well plates in FCS free CM media. Conversion of 11-dehydrocorticosterone to corticosterone (oxoreductase) in cultured rat CM and cFb was analysed by incubating cells with 100 nM 11-dehydrocorticosterone and tracer amounts of ^{3}H-11-dehydrocorticosterone (synthesised in-house, described in section 2.1.9) for two hours. Dehydrogenase activity (corticosterone to 11-dehydrocorticosterone) was assessed using 100 nM unlabelled corticosterone (Sigma-Aldrich Company Ltd,

Dorset, UK) diluted in serum-free medium and tracer amounts of ^{3}H-corticosterone (specific activity 74·0 Ci/mmol; NEN, Boston, MA, USA) at 37 ℃ for 8 h. Steroids were extracted from the medium with 7ml of dichloromethane, separated by thin-layer chromatography with chloroform:ethanol (92:8) as a mobile phase and the fractional conversion of steroids was calculated after scanning using a Bioscan 2000 radioimaging detector (Bioscan, Washington, DC, USA). Enzymatic activities were calculated and expressed as pmol/mg protein/hour or pmol/10^{6} cells/h. All assays were carried out in triplicate and conversion rates calculated from three individual experiments.

3.2.10 Proliferation assay

Fibroblast proliferation was assessed by incorporation of ^{3}H-thymidine as described in section 2.1.3. Isolated cFb in culture (less than 3 passages) were seeded into 12 well plates. The following day, cFb were treated with steroids (final concentration) DEX, corticosterone, 11-dehydrocorticosterone (1-100nM) in the presence or absence of RU486 (1µM). For proliferation assays, MyoFb were treated with corticosterone or 11-dehydrocorticosterone (1µM) in the presence or absence of GE (10µM). All treatments were performed in triplicate. Each experiment was repeated 3 times.

3.2.11 Patients

Left ventricular (LV) cardiac biopsies were obtained from patients with LV hypertrophy (LVH) secondary to aortic valve stenosis undergoing aortic valve replacement (AVR) or from transplant donor hearts (courtesy of Mr Domenico Pagano and Mr Neil Howell, Department of Cardiothoracic surgery, Queen Elizabeth Hospital, Birmingham).

Characteristics of aortic stenosis patients (N=17): male/female: 12/5; Age: 68±11; BMI: 29±5 kg/m^2; IVS: 17±3 mm; aortic gradient: 77±19 mmHg. Four patients had coronary artery disease, and all but one patient had a normal LV ejection fraction (LVEF).

Characteristics of heart donor patients (N=15): male/female: 7/8; Age: 51±12 years; BMI: 25±3 kg/m^2; IVS: 11±2mm; primary cause of death was intracranial bleed (N=8), head injury (N=5), infection (N=1) and intracranial tumour (N=1). Donors were part of a randomised controlled clinical trial (Venkateswaran et al., 2009). As part of this trial, patients were randomised to a 6.3±1.4h treatment with tri-iodothyronine (T3) (N=11) or placebo (N=4). Two patients received DEX and one donor received hydrocortisone prior to brain stem death.

Normal human liver (N=4) (Ahmed et al., 2008) and muscle mRNA (N=5) (Morgan et al., 2009) was obtained from biopsy material under ethical approval from other research programmes within the group evaluating corticosteroid hormone action in metabolic tissues (courtesy of Drs Ahmed and Sherlock). All patients or next of kin (transplant donors) gave written informed consent. The study protocol was approved by the Local Research Ethics Committee.

3.2.12 Collection of human left ventricular cardiac biopsies

Full-thickness free wall LV biopsy samples were obtained using Tru-Cut Biopsy Needles (Allegiance Healthcare Corp, McGaw Park, Ill) immediately prior to aortic cross clamp placement (in both patients undergoing aortic valve replacement and donors prior to heart explant). Biopsies were immediately snap frozen in liquid nitrogen and stored at -80ºC until further use.

3.2.13 Statistical analysis

Statistical analysis was performed using the software package SPSS for Windows Version 16 (SPSS, Inc., Chicago, IL, USA). Data are presented as means±SD. Real-time PCR analyses were performed following transformation of ΔCts values into arbitrary units through the equation $2^{-\Delta Ct}$. Statistical analyses were performed using a t-test/ANOVA for normally distributed data. p values <0.05 were accepted as statistically significant.

3.3 Results

3.3.1 Characterisation of steroid hormone signalling in cardiomyocytes

We first studied expression of key components involved in corticosteroid hormone signalling in rat heart and isolated rat CM in culture by conventional RT-PCR. Both the GR and MR were readily expressed in whole rat heart and CM (Figure 3-1). 11β-HSD1 was also expressed in rat heart and isolated CM. Based on visual comparison of band intensities, 11β-HSD1 appeared to be less abundantly expressed compared to the GR and MR, whereas 11β-HSD2 was undetectable (Figure 3-1).

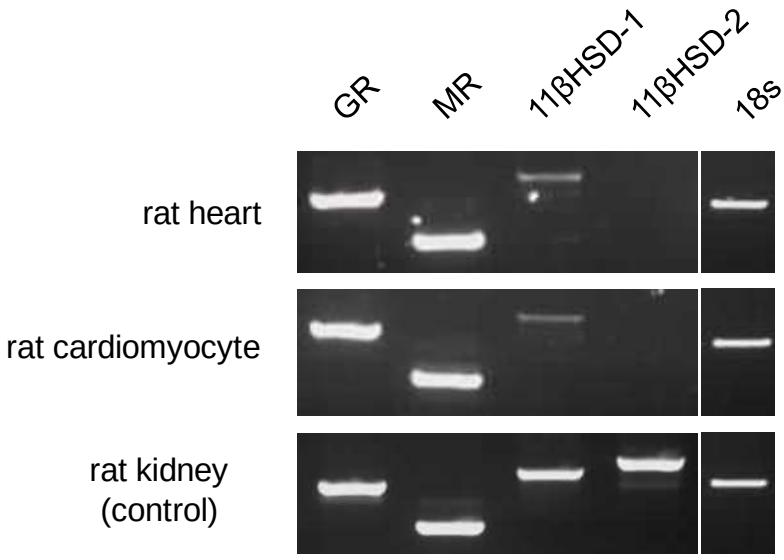

Figure 3-1 Expression of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) in whole rat heart and isolated rat cardiomyocytes by conventional RT-PCR.

Expression of the GR and MR was confirmed at a protein level by immunostaining. Expression levels of GR and MR was higher in extracts from whole rat hearts compared to isolated CM (Figure 3-2).

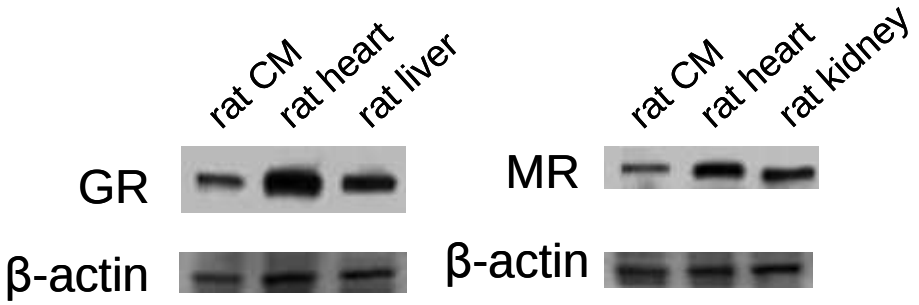

Figure 3-2 Western blot analysis of GR and MR in isolated cardiomyocytes (CM) and whole rat heart.

Relative expression levels of GR, MR, 11β-HSD type 1 and 2 were then compared in heart, liver, quadriceps muscle, kidney and gonadal fat of male and female adult

C57BL/6 mice by quantitative PCR (Figure 3-3). GR and MR were expressed at similar levels in all tissues with highest levels in heart and skeletal muscle. MR expression levels were 5-10 fold lower compared to GR in all tissues (Figure 3-3a and b). 11β-HSD1 was predominantly expressed in liver, less in kidney and fat and lowest in heart and skeletal muscle (Figure 3-3c), whereas 11β-HSD2 was exclusively expressed in kidney (Figure 3-3c). With the exception of GR and 11β-HSD1 which exhibited higher expression levels in the kidney and adipose tissue of male compared to female mice, there were no sex differences in expression levels.

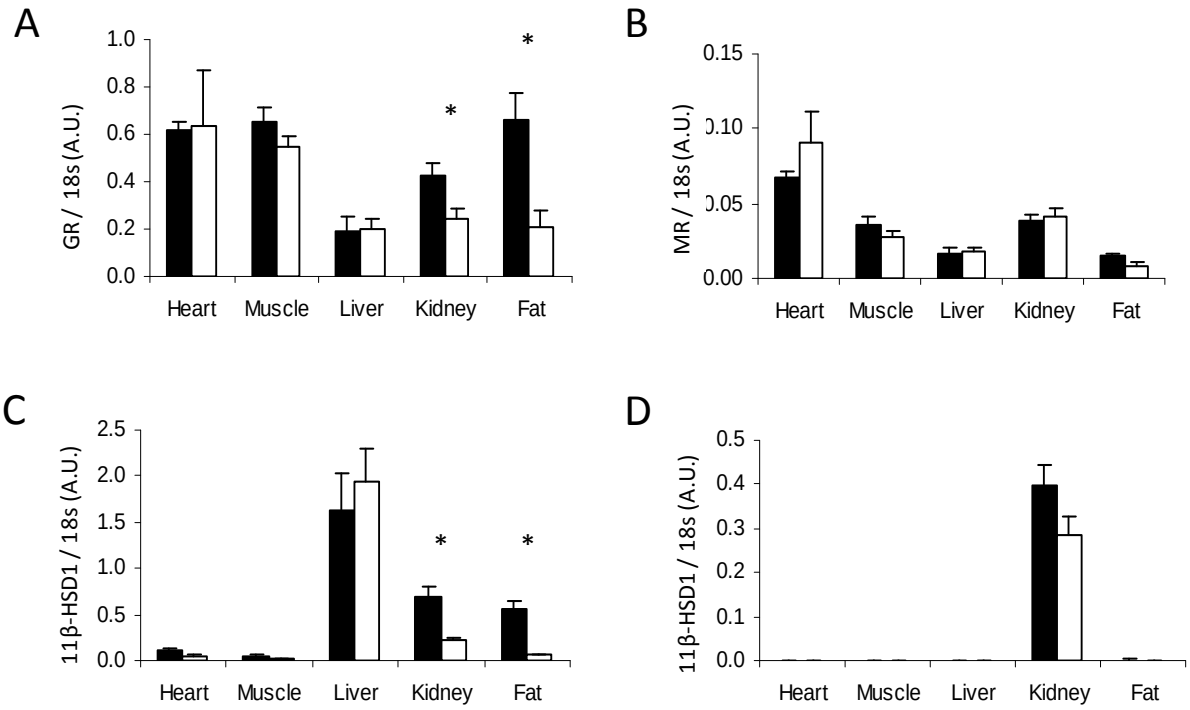

*Figure 3-3 Relative Expression levels of GR (**A**), MR (**B**), 11β-HSD type 1 (**C**) and 2 (**D**) in male (■, N=3) and female (□, N=3) C57BL/6 mice in heart, quadriceps muscle, liver, kidney, and gonadal fat normalised to 18s by quantitative PCR (data represents mean±SD; Gene expression levels between male and female mice were compared employing t-test; * p<0.05).*

Enzymatic activities of 11β-HSDs in CM were assessed by incubating CM with 11-dehydrocorticosterone (11-DHB) and corticosterone (B). In CM, 11β-HSD1 oxoreductase activity (conversion of 11-DHB to B) was readily detectable whereas dehydrogenase activity (conversion of B to 11-DHB) was at the lower limit of detection (Figure 3-4) consistent with the RT-PCR data which suggesting complete absence of 11β-HSD2 in CM (Figure 3-4).

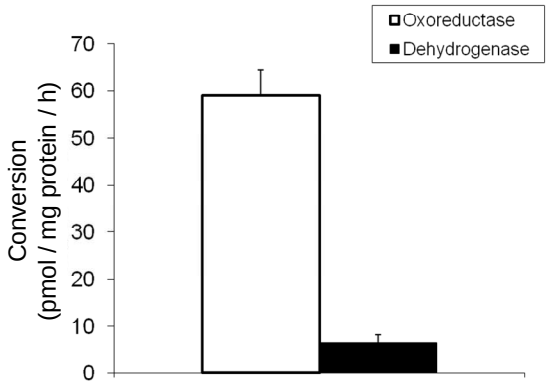

Figure 3-4 11β-HSD oxoreductase and dehydrogenase activity in isolated rat cardiomyocytes. Oxoreductase and dehydrogenase activity was determined by incubating cardiomyocytes with 11-dehydrocorticosterone and corticosterone, respectively. Enzymatic activities were calculated from the percentage conversion of each steroid. (data represents mean of 3 individual experiments ± SEM).

Kinetic properties of 11β-HSD1 oxoreductase activity were further characterised by incubating CM with increasing concentrations of 11-DHB (Figure 3-5a). Following Lineweaver-Burk transformation the apparent K_m of 11β-HSD1 in CM was calculated to be 415nM with a V_{max} of 216 pmol/10^6 cells/h (Figure 3-5b).

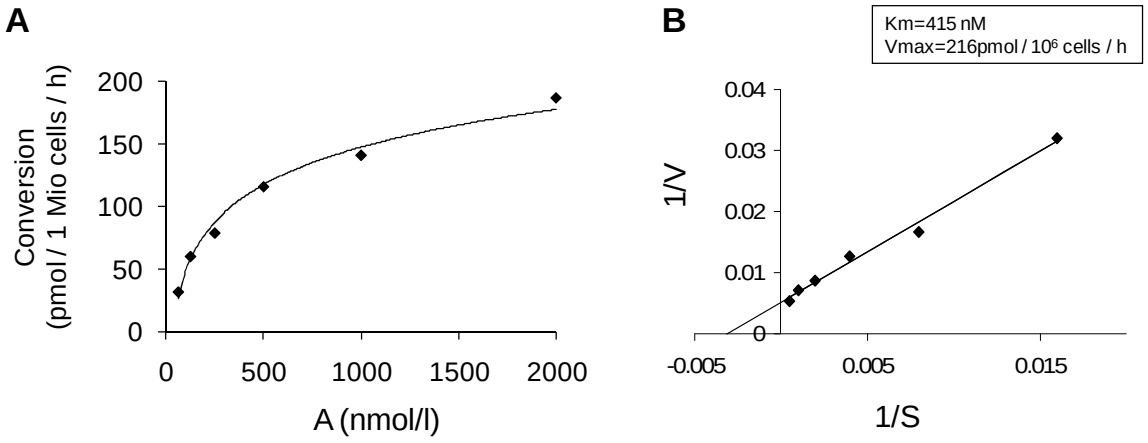

Figure 3-5 Enzymatic kinetics of oxoreductase activity of 11β-HSD1 in isolated rat CM. ***A*** *Concentration dependent conversion of 11-dehydrocorticosterone (11-DHB) to corticosterone (data represents mean of 3 individual experiments).* ***B*** *Lineweaver-Burk graph of 11β-HSD1 oxoreductase activity in cardiomyocytes normalised to 1x10^6 cells.*

Furthermore, we assessed 11β-HSD1 oxoreductase and dehydrogenase activity in cardiac explants from C57BL/6 (WT) and H6PDH knock out (H6PDH$^{-/-}$) mice. While WT mice predominantly exhibited oxoreductase and only little dehydrogenase activity, enzymatic activities in H6PDH$^{-/-}$ mice were reversed with a loss in oxoreductase and gain in dehydrogenase activity (Figure 3-6).

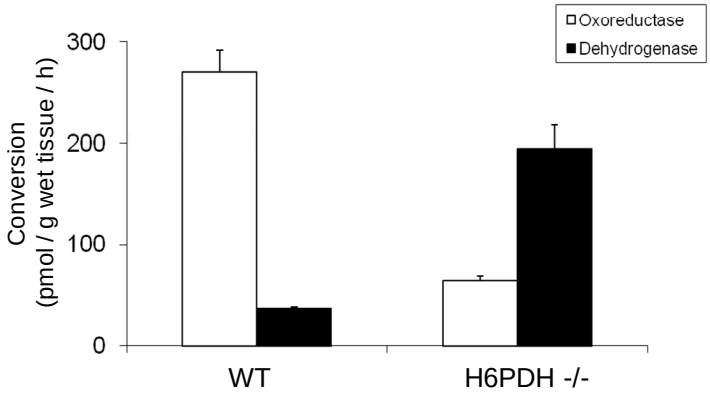

Figure 3-6 11β-HSD1 oxoreductase and dehydrogenase activities in heart explants from C57BL/6 WT and H6PDH$^{-/-}$ mice. Enzymatic activities were calculated from the percentage conversion of each steroid. (data represents mean of 3 individual experiments).

We next assessed the regulation of glucocorticoid target genes in cultured CM. Incubation of CM with 100nM DEX for 8h strongly induced expression of the angiotensin converting enzyme 1 (ACE1), whereas B and 11-DHB were less potent (Figure 3-7a). ACE1 induction by B and 11-DHB was dose dependent (Figure 3-7b and c) and was blunted by co-incubation with the GR antagonist RU486 (Figure 3-7b). ACE1 induction by 11-DHB was dependent on bioactive 11β-HSD1 as co-incubation with the 11β-HSD1 inhibitor GE blocked the response.

By contrast, expression of the vascular endothelial growth factor A (VEGFa) was repressed following an 8h incubation with 100nM DEX, B, and 11-DHB (Figure 3-8a). VEGFa repression by B and 11-DHB was dose dependent (Figure 3-8 b and c) and co-incubation of B with RU486 (Figure 3-8b), 11-DHB with GE but not B with GE blunted VEGFa repression (Figure 3-8c).

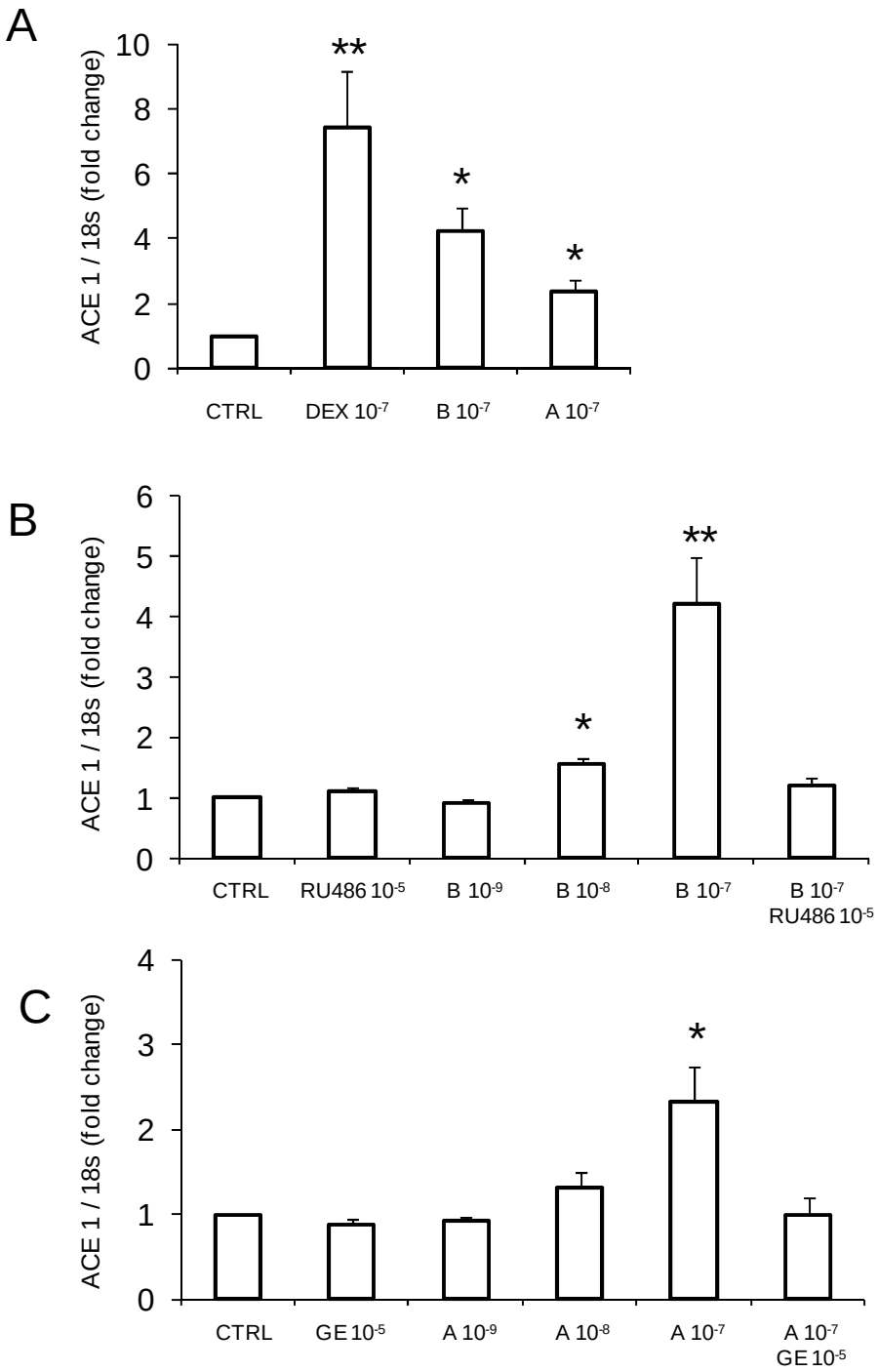

Figure 3-7 Induction of ACE1 gene expression by glucocorticoids in rat cardiomyocytes. ***A*** *ACE1 expression is up-regulated by dexamethasone (DEX), corticosterone (B) and 11-dehdrocorticosterone (A) (100 nM)* ***B*** *ACE1 expression is up-regulated by B in a concentration dependent manner (1-100 nM) ± RU486 (10μM)* ***C*** *ACE1 expression is up-regulated by B and 11-dehydrocorticosterone (A); Glycyrrhetinic acid (GE) (10μM) blunts induction bv 11-DHB but not B. (The data represents mean±SEM of 3 individual experiments each performed in duplicate; Treatment effects vs control (CTRL) were compared by one way ANOVA.* p<0.05; ** p<0.01).*

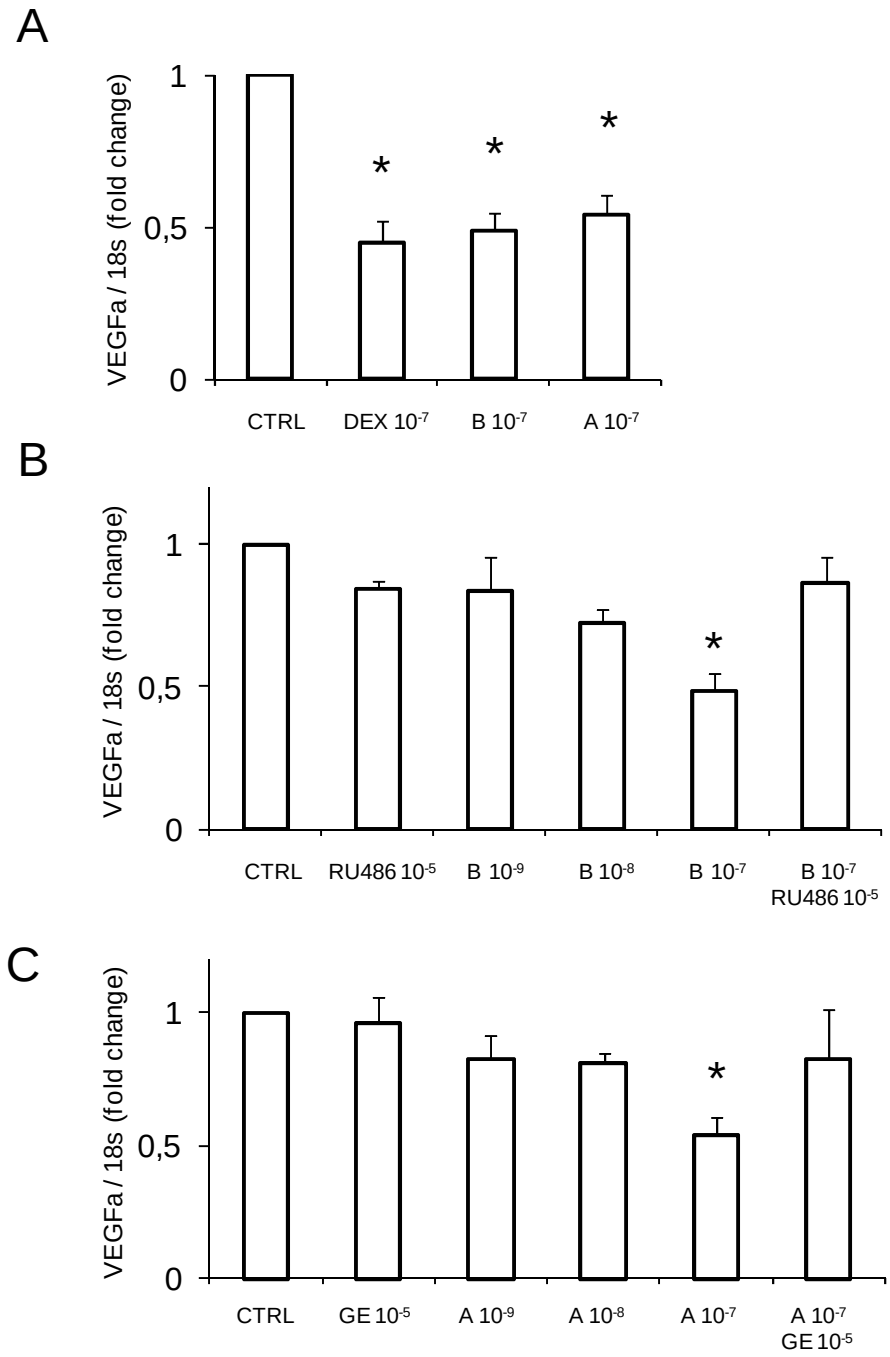

Figure 3-8 Repression of the VEGFa gene by glucocorticoids in rat cardiomyocytes. ***A*** *VEGFa is suppressed by dexamethasone (DEX), corticosterone (B) and 11-dehdrocorticosterone (A) (100nM)* ***B*** *VEGFa is repressed by B in a concentration dependent manner (1-100 nM); RU486 (10μM) blocked VEGFa induction by B.* ***C*** *VEGFa expression is repressed by 11-dehydrocorticosterone (A) and B; glycyrrhetinic acid (GE) (10μM) blunted the repression by 11-DHB but not B. (The data represents mean±SEM of 3 individual experiments each performed in duplicate; Treatment effects vs control (CTRL) were compared by one way ANOVA.* $p<0.05$; ** $p<0.01$).*

3.3.2 Characterisation of steroid hormone action in cardiac fibroblasts

We first assessed purity of primary cultures of rat cFb by assessing expression of cell specific markers (Figure 3-9). cFb readily expressed the fibroblast specific marker vimentin and α-smooth muscle actin (α-SMA), a marker which is expressed in activated fibroblasts suggesting that cFb had transformed into MyoFb. By contrast, the endothelial marker platelet/endothelial cell adhesion molecule 1 (PECAM-1) and atrial natriuretric factor (ANF) which are only expressed in endothelial cells and CM, respectively, were not expressed. These results suggest a pure colony of cardiac MyoFb with no evidence of contamination from endothelial cells or myocytes.

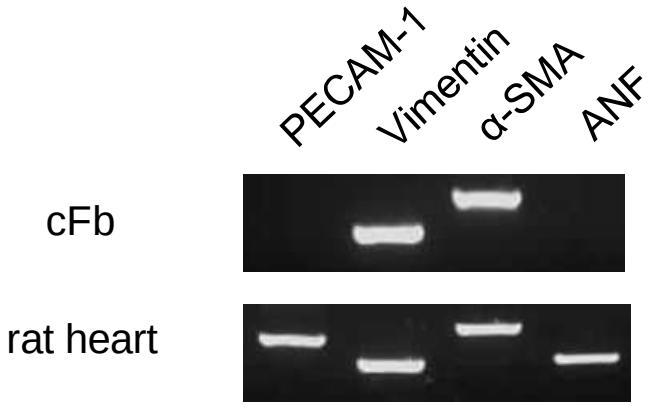

Figure 3-9 Characterisation of primary rat cardiac fibroblasts (cFb) in culture by conventional RT-PCR. cFb expressed the fibroblast marker vimentin and the myofibroblast marker α-smooth muscle actin (α-SMA), whereas the endothelial marker platelet/endothelial cell adhesion molecule 1 (PECAM-1) and the myocytes marker atrial natriuretric factor (ANF) were not expressed in cFb. Whole rat heart was used as a positive control.

We next assessed key components involved in steroid hormone signalling in cFb. We found both the GR and 11β-HSD1 to be highly expressed in cFb (Figure 3-10). The MR was also readily expressed albeit at lower levels compared to the GR. However, similar to CM (Figure 3-1), 11β-HSD2 was not expressed in cFb.

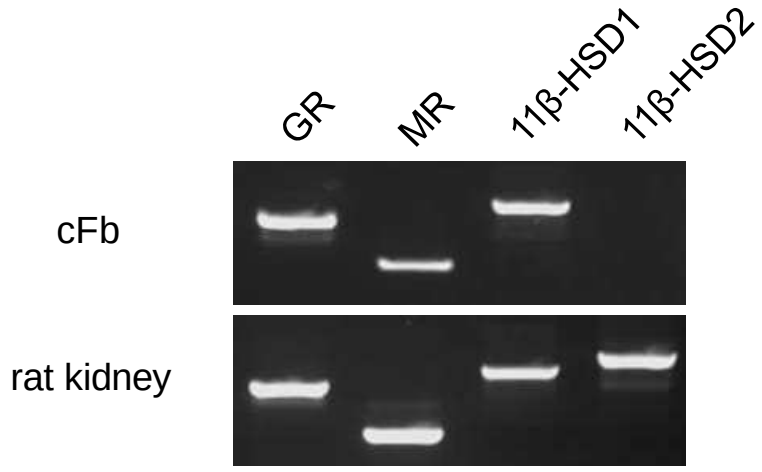

Figure 3-10 Expression of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type II (11β-HSD2) in cultured primary rat fibroblasts by conventional RT-PCR. Rat kidney served as a positive control.

We next assessed relative expression levels of 11β-HSD1 in CM and cFb in comparison to whole rat heart and rat liver by quantitative realtime PCR (qPCR). In agreement with the low expression levels of 11β-HSD1 in CM by conventional RT-PCR, qPCR showed a 10 fold lower expression level of 11β-HSD1 in CM cultures and whole heart compared to liver (Figure 3-11).

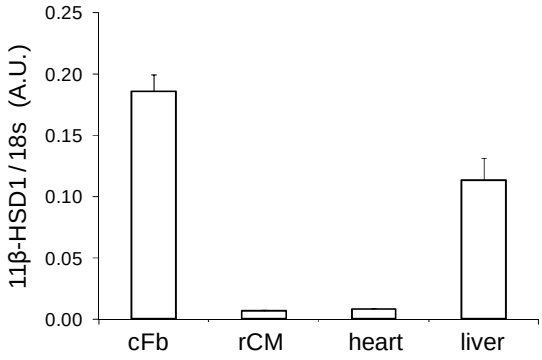

Figure 3-11 Relative expression levels of 11β-HSD1 in rat cardiomyocytes (rCM), cardiac fibroblasts (cFb), whole rat heart and rat liver by quantitative realtime PCR.

However by contrast, cFb showed high expression levels of 11β-HSD1 which was even higher than in liver.

We next studied functional activities of 11β-HSDs in cFb. Oxoreductase activity was high but only very little dehydrogenase activity was detectable (Figure 3-12), reflecting the high expression of 11β-HSD1 and absence of 11β-HSD2 observed in the conventional RT-PCR (Figure 3-10).

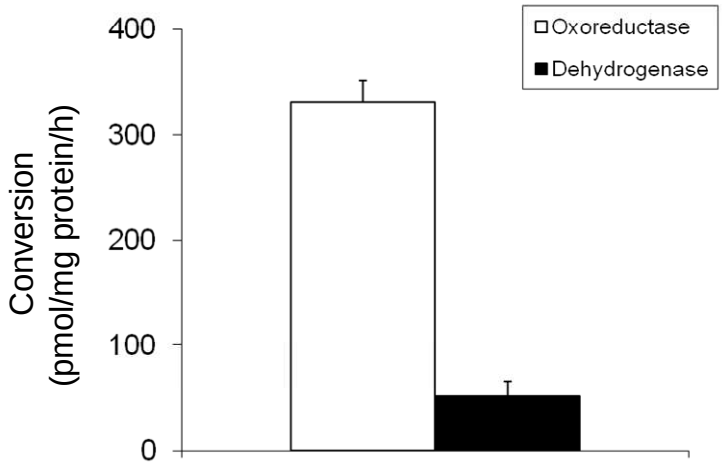

Figure 3-12 11β-HSD oxoreductase and dehydrogenase activities in cultured rat cardiac fibroblasts (cFb). Oxoreductase and dehydrogenase activities were determined by incubating cFb with 11-dehydrocorticosterone and corticosterone, respectively. Enzymatic activities were calculated from the percentage conversion of each steroid (data represents mean of 3 individual experiments).

Glucocorticoids are a known stimulator of 11β-HSD expression resulting in increased oxoreductase activity. Incubation of cFb with DEX resulted in a dose dependent induction of 11β-HSD expression (Figure 3-13a) and oxoreductase activity (Figure 3-13b) which was completely blunted by co-administration of the specific GR antagonist RU486.

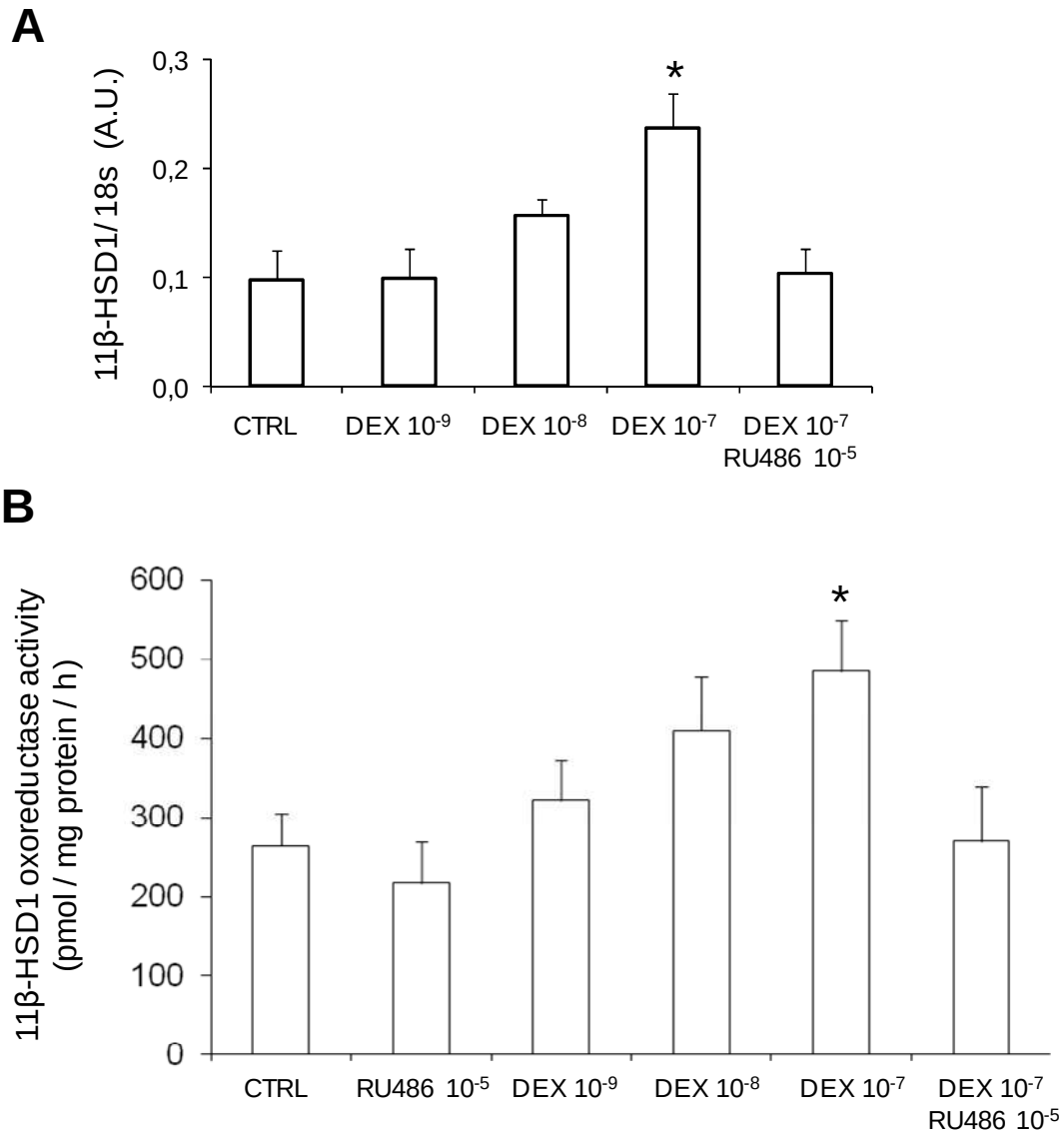

*Figure 3-13 Induction of 11β-HSD by glucocorticoids. Dexamethasone (DEX) dose dependently (1-100nM) induces 11β-HSD1 expression (**A**) and 11β-HSD oxoreductase activity (**B**) in cardiac fibroblasts. Treatment effects vs control (CTRL) were compared by one way ANOVA;* p<0.05.*

We next assessed the effects of glucocorticoids on cFb proliferation. Glucocorticoids repressed cFb proliferation (Figure 3-14a). DEX showed the strongest effects on proliferation, whereas corticosterone (B) and 11-dehydrocorticosterone (A) showed similar but less potent effects. As exemplified by DEX, repression of cardiac proliferation by glucocorticoids was dose dependent and was completely blocked by

co-administration of RU486 (Figure 3-14b). We next assessed the involvement of 11β-HSD in mediating the repressive effects of ‘B’ and ‘A’ by co-administration of the 11β-HSD inhibitor GE (Figure 3-14c). GE by itself had no effect on cFb proliferation (Figure 3-14c) whereas GE in the presence of ‘A’ but not ‘B’ blunted the repressive effects of glucocorticoids on proliferation suggesting that a functionally active 11β-HSD1 enzyme is required for repression of cFb proliferation by the biologically inactive glucocorticoid 11-dehydrocorticosterone.

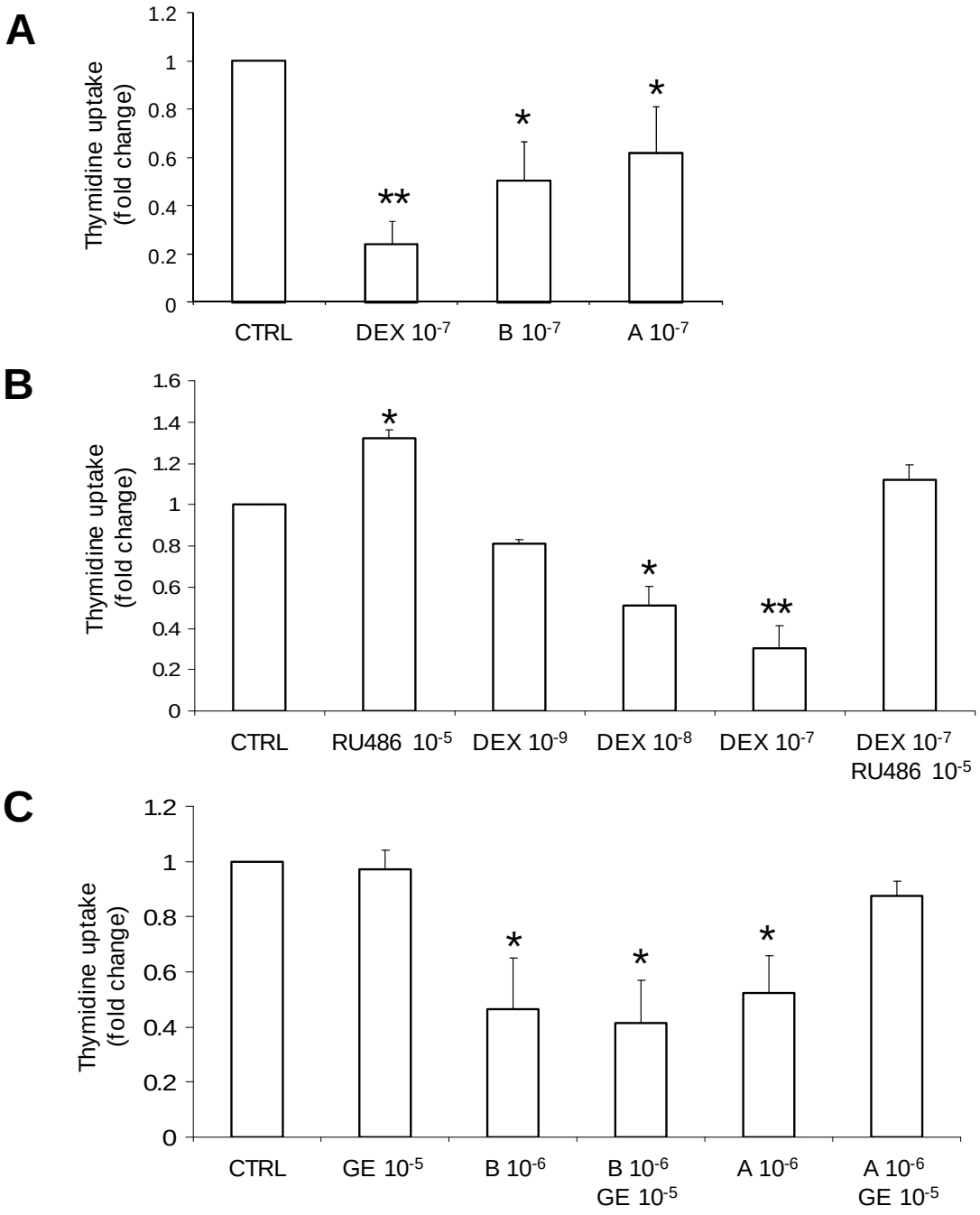

*Figure 3-14 Glucocorticoids repress proliferation of cardiac fibroblasts (cFb). Proliferation was measured by incorporation of labeled thymidine. **A** Treatment of cFb for 24h with 100nM dexamethasone (DEX), corticosterone (B) and 11-dehydrocorticosterone (A) reduces thymidine uptake. **B** DEX dose dependently (1-100nM) reduces thymidine uptake in cFb. **C** Glycyrrhetinic acid (GE) blunts the effects of A but not B. Treatment effects vs control (CTRL) were compared by one way ANOVA; * p<0.05; ** p<0.01.*

3.3.3 Expression studies in human left ventricular biopsies

We also characterised expression of key components involved in steroid hormone signalling in human tissues including skeletal muscle, heart and liver. Expression levels of the GR in human left ventricular biopsies from transplant donors was similar to liver but about four fold lower compared to skeletal muscle (Figure 3-15a). By contrast, MR expression levels were similar in heart and skeletal muscle and significantly higher compared to liver (Figure 3-15b). Expression levels of 11β-HSD1 were found to be 100 fold higher in human liver compared to both heart and skeletal muscle (Figure 3-15c).

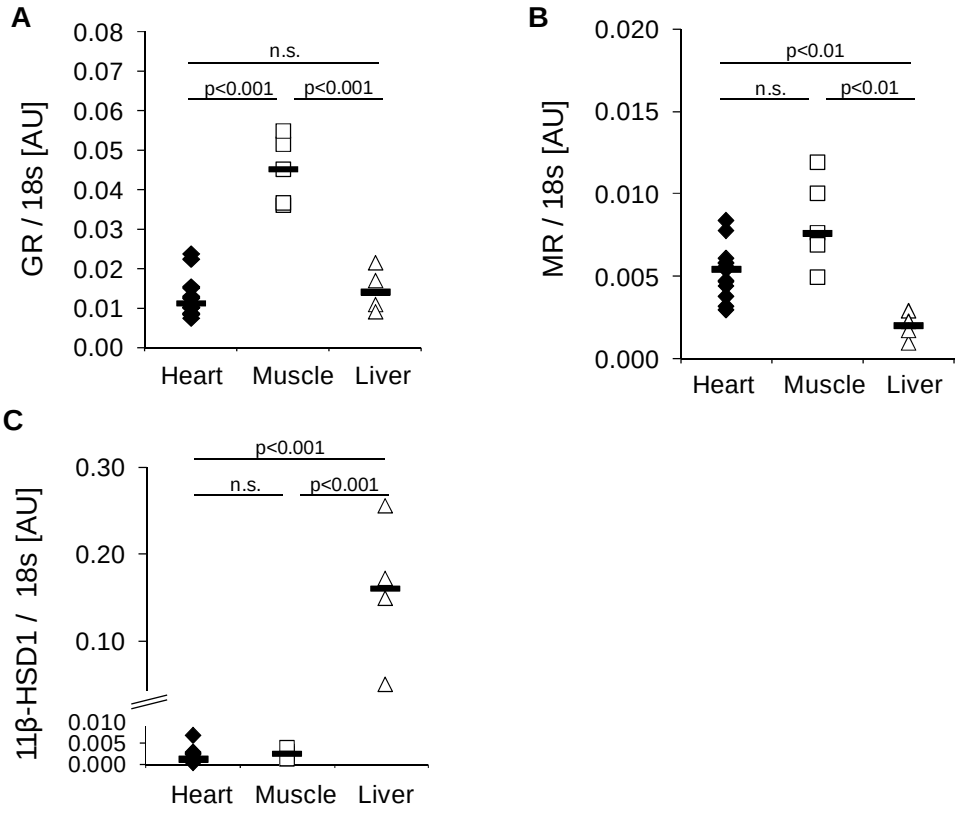

*Figure 3-15 Relative expression levels of the glucocorticoid receptor (GR) (**A**), the mineralocorticoid receptor (MR) (**B**) and 11β-HSD1 (**C**) in left ventricular biopsies of transplant donor hearts (N=8), human quadriceps muscle (N=5) and liver (N=4) biopsies by quantitative realtime PCR. Tissue specific expression levels were compared by Kruskal-Wallis ANOVA.*

We next assessed expression of GR, MR and 11β-HSD1 in cardiac hypertrophy. GR expression levels were about 50% higher in left ventricular biopsies from patients with marked hypertrophy (Figure 3-16a), whereas expression levels for MR were similar (Figure 3-16b). 11β-HSD1 showed a trend of decreased expression in hypertrophy compared to transplant donors but this was not statistically significant (Figure 3-16c).

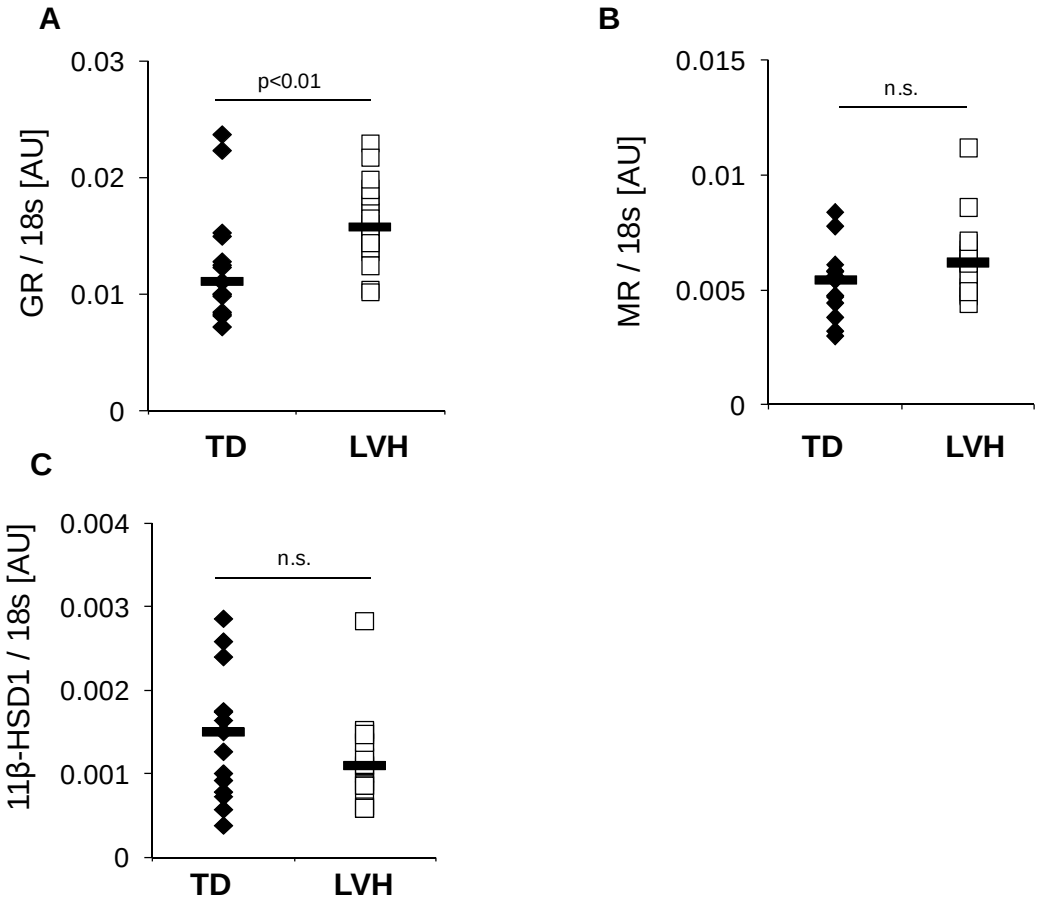

*Figure 3-16 Relative expression levels of the glucocorticoid receptor (GR) (**A**), the mineralocorticoid receptor (MR) (**B**) and 11β-HSD1 (**B**) in left ventricular biopsies of transplant donor hearts (TD) (N=15) and hypertrophied hearts (N=19) by quantitative realtime PCR. Gene expression levels between TD and LVH patients were compared by the Mann-Whitney-U test.*

Additionally, we assessed expression levels of collagen 1 and 3 and angiotensin converting enzyme 1 in left ventricular biopsies of patients with cardiac hypertrophy (LVH) with those non hypertrophied hearts from transplant donors (TD). Expression levels for collagen 1 and 3 and ACE-1 were significantly higher in LVH compared to TD hearts (Figure 3-17).

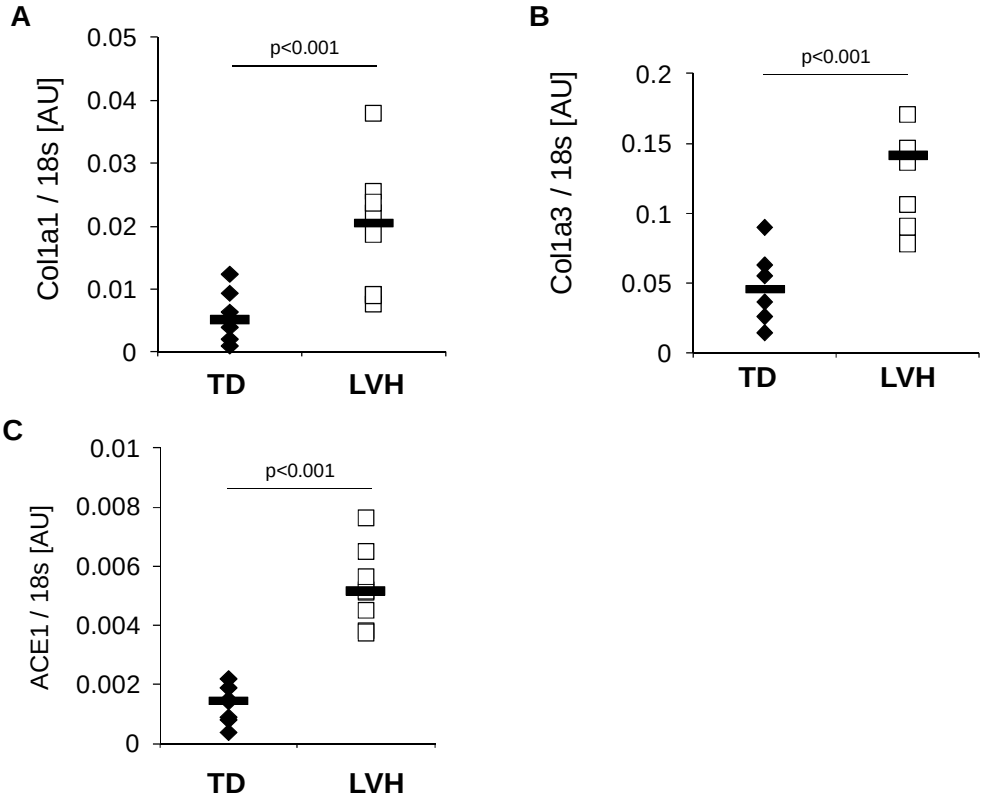

*Figure 3-17 Relative expression levels of the collagen I (Col1a1) (**A**), collagen IX (Col1a9) (**B**) and angiotensin converting enzyme 1 (ACE-1) (**C**) in left ventricular biopsies of transplant donor (TD) hearts (8) (N=8) and hypertrophied (LVH) hearts (N=7) by quantitative realtime PCR. Gene expression levels between TD and LVH patients were compared by the Mann-Whitney-U test.*

Given that ACE1 and GR were significantly up-regulated in LVH compared to TD and glucocorticoids up-regulated the ACE-1 gene in CM, we analysed if GR expression levels were associated with those of the ACE-1 gene. Correlation of ACE-1 and GR

in left ventricular biopsies from LVH samples showed a significant positive association (Figure 3-18) suggesting that glucocorticoid signalling via GR may contribute to cardiac hypertrophy via induction of the ACE-1 gene in humans.

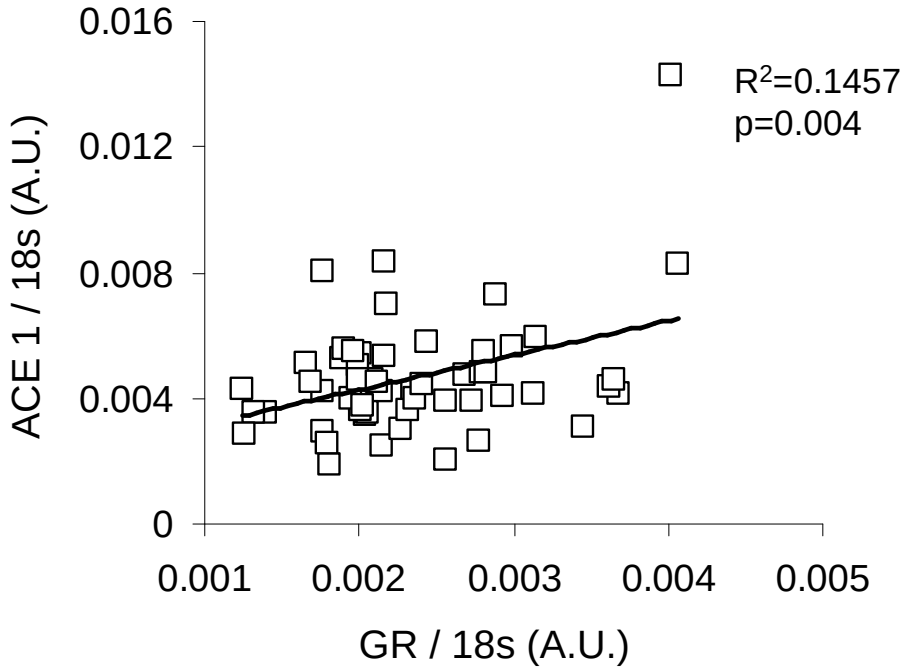

Figure 3-18 Correlations of angiotensin converting enzyme 1 (ACE1) and the glucocorticoid receptor (GR) in left ventricular biopsies of hypertrophied hearts (N=52) by quantitative realtime PCR.

3.4 Discussion

In this chapter we have characterised the expression and function of key components involved in glucocorticoid hormone action and glucocorticoid modulation by 11β-HSD1 in isolated adult rat CM and cardiac MyoFb.

To date, corticosteroid signalling has only been assessed in neonatal CM which are structurally, morphologically and functionally different from mature cells which are terminally differentiated cells and no longer have the ability to divide. Our study is the

first to address expression of key components involved in corticosteroid signaling and enzymatic activities of 11β-HSDs in isolated post-mitotic adult rat CM. In agreement with the findings by Sheppard *et al* from nrCM (Sheppard & Autelitano, 2002), we found expression of MR, GR and 11β-HSD type 1 but not type 2 in isolated rat CM. The absence of 11β-HSD2 suggests that the MR in CM, in contrast to classic Aldo target tissues, is not protected from glucocorticoids and is therefore a target for both mineralocorticoids and glucocorticoids given its similar affinities for both steroids. Relative expression levels of 11β-HSD1 were considerably lower in rodent hearts and isolated rat CM compared to liver tissue. However, despite its relatively low level of expression, 11β-HSD1 oxoreductase activity was readily present in isolated CM and murine cardiac explants. By contrast and consistent with the absence of 11β-HSD2 mRNA expression, 11β-HSD dehydrogenase activity was very low.

In addition to CM, we also assessed corticosteroid hormone action in cFb. Characterisation of primary cultures of cFb showed a pure cell population with no evidence for contamination with CM or endothelial cells. We found high expression levels of the MyoFb marker α-smooth muscle actin (α-SMA) suggesting that cFb had spontaneously differentiated into MyoFb in culture. MyoFb are the key cell type involved in cardiac remodeling and are the principal cells secreting ECM proteins including collagen I and III to stabilise the healing wound and form the infarct scar (described in section 1.8.4.2). Transformation of cFb into MyoFb in culture can be achieved by treatment with TGF-β1 (Leask, 2007) or, as previously reported by seeding fibroblasts at low density which results in spontaneous differentiation into MyoFb (Masur et al., 1996). Although activated MyoFb as opposed to resting cFb are key in the formation of the ECM in heart disease (Porter & Turner, 2009), so far steroid hormone action in MyoFb has not been characterised.

Cultured MyoFb readily expressed the GR and MR albeit at lower levels compared to the GR. Furthermore, MyoFb showed high expression levels of 11β-HSD1 whereas 11β-HSD2 was absent. Comparison of 11β-HSD1 expression showed that its levels in MyoFb were much higher than in CM and even exceeded those of liver tissue which is known to highly express 11β-HSD1 (Tannin et al., 1991). Enzymatic studies reflected expression data and showed high oxoreductase but negligible dehydrogenase activity in keeping with previous reports investigating fibroblasts from other sources (Hardy et al., 2006; Onyimba et al., 2006).

In human left ventricular biopsies, the relative expression patterns of the GR in relation to skeletal muscle and liver was different in man compared to mice. GR expression in human hearts was similar to liver tissue but skeletal muscle showed a 4 fold higher expression level, whereas GR expression in mice was similar in the heart and skeletal muscle and approximately 3 fold higher compared to liver. With regard to MR, we found a similar pattern of expression in mice and man, while the relative abundance of 11β-HSD1 in heart compared to liver was much less in man (1-2%) compared to mice (10%). These expression studies suggest and confirm that there are no fundamental but relative differences in the expression level of GR, MR and 11β-HSD1 between mice and man.

11β-HSD1 is a bidirectional enzyme and its oxoreductase activity critically depends on the presence of the co-factor NADPH. H6PDH is an enzyme expressed in the endoplasmic reticulum which regenerates NADPH from $NADP^+$ by converting glucose 6-phosphate to 6-phosphogluconolactonate. We have recently shown that the enzymatic activity of 11β-HSD1 in mice with a targeted deletion of the H6PDH changes from an oxoreductase to a dehydrogenase in liver microsomes (Lavery et al., 2006). Here we show that cardiac explants from $H6PDH^{-/-}$ mice exhibit a similar change, namely reversal of oxoreductase to dehydrogenase activity. Interestingly and

similar to mice with a deletion of the HSD11B1 gene (Kotelevtsev et al., 1997), H6PDH$^{-/-}$ mice do not exhibit any obvious structural or functional cardiac changes at rest (Lavery et al., 2006) but the role of 11β-HSD1 in the pathogenesis and modulation of heart disease so far has not been fully elucidated. A previous report suggests that 11β-HSD1 may play a modulatory role in ischaemic heart disease. In this animal model of MI, mice with a targeted deletion of the 11β-HSD1 gene (11β-HSD1$^{-/-}$ mice) exhibited increased neovascularisation and improved cardiac function compared to WT mice (McSweeney et al., 2010; Small et al., 2005). Similarly, administration of the GR antagonist RU486 in this model also augmented angiogenesis following MI suggesting that not loss of 11β-HSD1 per se but reduced glucocorticoid levels, caused increased angiogenesis in this model (Small et al., 2005).

A number of studies have linked glucocorticoid hormone action with cardiac hypertrophy. In Cushing's syndrome, a condition characterised by glucocorticoid excess, a high percentage of patients show left ventricular hypertrophy which however, in part may be secondary to concomitant high blood pressure (Muiesan et al., 2003; Muiesan et al., 2008). In neonatal rats (de Vries et al., 2002; La Mear et al., 1997) and fetal sheep (Lumbers et al., 2005) glucocorticoids have been shown to induce cardiac hypertrophy. These findings are supported by *in vitro* studies, which show that DEX treatment of isolated nrCM increases markers of CM hypertrophy including protein content per cell and cell size (Whitehurst et al., 1999). This response is dependent on L-type Ca2+ channels as co-administration of the L-type specific antagonist nifedipine was able to block the effect. Lister and colleagues investigated expression and function of components involved in corticosteroid signalling in a model of pharmacologically induced hypertrophy in nrCM *in vitro* (Lister et al., 2006). Both phenylephrine and the protein kinase C activator PMA

induced expression of the atrial natriuretic peptide (ANP), a marker gene of hypertrophy. Similarly, expression levels of GR and MR increased by 2 fold following both treatments, whereas expression and activity of 11β-HSD1 was only induced following PMA but not phenylephrine treatment of CM (Lister et al., 2006). Ang II which is generated by ACE from Ang I, is critically involved in various aspects of cardiac remodelling in heart disease (Sun, 2009). In keeping with a modulatory role of local ACE1 expression in cardiac hypertrophy, ACE1 expression has been shown to be increased in experimental cardiac hypertrophy (Schunkert et al., 1990). Furthermore, overexpression of ACE1 in CM augments hypertrophy induced by pressure overload suggesting that up-regulation of ACE1 plays a causative rather than consequential role in the pathogenesis of cardiac hypertrophy (Tian et al., 2004). Expression of ACE1 so far has not been assessed in cardiac hypertrophy in humans. For the first time, we found the ACE1 gene to be up-regulated in left ventricular biopsies from patients with cardiac hypertrophy secondary to aortic stenosis compared to normal hearts from transplant donors in keeping with the experimental data.

ACE1 expression is modulated by corticosteroids. In nrCM it was previously shown that Aldo up-regulates ACE1 expression (Harada et al., 2001) and that glucocorticoids augment ACE activity (Barreto-Chaves et al., 2000). Our data confirm these previous findings and show dose dependent up-regulation of the ACE1 gene via GR in CM.

Local glucocorticoid generation in CM can be modulated by 11β-HSD1 by activating 11-dehydrocorticosterone to corticosterone thereby facilitating glucocorticoid hormone action. Mazancova *et al* have addressed 11β-HSD1 expression and activity in hearts from various rat strains including spontaneous hypertensive rats (SHR), WKY, Dahl salt-sensitive (DS) and Dahl salt-resistant (DR) rats (Mazancova et al.,

2005). The main finding of their study was that expression levels and activity of 11β-HSD1 considerably varied in different rat strains but did not change when DS rats were fed a high salt diet which induced high blood pressure, and as a consequence cardiac hypertrophy, suggesting that local glucocorticoid generation by 11β-HSD1 does not contribute to cardiac hypertrophy. This notion is supported by our findings in human hearts which did not show a significant change in 11β-HSD1 expression in hypertrophied compared to transplant donor hearts. Therefore similar to rodents, 11β-HSD1 does not seem to play a major role in cardiac hypertrophy in humans.

However, a pathophysiological role for glucocorticoid hormone action in the regulation of the ACE1 gene in cardiac hypertrophy is endorsed by our findings in human left ventricular heart biopsies. Compared to transplant donors, patients with left ventricular hypertrophy showed increased GR and ACE1 expression underlining the implication of glucocorticoid hormone action and local Ang II production, respectively, in the pathophysiology of cardiac hypertrophy. The potential regulatory role of glucocorticoid action in the regulation of ACE1 is further underlined by the positive correlation of GR and ACE1 expression in hypertrophied hearts. However, a definitive answer to the involvement of GR mediated glucocorticoid hormone action in cardiac hypertrophy can only be expected from CM specific GR knock out mice which at the present time have not been reported.

In addition we assessed the effects of glucocorticoids on the expression of VEGFa which has previously been shown to be down-regulated by glucocorticoids (Iwai et al., 2004; Machein et al., 1999). In CM, glucocorticoids repressed VEGFa expression via the GR which in case of 11-dehydrocorticosterone was dependent on 11β-HSD1 bioactivity. It is therefore tempting to speculate that augmented angiogenesis in 11β-HSD1$^{-/-}$ mice following MI (Small et al., 2005) might at least in part be explained by

an increased expression of the VEGFa gene as a result of reduced local glucocorticoid levels.

11β-HSD1 expression and activity has previously been found to be up-regulated by glucocorticoids suggesting a potential feed-forward mechanism of action (Sai et al., 2008; Sun & Myatt, 2003; Yang et al., 2009). In agreement with these studies we found a dose dependent up-regulation of 11β-HSD1 expression and activity mediated via the GR in MyoFb. Moreover, we assessed the effects of glucocorticoids and 11β-HSD1 on MyoFb proliferation. Previous reports suggested that glucocorticoids and mineralocorticoids have opposing roles on fibroblast proliferation. While the effects of Aldo on fibroblast proliferation have been conflicting (Rombouts et al., 2001; Stockand & Meszaros, 2003), glucocorticoids have been shown to be potent inhibitors of fibroblasts proliferation (Durant et al., 1986) but so far this has not been assessed in cardiac MyoFb. We here show that glucocorticoids suppress MyoFb proliferation in a dose dependant manner. This effect is mediated via the GR and in the case of 11-dehydrocorticosterone is dependent on biologically active 11β-HSD1. Given the high expression levels of 11β-HSD1 in MyoFb we conclude that 11β-HSD1 may be an important autocrine mechanism to facilitate and modulate glucocorticoid hormone action in MyoFb and thereby repress MyoFb proliferation. McSweeney and colleagues recently reported that 11β-HSD1 knock out mice following MI had thicker infarct scars and improved cardiac function compared to WT animals (McSweeney et al., 2010), which may be explained by increased proliferation of MyoFb in 11β-HSD1$^{-/-}$ mice resulting from reduced cellular glucocorticoid levels. By contrast, systemic administration of glucocorticoids appears to delay healing in animal models of MI (El-Helou et al., 2008; Shizukuda et al., 1991). In keeping with these findings, glucocorticoid treatment of patients with MI in the early post infarct period has been associated with an increased risk of rupture of the left ventricular free wall and thus,

systemic glucocorticoid treatment following MI should be avoided (Bulkley & Roberts, 1974; Sholter & Armstrong, 2000). Given the high activity of 11β-HSD1 in MyoFb treatment with specific 11β-HSD1 inhibitors may provide a novel therapeutic way to improve cardiac remodelling following MI. These drugs are currently tested in patients with diabetes (Ge et al.) and the metabolic syndrome (Stewart & Tomlinson, 2009) but may also prove beneficial in cardiovascular disease such as ischaemic heart disease (Hadoke et al., 2009).

In summary, adult rat CM and MyoFb readily express corticosteroid hormone receptors and 11β-HSD type 1, whereas 11β-HSD type 2 is not expressed. Consistent with this expression pattern, CM and MyoFb almost exclusively exhibit 11β-HSD oxoreductase but not dehydrogenase activity. Similar to other tissues, we found that 11β-HSD type 1 can facilitate glucocorticoid hormone action and thereby modulate target gene expression in CM and MyoFb and impact on proliferation of cFb. However, to date the modulatory role of 11β-HSD1 in the pathophysiology of heart disease and the potential therapeutic implications of 11β-HSD1 inhibitors in cardiovascular disease remain unclear. Importantly, complete absence of 11β-HSD type 2 in CM and fibroblasts leaves the MR unprotected from glucocorticoid binding and action raising the question of specificity. In an attempt to identify differentially regulated genes which may give further insights into corticosteroid hormone action in these cells we have performed a microarray screening experiment in CM (see Chapter 4).

Chapter 4 - Identification of Differentially Regulated Genes by Gluco- and Mineralocorticoids in Cardiomyocytes

4.1 Introduction

Both glucocorticoid and mineralocorticoid excess confers cardiovascular injury but the differential effects of corticosteroids via their cognate receptors and the downstream effects on target gene regulation remain largely unknown. It is clear from experimental and clinical studies that only blockade of the MR (Brilla et al., 1993; Pitt et al., 2003a; Pitt et al., 1999) but not GR (Rickard et al., 2006; Rickard et al., 2007) is cardioprotective, suggesting that the MR plays a pivotal role in mediating adverse steroid effects. The MR exhibits similar affinities for Aldo and corticosterone (Edwards et al., 1988; Funder et al., 1988), which is explained by the high homology of the receptors (described in section 1.4.1) and structural similarities of corticosteroids (described in section 1.3.2). In classical mineralocorticoid target tissues, MR specificity for Aldo is ensured at a pre-receptor level by the enzyme 11β-HSD2 which selectively inactivates corticosterone to 11-dehydrocorticosterone (described in section 1.7.2.2) leaving Aldo the principal MR ligand in classical MR target tissues. In non-classical tissues which do not express 11β-HSD2, however, both Aldo and corticosterone must be considered MR ligands which raises the question of ligand specificity. The ability of Aldo to induce cardiac fibrosis in animal models (described in section 1.9.2.1) and cardiac hypertrophy in primary hyperaldosteronism (described in section 1.9.1.1.1) independently of blood pressure, suggests that Aldo has direct cardiac effects, whereas the role for glucocorticoids is less clear. In an animal model of cardiac fibrosis, Young and colleagues recently reported overlapping effects of mineralocorticoids and glucocorticoids via MR (Young et al.). Molnar and colleagues studied the downstream effects of corticosterone via MR in vascular smooth muscle cells (VSMCs) which in contrast to CM express 11β-HSD2. Nevertheless, corticosterone even at very low concentrations induced rapid

MR signalling involving the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase-dependent pathway (Molnar et al., 2008). Mihailidou and colleagues recently studied the effects of corticosteroids in an *ex vivo* model of MI and found that cardiac damage is similarly aggravated by Aldo and cortisol via activation of MR (Mihailidou et al., 2009). Although these studies provide evidence that glucocorticoids may indeed induce similar effects via MR, the significance of these findings in the pathophysiology of heart diseases remains unclear. In contrast to these findings, a transgenic mouse model over-expressing 11β-HSD2 which results in artificial glucocorticoid inactivation at the pre-receptor level suggests that glucocorticoids may be cardioprotective (Qin et al., 2003). These mice develop severe heart failure and cardiac fibrosis and as a consequence die at a young age suggesting that loss of tonic MR blockade by glucocorticoids facilitates MR activation by Aldo which in turn triggers the detrimental phenotype observed in these mice. On aggregate, these data suggest, that signalling via MR is detrimental because blockade of the MR attenuates these effects whereas signalling via GR appears to be of minor importance. However, the role of glucocorticoid binding to the MR remains unclear.

In an attempt to discover selective Aldo target genes, three studies employing whole genome microarrays and one study using a serial analysis of gene expression (SAGE) library have been conducted. Turchin *et al*, injected mice with a bolus of Aldo and gene expression in the heart was investigated at various time points (0.5, 1, 2, 3, 4, 5 and 12h) to study temporal expression patterns of Aldo regulated target genes (Turchin et al., 2006). As expected, the authors observed a rapid rise of plasma Aldo levels following the injection but plasma levels of corticosterone exhibited a similar stress induced pattern as the animals were not adrenalectomised prior to Aldo injection, questioning the specificity of their findings. Fejes-Toth *et al*, chose a

different approach and investigated early changes in the transcriptome of H9C2 cells, which is a myoblast cell line derived from embryonic rat heart, after 2h in response to a physiological concentration of Aldo (1nM) (Fejes-Toth & Naray-Fejes-Toth, 2007). In this study 12 known genes and three expressed sequence tags (ESTs) were identified by microarray analysis to be regulated by Aldo of which 8 (tenascin-X, ADAMTS1, PAI-1, UPAR, hyaluronic acid synthase-2, RGS2, adrenomedullin and orosomucoid) were confirmed by subsequent RT-PCR studies. However, the physiological relevance of these findings must be questioned; H9C2 cells may be an artificial model as they have lost expression of the MR which had to be stably expressed in these cells prior to these studies. Finally, Muller *et al* studied the effects of high doses of Aldo (1μM) on the transcriptome nrCM employing a SAGE library (Muller et al., 2007). In this study, 101 genes were found to be regulated by Aldo after 4h. In their subsequent analysis, they focused on only one Aldo regulated gene, ecto-ADPribosyltransferase-3 (Art3), and showed by using neonatal CM isolated from MR and GR knock out mice that Art3 was solely regulated by Aldo via the GR but not the MR. The fact that the authors identified Art3 as an Aldo target gene being regulated via GR is not surprising as Aldo at very high concentrations is known to also bind and activate the GR. Therefore the implications of this study with regard to identification of Aldo target genes induced via the MR appear to be flawed by the experimental design. Thus, these approaches to elucidate novel Aldo regulated genes appear to be limited by methodological problems and hence, the relevance of these results must be questioned. Yoshikawa and colleagues studied differential gene regulation in nrCM following a three hour treatment of the highly selective GR agonist, cortivazol, as well as corticosterone and Aldo to define specificity of glucocorticoid hormone action via GR in CM (Yoshikawa et al., 2009). A high degree of overlap of the three steroids in gene regulation was found for up-regulated, but not

for down-regulated genes. Perhaps, not surprisingly, the authors found that glucocorticoids in comparison to Aldo were the major determinants of GR-mediated gene expression in this study. Unfortunately, subsequent analysis of microarray results focused on the specific effects of glucocorticoids via GR whereas regulation of Aldo target genes via MR was not further explored.

In summary, these studies have not provided conclusive answers regarding differential downstream signalling of glucocorticoids as opposed to mineralocorticoids in CM. In an attempt to do so, we studied gene regulation in adult rat CM using the steroids Aldo, corticosterone and DEX and studied differential gene regulation in CM in the presence or absence of the selective GR and MR antagonists RU486 and RU28318, respectively.

4.2 Materials and Methods

4.2.1 Experimental set up

The experimental set up to study differentially regulated genes is shown in Figure 4-1. Untreated cells (panel A) and the specific GR (panel B) and MR (panel C) antagonist served as controls. To study genes that are regulated via GR the GR agonist DEX was chosen (panel D) and GR specificity was ensured by co-treatment of DEX with the GR antagonist RU486 (panel E). To study gene regulation via MR, the MR agonist Aldo was chosen (panel F) and specificity was ensured by co-treatment of Aldo with the MR antagonist RU28318 (panel G). In order to study differential effects of corticosterone (B) via GR and MR, CM were treated with corticosterone only (panel H), with corticosterone in the presence RU486 to identify genes regulated by corticosterone via MR (panel I) or corticosterone in the presence of RU28318 to identify genes regulated by corticosterone via GR (panel J).

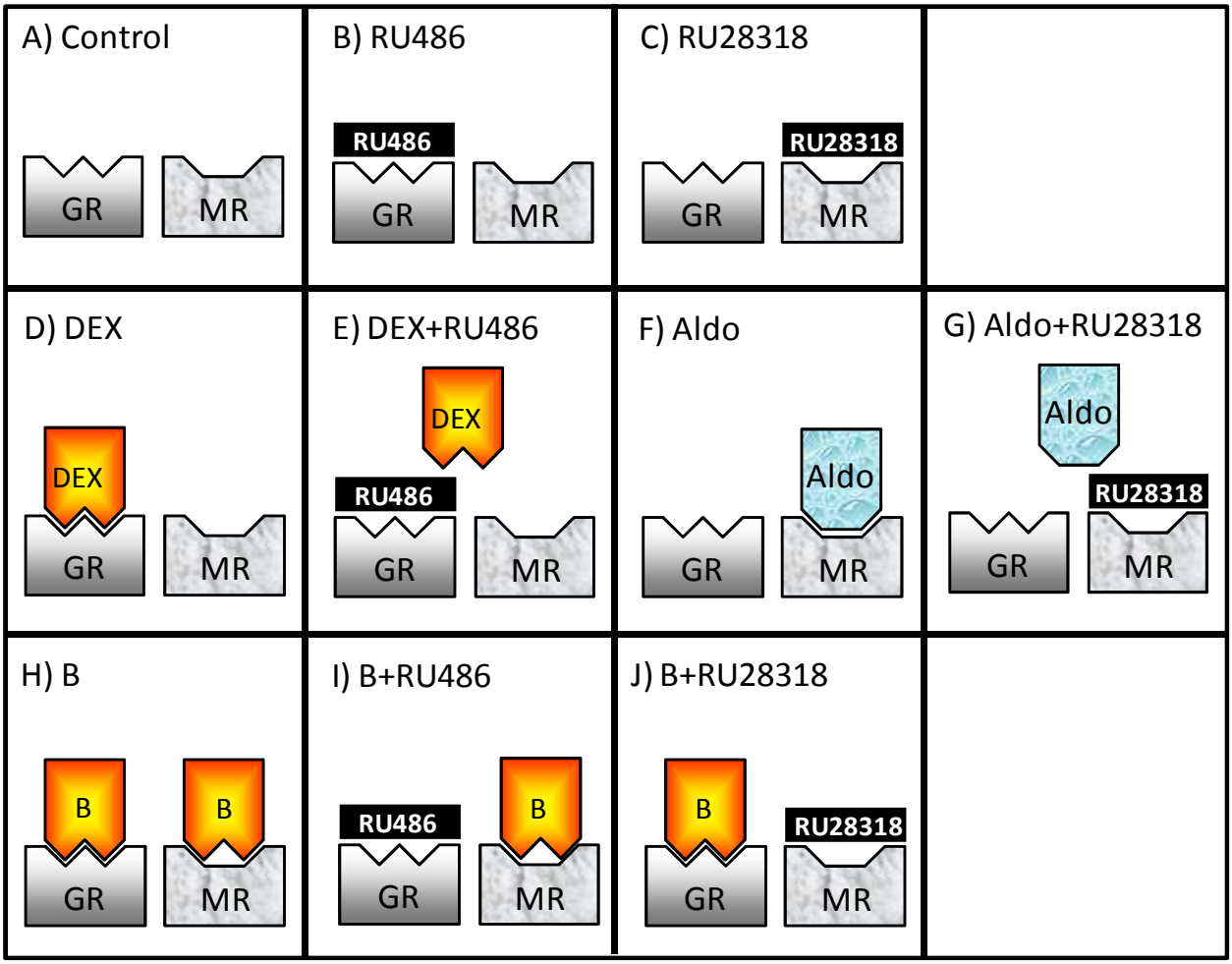

Figure 4-1 Schematic overview of treatments to study differential gene regulation in cardiomyocytes employing microarray technology. Panels A-C: Controls; panels D-G: gene regulation by dexamethasone (DEX) ± RU486 and aldosterone (Aldo) ± RU28318; panels H-J: differential signalling of corticosterone (B) via MR and GR.

Isolated CM from one rat heart were split and subjected to the 10 different treatments (Figure 4-1). The experiment was repeated three times (biological replicates).

4.2.2 Isolation and culture of cardiomyocytes

For CM isolation male Sprague-Daley with a body weight of 250 to 300g were used. Rats were fed regular chow with no restrictions to food or water intake. CM were freshly isolated from rat hearts as described in section 2.1.1.1. CM were counted in a haemocytometer and approximately 200,000 rod shaped CM per well were incubated

in CM culture media in the presence of 10% FCS in 6-well plates for 1h to let CM attach and adhere to the culture plate (Corning, Surrey, UK). Non-adherent CM were aspirated and the adherent CM were washed once with M199 media and subsequently incubated in 1.5 ml / well CM culture media.

4.2.3 Treatment of rat cardiomyocytes

Before treatment of rat CM, steroids and antagonists were diluted from 10mM stock solutions. Steroids (DEX, Aldo and corticosterone (B)) were first diluted in CM culture media 1:1000 to yield a concentration of 10µM of which 15µl were added per well to yield a final concentration of 100nM. Antagonists (RU486, RU28318) were initially diluted 1:10 to yield a concentration of 1µM of which 15µl were added per well to yield a final concentration of 10µM. Ethanol traces from stock solutions were compensated by addition of 15µl of 1:10 diluted ethanol to all treatments with no antagonists.

4.2.4 RNA isolation, labelling and hybridisation

RNA of CM in culture was performed as described in section 2.1.5. Quality and purity of RNA was assessed on a Bioanalyser-2100 (Agilent, Birkshire, UK) and a Nanodrop (ND-1000) spectrophotometer (Thermo Scientific, Cheshire, UK). RNA integrity numbers (RIN) were above 8 for all samples, the $OD_{260/280}$ indicating protein contamination was above 1.8 and the $OD_{260/230}$ indicating solvent contamination was found to be between 1.5 and 2.5 suggesting intact and pure RNA.

4.2.4.1 Preparation of one-color Spike mix

In order to compare microarray results from different slides an internal standard was added to the RNA sample prior to the reverse transcription and labeling reaction. The

stock solution of the one-color Spike-Mix was diluted 1:5000 with the supplied dilution buffer and 3µl were added to 7.3µl of the RNA sample and 1.2µl T7 promoter, incubated at 65ºC for 10 min and then placed on ice for 5 min.

4.2.4.2 cDNA synthesis

For cDNA synthesis 4µl [5x] first strand buffer, 2µl [0.1M] dithiothreitol (DTT), 1µl [10mM] dNTP, 1µl MMLV-RT and 0.5µl RNase inhibitor were added to each RNA sample from step 4.2.4.1 and incubated at 40ºC for 2h. Subsequently the samples were incubated at 65ºC for 15min to inactivate the reverse transcriptase.

4.2.4.3 cRNA synthesis

For synthesis of labeled cRNA 15.3µl nuclease free water, 20µl [4x] transcription buffer, 6µl [0.1M] DTT, 8µl NTP mix, 6.4µl [50%] polyethylenglycol (PEG), 0.5µl RNase inhibitor, 0.6µl inorganic pyrophosphate, 0.8µl T7 RNA polymerase and 2.4µl Cyanine 3-CTP were added to the cDNA from step 4.2.4.2 and incubated at 40ºC for 2h

4.2.4.4 cRNA purification

The labeled cRNA was purified using the Qiagen RNeasy Mini kit according the manufacturer's manual. In brief, 20µl of ddH_20, 350µl RLT buffer, 250µl 70% ethanol were added to the cRNA, transferred to a Qiagen RNA column and centrifuged for 30 seconds at 13,000 rpm in order to bind the RNA to the matrix of the column. The cRNA was then washed twice with 500µl RPE buffer and eluted with 30µl ddH_20 at 13,000 rpm for 1 minute.

4.2.4.5 Quantification of cRNA concentration and specific activity

The concentration of the purified cRNA and incorporation of Cyanine 3 (Cy3) was measured using a NanoDrop ND-1000 UV-VIS spectrophotometer. The total amount of labeled cRNA and the specific activity of the cRNA probe were calculated according to the following formulas:

cRNA yield = cRNA [µg/µl] * total volume [µl]

Specific activity [pmol/µg] = Cy3 concentration [pmol/µl] / concentration of cRNA [µg/µl]

The labeling reaction resulted in sufficient amounts of cRNA (total amount > 1.65µg) required for microarray hybridisation. The specific labeling activities indicating incorporation of Cy3 into the cRNA ranged between 12 and 18 pmol/µg for all 30 samples, well above the minimum activity recommended by the manufacturer (9 pmol Cy3/µg cRNA).

4.2.4.6 Hybridisation of labeled cRNA with microarrays

Prior to hybridisation of the cRNA probes to the microarrays, 1.65µg of cRNA was fragmented by adding 11µl [10x] blocking agent and 2.2 µl [25x] fragmentation buffer in a total volume of 55µl at 60ºC for exactly 30min. The fragmentation reaction was stopped by adding 55µl [2x] GE hybridisation buffer. The samples were centrifuged for 1 min at 13,000 rpm and kept on ice until loading. Following fragmentation 55µl of ddH_20 was added to the cRNA.

For hybridisation of microarrays, 100µl of the cRNA containing hybridisation solution was applied onto gasket slides. Microarray slides were then carefully laid on top of the gasket slides so that the hybridisation solution was held within the rubber wall of

the gasket slide. The sandwich-pairs were then fitted into hybridisation chambers and incubated in a hybridisation oven for 17h at 65ºC rotating at 10 rpm.

The following day the hybridisation chamber was disassembled, the slides removed and the microarray slides were dismantled from the gasket slides submerged in wash buffer #1. Subsequently the microarray slides were washed for 60 seconds in wash buffer #1 at RT and then transferred and incubated for 60 seconds in wash buffer #2 at RT and another 60 seconds in the same buffer pre-warmed to 37ºC. Following the second wash, the microarray slides were dried at RT and scanned.

4.2.4.7 Scanning of microarrays and feature extraction

The microarray slides were placed in a slide holder and fitted into the carousel of an Agilent High-Resolution Microarray Scanner (Thermo Scientific, Cheshire, UK) and scanned at a resolution of 5µm. The scanned image was then processed using the Agilent feature extraction (FE) software (Version 9.5.3). The FE software translates the intensity of fluorescence of every individual gene spot into a number representing the expression level and allocates this intensity to the corresponding probe name.

4.2.5 Normalisation of expression data and quality assessment

Assessment and analysis of microarray data was done employing the software “R: A language and environment for statistical computing” (http://www.r-project.org/) using packages from Bioconductor, open source software for bioinformatics. (http://www.bioconductor.org/). Of all 30 microarrays, 29 showed a very similar density spectrum (Figure 4-2a). For unknown reasons, one array (treatment: Aldo+RU28318) showed a very different density spectrum from all other 29 arrays and was therefore excluded from all subsequent analysis. The remaining 29 arrays were then normalised using quantile normalisation which is based on the magnitude

(quantiles) of measures in order to achieve the highest degree of comparability between microarrays. Density plots following quantile normalisation are depicted in Figure 4-2b which shows a perfect fit of all 29 individual density plots.

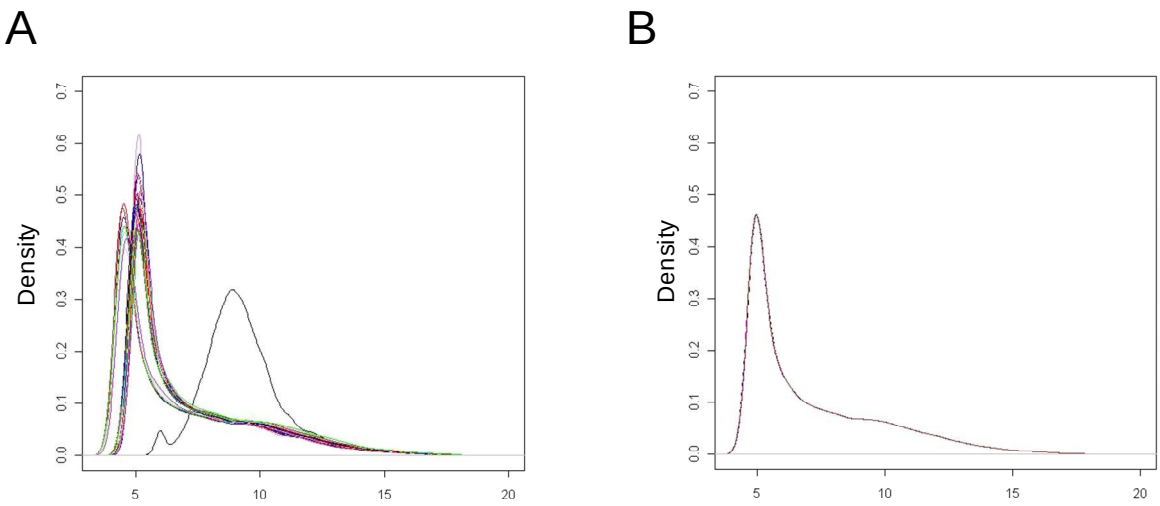

*Figure 4-2 Density plots of scanned microarrays before **A** and after **B** quantile normalisation. Each line represents the distribution of features (x-axis: expression strength, y-axis: relative quantity) of one microarray. One array showed a considerably different density spectrum and was excluded from all subsequent analysis.*

In a typical microarray experiment, it becomes impossible to make a visual inspection of the relationship between genes or conditions in such a multi-dimensional matrix. Therefore the expression states are defined by a core set of independent features that allow direct comparison. Principal components analysis (PCA) is a statistical technique for determining the key variables in a multidimensional data set that explains the differences in the observations. To achieve a graphical visualisation, PCA reduces data dimensionality by performing a covariance analysis between factors. The variance accounted for by each of the components is its associated eigenvalue which is the variance of a component over all genes. Consequently, the

eigenvectors with large eigenvalues are the ones that contain most of the information whereas eigenvectors with small eigenvalues are uninformative. The principal component 1 (PC_1) individuates the direction along the greatest variation. PC_2 the direction with maximum variation left in data orthogonal to the direction (i.e. vector) of PC_1, PC_3 the direction with maximum variation left in data orthogonal to the plane of PC_1 and PC_3 (rarely used), and so on to the component PC_n.

Figure 4-3a shows PCA eigenvalues of the present study. The first component exhibits the largest eigenvalue that indentifies the main direction of the data variance. Other eigenvalues are modest (the second one) or negligible. Figure 4-3b shows the data set of all microarrays, each represented by a box, projected onto the plane individuated by the axis of PC_1 (horizontal) and PC_2 (vertical). Microarray distribution did not appear widespread or clustered indicating similar global features among microarray hybridisations.

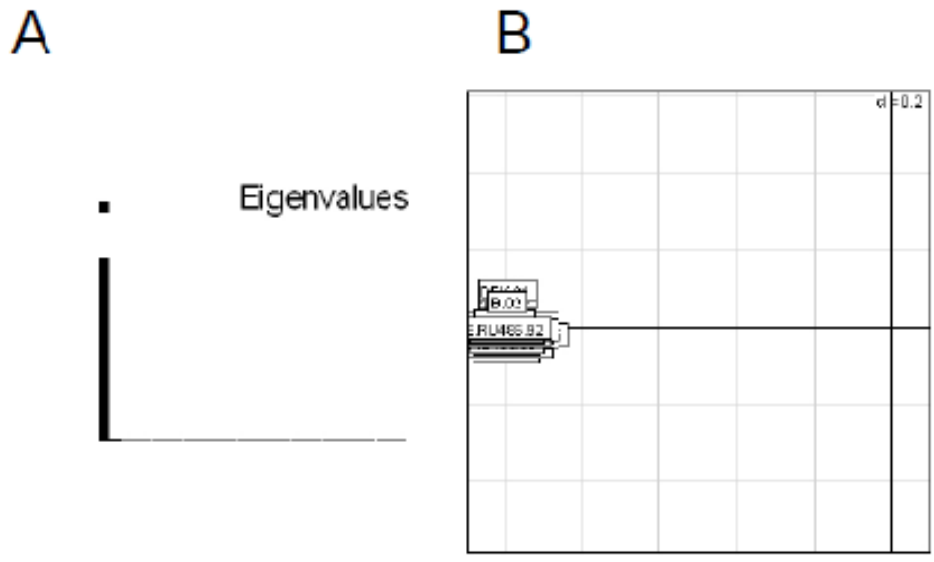

Figure 4-3 Principal component analysis of the study dataset. ***A*** *plot of the eigenvalues; iteratively, the direction with largest variance is selected as the ith principal component (orthogonality constraint).* ***B*** *Projection of microarray samples*

Associations of treatment effects were visualised using the correspondence analysis (CA). Like PCA, CA reveals the principal axes of this high-dimensional space, enabling projection into a subspace of low dimensionality that accounts for the main variance in the data. Unlike PCA, CA is able to account for the genes in a hybridisation-dimensional space and the hybridisations in a gene-dimensional space at the same time (biplots). CA attempts to separate dissimilar objects (genes or sample hybridisations) from each other; similar objects are clustered together resulting in small distances. In contrast, the distance between a gene and a sample hybridisation cannot be directly interpreted.

In CA, points are depicted such that the sum of the distances of the points to their centroid (called ‘‘total inertia’’) is proportional to the value of the χ^2 statistic of the microarray data. For a positive association like up-regulation of a gene in a particular condition, the two points will lie on the same side of the centroid, with the larger the distance to the centroid, the stronger the association. A negative association like down-regulation will cause the sample-point and the gene-point to lie on opposite sides of the centroid. Figure 4-4 shows a projection of microarray samples. Along the y-axis clustering of the three CM preparations can be observed, whereas clustering of treatments are observed along the x-axis.

Analysis of microarray data by CA shows some details masked in PCA. The moderately widespread clusters of DEX and B shows the highest distances from the origin of the centroid, indicating relative strong associations and low affinity with the others samples. On the contrary the other samples show affinities independently from the treatment resulting in three main grouped sets in which they are relatively close to each other (Figure 4-4).

Statistical analysis to select differentially expressed genes was performed using the “limma“ (Linear Models for Microarray Analysis) package. The core of the limma package is an implementation of the empirical Bayes linear modeling approach of Smyth (Smyth, 2004) and can be used for stable analysis even for smaller sample sizes.

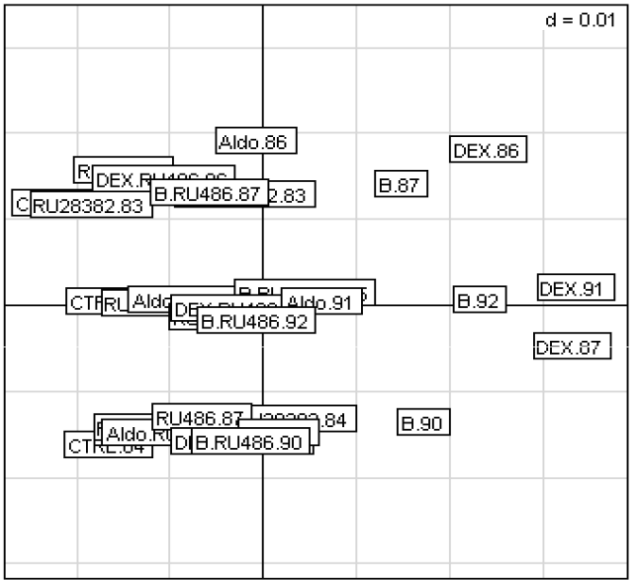

Figure 4-4 Correspondence analysis of microarrays. Microarray samples are projected into a 2 dimensional space and show clustering along the x-axis according to treatment and along the y-axis according to cardiac preparations.

4.2.6 Visual analysis of treatment effects by MA plots

Following normalisation and quality assessment mean gene expression strength for each feature was calculated from the three biological replicates. Treatment effects in comparison to untreated cells (control) were assessed graphically by MA plots using R software (Figure 4-5 and Figure 4-6). Each dot represents one feature. The distance from the X-axis reflects the logarithm of the fold change of the respective feature in comparison to untreated cells (control) and the distance from the y-axis

reflects its expression strength. The dot colour indicates significance (red and green dots represent significantly up- and down-regulated features; blue colour indicates non significant regulation). Figure 4-5 shows DEX and Aldo effects on gene regulation in the presence or absence of specific antagonists. Visual analysis of MA plots suggest that both DEX and Aldo treatment results in many significantly up- and down-regulated genes which is greatly diminished in the presence of the respective steroid hormone receptor antagonist suggesting by and large specific up-regulation of DEX genes via GR and Aldo genes via MR.

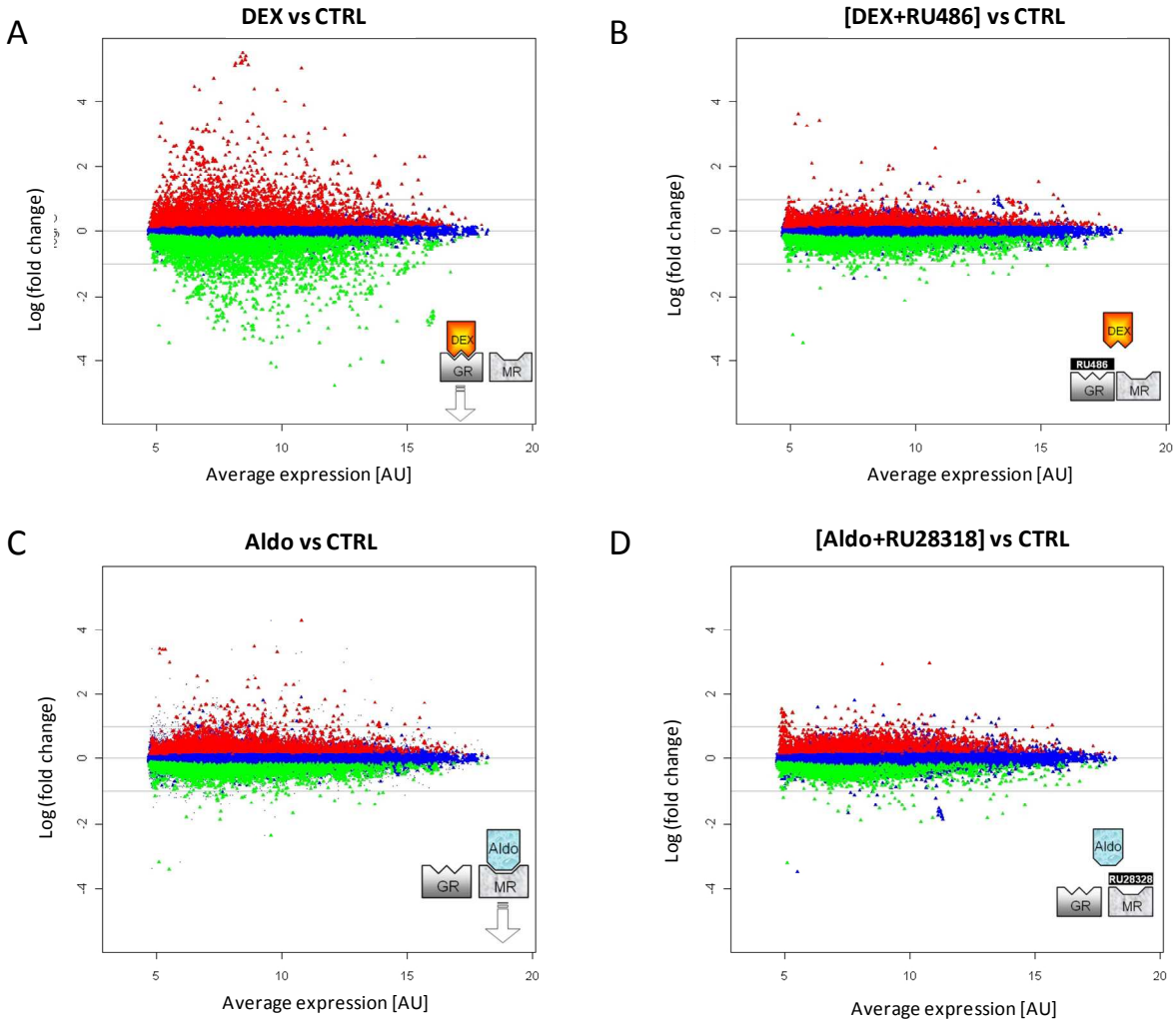

Figure 4-5 MA plots of dexamethasone (DEX) and aldosterone (Aldo) regulated genes. ***A*** *DEX regulated genes.* ***B*** *DEX regulated genes in the presence of RU486.* ***C*** *Aldo regulated genes.* ***D*** *Aldo regulated genes in the presence of RU28318. Average expression strength (mean of three microarray experiments) is depicted on the x-axis and Log (fold change) in comparison to control (CTRL) on the y-axis. Significantly up-regulated genes are depicted in red, significantly down-regulated genes in green and non-significantly regulated genes in blue colour.*

A similar analysis was down for corticosterone treatments in the presence or absence of the GR antagonist RU486 and MR antagonist RU28318 (Figure 4-6). Visual analysis suggests that blockade of steroid hormone receptors greatly diminishes the magnitude of gene regulation suggesting that GR and MR are not redundant and both required for a full biological effect of corticosterone.

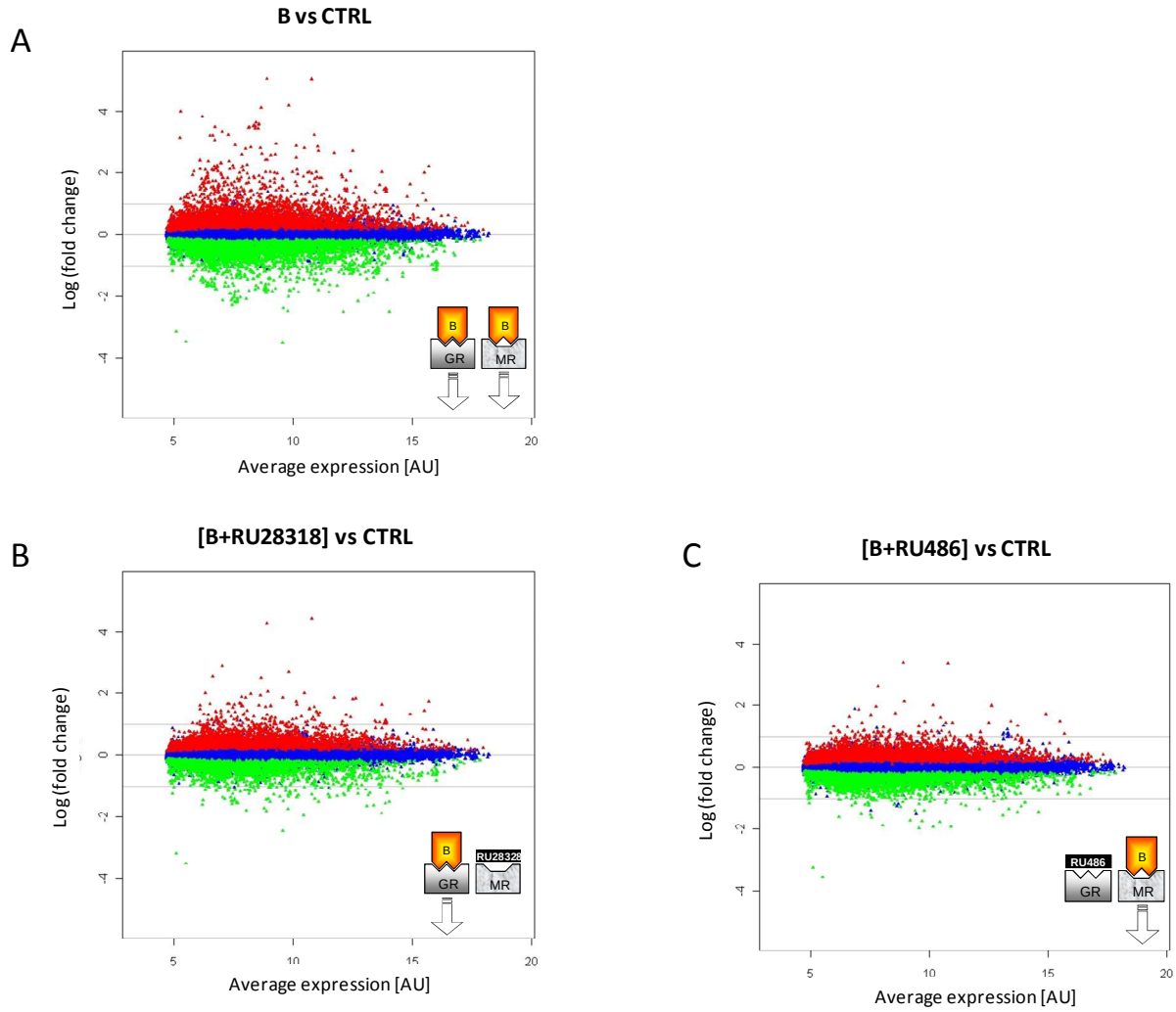

Figure 4-6 MA plots of corticosterone (B) regulated genes. ***A*** *B regulated genes in the absence of antagonists.* ***B*** *B-regulated genes in the presence of RU28318.* ***C*** *B-regulated genes in the presence of RU486.*

4.3 Results

For simplification purposes, the term *gene* instead of *features* will be used although strictly speaking this is incorrect because some genes are represented by multiple features on every microarray. Gene regulation (up or down) was defined if gene expression in response to treatment was significantly ($p<0.05$) increased or decreased by at least 2 fold compared to control. These genes are referred to as 'regulated genes'. Venn diagrams were chosen to depict differential expression

patterns of genes. Non overlapping parts of circles indicate the number of genes that were exclusively regulated by one but not any other treatment and are referred to as ‘selective genes’. Because some of these selective genes were also significantly regulated ($p<0.05$) yet to a lesser degree ($2.0 < FC < 1.5$) by one or both of the other treatments and hence did not fulfil the criteria of a selectively regulated gene, we subtracted these genes from the number of selective genes and thereby calculated the number of ‘highly selective genes’ which may be a better reflection of true differential regulation. The total number of these highly selective genes is shown in brackets behind the total number of selective genes in the Venn diagrams.

4.3.1 Differential gene regulation by corticosterone via the glucocorticoid and mineralocorticoid receptor

We first studied if gene regulation was receptor dependent by analysing the transcriptomes induced by corticosterone via GR (B/GR), MR (B/MR) or both receptors (B/GR+MR) employing the specific steroid hormone receptor antagonists RU28318, RU486 (Figure 4-7). Comparison of corticosterone regulated genes in the absence of antagonists with regulated genes after blocking the GR or MR with RU486 or RU28318, respectively, showed a great reduction of the total number of up-regulated genes (B only: 328; B+RU486: 75; B+RU28318: 115) and down-regulated genes (B only: 215; B+RU486: 53; B+RU28318: 86), suggesting that both receptors are required for a maximal effect on transcriptional regulation. Moreover, we found only a small subset of genes to be selectively up- or down-regulated in the presence of RU486 (up: 15; down: 10) or RU28318 (up: 2; down: 12). When applying the criteria of highly selective genes only 3 and 2 genes were up-regulated and 0 and 4 genes were down-regulated in the presence of RU486 reflecting B/MR signalling or in the presence of RU28318 reflecting B/GR signalling, respectively. These data

suggest that signalling of corticosterone via GR or MR does not result in a differential transcriptome.

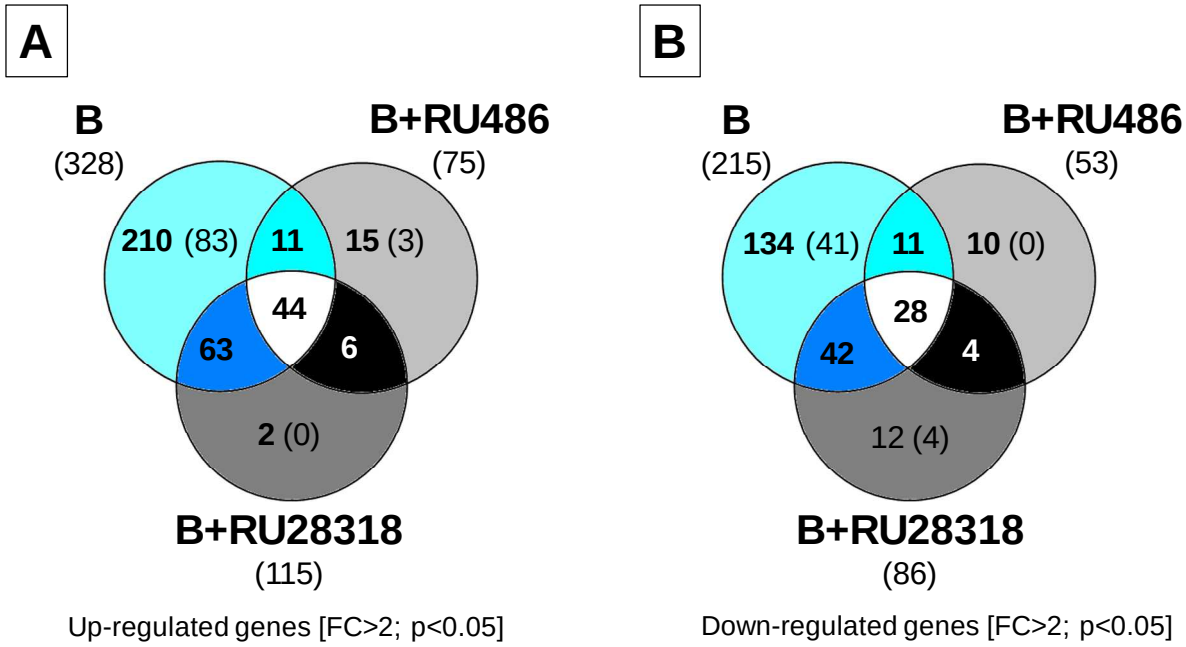

Figure 4-7 Venn diagram of corticosterone (B), B+RU486 and B+RU28318 regulated genes (fold change (FC)>2, p<0.05) (concentration: B 100nM; RU486, RU28318 10µM). ***A*** *Up-regulated genes: B: 328, B+RU486: 75 and B+RU28318: 115; selectively up-regulated genes by B: 210, B+RU486: 15 and B+RU28318: 2 (in brackets: number of highly selective genes (see text)).* ***B*** *Down-regulated genes: B: 215, B+RU486: 53 and B+RU28318: 86; selectively up-regulated genes by B: 134, B+RU486: 10 and B+RU28318: 12.*

4.3.2 Differential gene regulation by aldosterone compared to corticosterone via the mineralocorticoid receptor

We next assessed ligand specificity at the MR by comparing the transcriptomes induced by Aldo via MR (Aldo/MR) with that of corticosterone via MR (B/MR), which was achieved by blocking corticosterone signalling via GR employing RU486 (Figure 4-8). Both treatments resulted in a significant overlap of regulated genes which was more pronounced for down-regulated genes (21 of 37 Aldo and 53 B+RU486

regulated genes) than for up-regulated genes (30 of 124 Aldo and 76 B+RU486 regulated genes). Nevertheless, 94 and 46 genes were selectively up-regulated and 16 and 31 were selectively down-regulated by Aldo/MR and B/MR, respectively. These results suggest that signalling of Aldo and corticosterone via MR results in a common as well as a differential transcriptome suggesting that interaction of Aldo and corticosterone with the MR induces a ligand specific transcriptome via the MR.

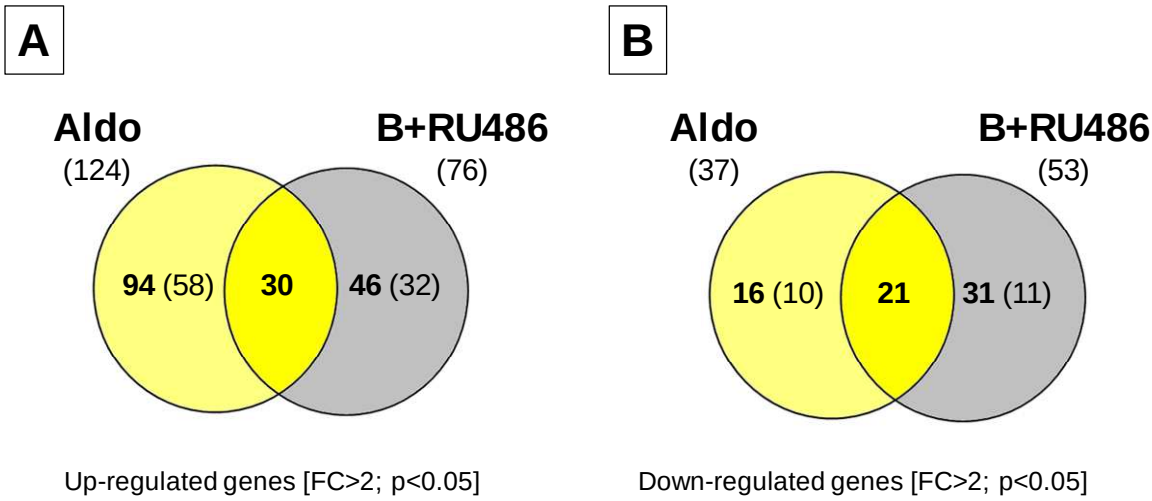

Figure 4-8 Venn diagram of aldosterone (Aldo) and corticosterone+RU486 (B+RU486) regulated genes (fold change (FC)>2, p<0.05). (concentration: A, B 100nM; RU486 10µM). ***A*** *Up-regulated genes: Aldo: 124, B+RU486: 76; selectively up-regulated genes by Aldo: 94, B+RU486: 46 (in brackets: number of highly selective genes (see text)).* ***B*** *Down-regulated genes: Aldo: 37, B+RU486: 53; selectively up-regulated genes by Aldo: 16, B+RU486: 53.*

4.3.3 Differential gene expression by DEX, Aldo and B

To address receptor specific gene regulation via GR, MR or both receptors we treated CM with the GR agonist DEX, the MR agonist Aldo or corticosterone, respectively (Figure 4-9). The total number of up- and down- regulated genes was highest for DEX (up: N=503; down: N=554), followed by B (up: N=328; down: N=215)

whereas Aldo showed the smallest response (up: N=124; down: N=37), suggesting, firstly, that DEX is a more potent GR agonist than B and, secondly, that GR activation by DEX induces a stronger response than MR activation by Aldo. There was a high degree of genes regulated by all three treatments (up: 97; down: 27) suggesting a high degree of redundancy for the different ligands interacting with GR and/or MR. In addition, all three treatments also induced a subset of genes that were not regulated by any of the other treatments. However, the number of highly selectively regulated genes was considerably smaller compared to the number of selective genes (DEX: up: 235 vs 61; down: 356 vs 90; B: up: 66 vs 37; down: 20 vs 9; Aldo: up: 17 vs 12; down: 1 vs 1) suggesting that only a subset from these genes were truly differentially regulated. In contrast to DEX and B, the total number of highly selective Aldo genes was similar to that of selective Aldo genes (up: 17 vs 12; down: 1 vs 1). Treatment with DEX and B resulted in a huge overlap of regulated genes (up: N=260 (163+97); down: N=192 (165+27)). With regard to Aldo, a high percentage of genes was also regulated by either DEX and/or B, hence, the number of selectively regulated genes (up: 17, down: 1) by Aldo in relation to the total number of Aldo regulated genes (up: N=124, down: 37) was small.

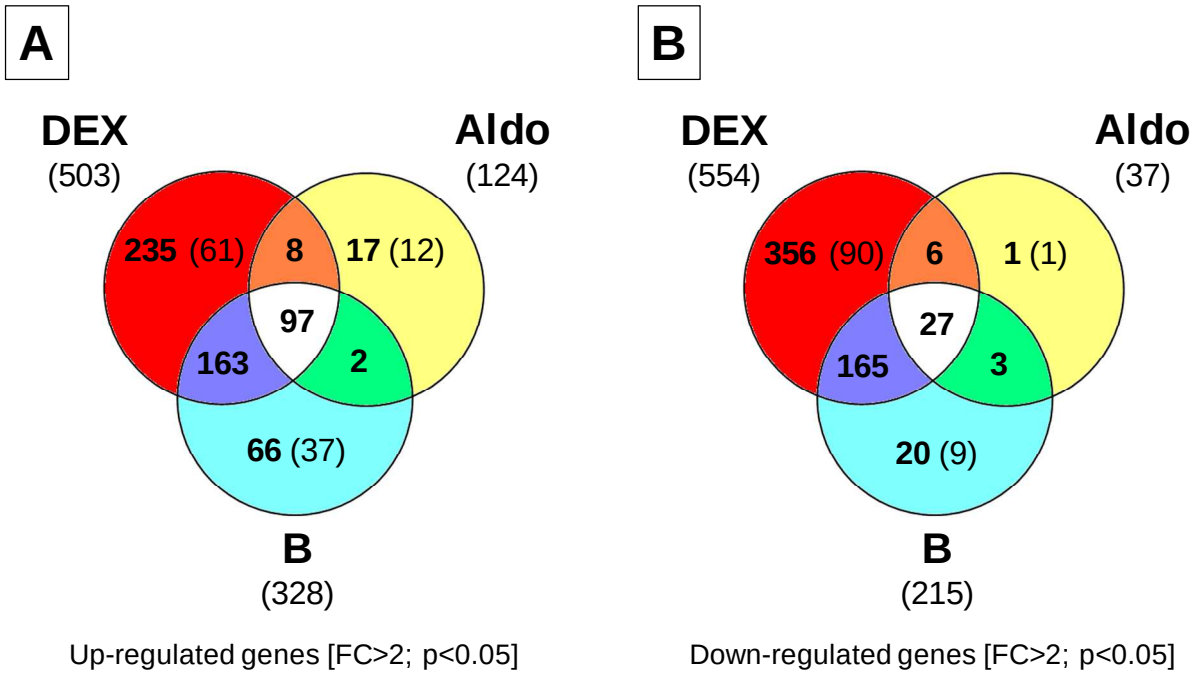

Figure 4-9 Venn diagram of dexamethasone (DEX), aldosterone (Aldo) and corticosterone (B) regulated genes (fold change (FC>2, p<0.05) (DEX, Aldo, B concentration: 100nM). ***A*** *Up-regulated genes (in brackets total number of features): DEX: 503, Aldo: 124, B: 328; selective up-regulated genes by DEX: 235, Aldo: 17 and B: 66 (in brackets are shown the number of highly selective regulated genes (see text)).* ***B*** *Number of down-regulated genes: DEX: 554, Aldo: 37 and B: 215 and selectively down-regulated genes: DEX: 356, Aldo: 1 and B: 20.*

Table 4-1 shows a list of genes selectively up- and down-regulated by Aldo but not corticosterone using the fold change cut-off of 2. Regulation by DEX was not an exclusion criteria because these genes may be relevant to physiology given the synthetic nature of DEX. Out of the total 25 (17 Aldo, 8 DEX+Aldo genes) up- and 7 (1 Aldo, 6 Aldo+DEX genes) down-regulated genes by Aldo (Figure 4-9), 6 (up) and 1 (down) probes bound to intronic gene regions, 7 (up) probes bound to sequences in the rat genome that did not belong to genes, 1 (down) probe did not match any sequence in the rat genome and 2 (up) and 2 (down) probes were not blocked by RU28318 and are not shown in Table 4-1.

Table 4-1 List of up- and down-regulated genes by aldosterone (FC≥2).

Aldo		Aldo/ RU28318		B		Expr	Gene Symbol	Gene Name
FC	P	FC	P	FC	p			
10.4	0.029	-1.2	0.902	-1.0	0.996	5.0	LPAR4	Lysophosphatidic acid receptor 4
9.6	0.027	-1.0	0.969	-1.0	0.980	4.8	Fbxo48	F-box protein 48
7.9	0.047	-1.3	0.825	-1.2	0.868	5.4	OLR760	Olfactory receptor 760
5.6	0.002	2.5	0.117	2.6	0.068	7.3	Rps26	ribosomal protein S26
3.1	0.021	1.0	0.951	1.0	0.974	4.9	DUSP8	Dual specificity phosphatase 8
2.6	0.027	1.1	0.866	1.0	0.923	10.5	VPS4A	Vacular protein sorting 4, yeast, homolog of, A
2.5	0.029	1.0	0.986	1.0	0.941	9.2	PURA	Purine-rich element-binding protein A
2.3	0.000	1.0	0.863	-1.2	0.234	8.1	Usp25	Ubiquitin specific peptidase 25
2.2	0.000	1.3	0.083	1.2	0.119	7.3	Pnn	Pinin
2.1	0.041	1.8	0.138	1.7	0.132	7.4	Unknown	Hypothetical LOC503134
-2.0	0.000	1.0	0.806	-1.4	0.000	10.6	Fst	Follistatin

FC=fold change, p=level of significance. Expr: expression strength.

To allow detection of less strongly regulated yet potentially more pathophysiologicaly relevant genes we lowered the fold change cut-off for regulated genes from 2 to 1.4 fold. The lower and less stringent cut-off of 1.4 fold was chosen arbitrarily and was based on the assumption that a change in gene expression in response to treatment of 1.4 fold may still be of biological relevance. Table 4-2 shows a selection of genes that were significantly up- or down-regulated by Aldo (FC≥1.4) but not corticosterone (p>0.05), blocked by co-administration of the MR antagonist RU28318 and are likely to be of biological relevance after careful review of the literature.

Table 4-2 List of up- and down-regulated genes by aldosterone (FC≥1.4).

Aldo		Aldo/ RU28318		B		Expr	Gene Symbol	Gene Name
FC	P	FC	P	FC	p			
1.5	0.014	1.0	0.895	1.0	0.966	4.7	**AQP4**	Aquaporin 4
1.4	0.001	-1.1	0.204	1.0	0.754	9.6	**STIM1**	Stromal interaction molecule 1
1.4	0.001	-1.0	0.835	1.1	0.333	11.4	**SIK1**	Salt-inducible kinase 1
1.4	0.005	1.1	0.269	1.2	0.120	10.9	**RAB1**	RAB1, member RAS oncogene family
1.4	0.016	-1.0	0.845	1.1	0.319	11.0	**SPEC1**	Small protein effector of Cdc42
-1.4	0.014	1.1	0.340	1.1	0.631	8.8	**CDKN1C**	Cyclin-dependent kinase inhibitor 1C
-1.4	0.000	-1.1	0.433	-1.2	0.078	13.8	**PHB**	Prohibitin
-1.4	0.050	-1.0	0.879	-1.1	0.709	9.3	**ZFHX3**	Zinc finger homeobox 3
-1.5	0.004	1.2	0.208	1.1	0.343	7.6	**SPEG**	The striated preferentially expressed gene
-1.8	0.009	-1.0	0.930	-1.2	0.313	8.9	**TBC1D20**	TBC1 domain family, member 20

FC=fold change, p=level of significance. Expr: expression strength; AU: arbitrary units.

4.4 Discussion

Here we studied differential gene regulation by corticosteroids in primary cultures of isolated adult rat CM employing microarray technology in an attempt to shed light on differential effects of corticosterone and Aldo signalling via GR and MR and thereby identified several genes selectively regulated by Aldo via the MR.

DEX, Aldo and corticosterone revealed a great overlap in their gene expression patterns consistent with a high degree of redundancy in corticosteroid hormone action. DEX exhibited the greatest effect on gene regulation indicated by the number of up- and down-regulated genes, followed by corticosterone and Aldo. This finding is consistent with the findings by Yoshikawa and colleagues who found in a similar

experimental set up that overall gene regulation via GR compared to MR was stronger (Yoshikawa et al., 2009).

Our results indicate that corticosteroid hormone signalling is ligand rather than receptor dependent. We found that gene regulation by corticosterone via GR or MR does not induce a differential transcriptome, indicating receptor redundancy in the context of corticosterone signalling. Presence of GR and MR resulted in the greatest transcriptional response (number of regulated genes), whereas the blockade of either receptor greatly reduced the total number of regulated genes, suggesting that GR and MR act synergistically and are both required for a maximal effect on gene regulation. This observation is likely to be attributable to formation of GR-MR heterodimers following binding of corticosterone. Previous studies have shown that interaction of GR and MR does occur both *in vitro* and *in vivo* (Liu et al., 1995; Savory et al., 2001; Trapp et al., 1994). Our findings are in good agreement with the study by Trapp *et al* who found an augmented transcriptional response of glucocorticoid target genes by GR-MR heterodimers compared to GR homodimers (Trapp et al., 1994). However by contrast, Liu *et al* reported attenuation of gene regulation by GR-MR heterodimers compared to GR homodimers (Liu et al., 1995). The conflicting results of these two studies are most likely related to the different gene context. Our results indicate firstly, that gene regulation by corticosterone via GR or MR is not fundamentally different and secondly suggest, that formation of GR-MR heterodimers occurs in CM and is required for a maximal effect. The biological meaning of these findings remains unclear at the present time. But CM specific GR and MR knock out mice may provide models to clarify this question in the future.

We next addressed if gene regulation via MR was ligand-dependent by comparing the transcriptomes induced by Aldo via MR (Aldo/MR) with that of corticosterone via MR (B/MR). As expected, there was a considerable overlap in the transcriptomes

induced by both ligands. However, we also identified a subset of genes that were only regulated by one or the other ligand, indicating that gene regulation of corticosteroids in CM, at least in part is ligand-dependent. The differential transcriptome induced by Aldo and corticosterone via MR raises the question of the underlying molecular mechanisms. In contrast to the kidney, CM do not express 11β-HSD2 (see Chapter 3) thus pre-receptor metabolism cannot be accounted for the effects observed in our study. At the receptor level, ligand binding to the MR may induce differential conformational changes of the receptor that may be different for Aldo and corticosterone. Aldo dissociates more slowly from the MR and induces greater transactivation than cortisol (Farman & Rafestin-Oblin, 2001; Lombes et al., 1994). Moreover, the interaction between the MR N-terminal domain and the C-terminal hinge and ligand binding domain (or the N/C–interaction), which stabilises the ligand-receptor complex (Centenera et al., 2008; Li et al., 2006) is much stronger in the presence of Aldo than cortisol, offering another mechanism of ligand specificity (Rogerson & Fuller, 2003). Furthermore, at the post-receptor level, an expanding library of nuclear receptor co-regulators has been identified (Lonard & O'Malley B, 2007). These co-regulators play a central role in modulating gene expression and are thought to confer ligand- and tissue-specificity due to their structural and functional diversity and tissue specific expression patterns, respectively (described in section 1.4.2). To date, interaction with the MR has only been demonstrated for 11 co-activator and 5 co-repressor proteins (Yang & Young, 2009). From these co-regulators only one, the subunit C of the nuclear transcription factor Y (NF-YC), has been shown to repress MR dependent gene transcription in an Aldo-specific fashion as co-repression by cortisol was not detected (Murai-Takeda et al.). In conclusion, ligand specificity in the absence of 11β-HSD2 can be explained by molecular

mechanisms at the receptor and post-receptor level which to date remain incompletely understood.

We next analysed the Aldo specific transcriptome with the aim to identify selective Aldo target genes that may provide molecular insights into the adverse effects of Aldo in cardiac remodelling. Applying the fold change cut-off of 2 for regulated genes we detected a small number of genes to be specifically regulated by Aldo. However, after careful review of the literature only the purine rich element binding protein α (PURA) and the lipoprotein a receptor type 4 (LPAR4) appeared to be candidate genes of pathophysiological relevance with regard to Aldo action.

We found PURA to be selectively up-regulated by Aldo. PURA has previously been reported to bind to the purine-rich negative regulatory (PNR) element located in the first intron of the α-myosin heavy chain (α-MHC) gene (Gupta et al., 1998) and thereby represses α-MHC transcription and translation (Gupta et al., 2003). Down-regulation of α-MHC and concomitant up-regulation of the β-MHC gene is a key feature of heart failure pathology, which may be modulated by Aldo via up-regulation of PURA in failing hearts.

We also found the lysophosphatidic acid receptor 4 (LPAR4) to be selectively up-regulated by Aldo. Lysophosphatidic acid (LPA) is a natural lipid with a broad range of cellular actions (Choi et al.). In the CVS LPA is involved in angiogenesis and in the pathogenesis of atherosclerosis. Treatment of CM with LPA has been shown to induce a hypertrophic response *in vitro* (Hilal-Dandan et al., 2004). Therefore, augmented LPA signalling induced by up-regulation of LPAR4 by Aldo may participate in cardiac hypertrophy and remodelling.

In an attempt to identify Aldo target genes which may be less strongly regulated yet potentially more relevant to Aldo pathophysiology we lowered the fold change cut-off for regulated genes from 2 to 1.4 and thereby identified candidate genes which are

likely to play a role in Aldo pathophysiology. Aldo induces cardiac hypertrophy *in vitro* and *in vivo* (described in section 1.9.3.5.2). In our microarray study, we found several genes that have previously been linked to cardiac hypertrophy to be selectively regulated by Aldo but not corticosterone including RAB1, the stromal interaction molecule 1 (STIM1), the small protein effector of Cdc42 (SPEC1) and the striated preferentially expressed gene (SPEG).

RABs are guanosine triphosphatases (GTPases) that belong to the Ras-related small GTPases super-family. Rab1 is localised in the ER and the Golgi apparatus and regulates export and transport of newly synthesised proteins from the endoplasmic reticulum (ER) to the Golgi. Overexpression of RAB1 in nrCM has been shown to induce hypertrophic growth with an increase in total protein synthesis, cell size and sarcomeric organisation (Filipeanu et al., 2004). Consistent with these findings, overexpression of RAB1 in a transgenic mouse model results in cardiac hypertrophy and heart failure (Wu et al., 2001). In addition, TBC1D20 has recently been identified as a RAB GTPase-activating protein (GAP) (Sklan et al., 2007). We identified both RAB1 and TBC1D20 to be selectively up-regulated by Aldo suggesting that increased RAB1 expression and TBC1D20 dependent activation may provide a molecular mechanism by which Aldo induces cardiac hypertrophy independent of blood pressure.

Cardiac hypertrophy depends on sustained Ca^{2+} entry into CM. Ca^{2+} homeostasis is regulated by the transient receptor potential canonical channel 1 (TRPC1) via store-operated Ca^{2+} (SOC) channels and thereby plays a pivotal role in cardiac hypertrophy by subsequent activation of the nuclear factor of activated T-cells (NFAT). It was recently shown that knock down of the stromal interaction molecule 1 (STIM1) inhibits up-regulation of TRPC1 and thereby attenuates the hypertrophic response induced by endothelin-1, Ang II and phenylephrine (Ohba et al., 2009). We

identified STIM1 to be selectively down-regulated by Aldo which provides another molecular mechanism by which Aldo may induce cardiac hypertrophy.

Cell division control protein 42 (Cdc42) is member of the Rho family of small GTPases which is involved in cell cycle regulation. Overexpression of Cdc42 in cardiomyocytes enhances myocyte growth, while overexpression of a dominant negative Cdc42 mutant has the opposite effect by modulating sarcomere assembly *in vitro* (Nagai et al., 2003). In agreement with these findings, CM specific deletion of Cdc42 *in vivo* renders the mouse heart more capable of hypertrophic growth following pressure overload, neuroendocrine agonist infusion, and exercise, suggesting that activation of Cdc42 antagonises cardiac growth (Maillet et al., 2009). Cdc42 activation is inhibited by the small protein effector of Cdc42 (SPEC1) through direct interaction with Cdc42 (Pirone et al., 2000) which we identified to be selectively up-regulated by Aldo.

Furthermore, the striated preferentially expressed gene (SPEG) is a member of a family of myosin light-chain kinases (MLCKs) that are required for cytoskeletal remodelling. SPEG knock out mice are characterised by CM hypertrophy, myofibril degeneration, and a marked decrease in cardiac function resulting in dilated cardiomyopathy (Liu et al., 2009a). We found SPEG to be down-regulated by Aldo which thereby may play a role in myocyte dysfunction and the development of heart failure. On aggregate, our microarray experiment has identified several novel selective Aldo target genes that may explain the direct, blood pressure independent effects of Aldo on cardiac hypertrophy.

Aldo excess in primary hyperaldosteronism has been associated with an increased risk of atrial fibrillation (Milliez et al., 2005). Moreover, drugs interfering with the RAAS such as ACEi and ARBs reduce the incidence and recurrence of atrial fibrillation (Iravanian & Dudley, 2008). A genome wide association study has recently

identified an intronic single nuclear polymorphism (SNP) in the zinc finger homeobox 3 (ZFHX3) gene to increase the risk of atrial fibrillation (Benjamin et al., 2009). ZFHX3 regulates myogenic and neuronal differentiation and has been reported to be a tumour suppressor gene in several cancers whereas its function in cardiac tissue is largely unknown. We identified ZFHX3 to be selectively down-regulated by Aldo which may provide a mechanism by which Aldo exerts proarrhythmic effects.

Prohibitins (PHB) are ubiquitous, evolutionarily conserved proteins involved in mitochondrial structure and function. Overexpression of prohibitin 1 (PHB1) in CM protects mitochondria from oxidative stress or hypoxia induced injury and thereby attenuates CM apoptosis *in vitro* (Liu et al., 2009b; Muraguchi et al.). We identified PHB1 to be selectively down-regulated by Aldo which may confer susceptibility to oxidative stress resulting in CM apoptosis.

Aquaporins are water channels that play a pivotal role in water flux across membranes. Ischemia of the myocardium is characterised by swelling of CM resulting in cardiac dysfunction. In an animal model of MI, AQP4 expression was increased in ischaemic CM and correlated with infarct size (Warth et al., 2007). We found AQP4 to be selectively up-regulated by Aldo which may augment cell swelling following MI. Furthermore, attenuation of AQP4 expression may be a one mechanism by which MR antagonists provide cardio protection post MI.

Furthermore, we identified two genes, the salt-inducible kinase 1 (SIK1) and the cyclin-dependent kinase inhibitor 1C (CDKN1C, also known as $p57^{Kip2}$), to be selectively regulated by Aldo in our microarray screen. SIK1 is a serine/threonine kinase which is a member of the adenosine monophosphate kinase (AMPK) family of kinases. It participates in the regulation of Na^+/K^+-ATPase activity under sodium stress conditions in a calcium dependent manner (Sjostrom et al., 2007). $P57^{Kip2}$ is a member of the cyclin-dependent kinase inhibitor family which is involved in the

control of cell proliferation and differentiation. SIK1 and $p57^{Kip2}$ have recently been shown to play an important role during cardiac development (Romito et al.). Using a model of embryonic stem cell-derived embryonic bodies, absence of SIK1 resulted in a lack of $p57^{Kip2}$ up-regulation and a delay of terminal cardiac differentiation which could be rescued by constitutive overexpression of $p57^{Kip2}$. These findings suggest that SIK1 is one of the key regulators in the cascade of events leading to cardiomyogenesis through the modulation of $p57^{Kip2}$ expression. The biological role of SIK1 and $p57^{Kip2}$ in adult cardiomyocytes is less clear. Overexpression of $p57^{Kip2}$C in CM in a transgenic mouse model has recently been shown to result in resistance to myocardial ischemia/reperfusion injury and improve recovery of cardiac function in adult mice (Haley et al., 2008) whereas the role of SIK1 in CM beyond development remains unknown.

In summary, our microarray experiment identified several genes which are selectively regulated by Aldo. These genes may provide novel molecular mechanisms through which Aldo exerts its detrimental effects on the heart.

Several caveats related to the experimental set up need to be kept in mind when interpreting the differential corticosteroid hormone effects in this study. Despite the preferential affinity of DEX for GR and Aldo for MR both steroids show some cross reactivity with the respective other corticosteroid hormone receptor (Coirini et al., 1985) and hence, cannot be regarded as selective agonists. Cross reactivity may particularly be of relevance with regard to Aldo given the supra-physiological concentration of 100nM that was used in this study. The reason for choosing such a high Aldo concentration was because preliminary studies showed no or only very little effect on target gene regulation when Aldo was used at high (1-10nM) physiological concentrations. This finding is consistent with many other reports in the literature which only observed significant transcriptional responses by Aldo at a minimum

concentration of 10nM or above (Boixel et al., 2006; Doi et al., 2008; Harada et al., 2001; Lalevee et al., 2005; Lee et al., 2004; Lopez-Andres et al., 2008; Okoshi et al., 2004). However reassuringly, gene regulation by cross reactivity of Aldo with GR appeared to be of minor significance in our study because only 5 of the 25 Aldo selective genes remained significantly ($p<0.05$) up-regulated in the presence of the MR antagonist RU28318 suggesting that the remainder of genes were regulated via MR.

We chose an incubation period of 8h in this study although transcriptional regulation of steroid target genes has been observed as early as 30 minutes following treatment and then may return to baseline levels within hours (Kellner et al., 2003). The identified genes in this study must therefore not be regarded as direct corticosteroid target genes because they firstly, are likely to represent secondary or maybe even tertiary gene regulatory events and secondly, may result from an initial non-genomic steroid effect (described in section 1.4.3) which subsequently triggers downstream transcriptional events. However, given our overall aim of discovering differentially regulated genes by corticosteroids relevant to cardiovascular disease, discrimination of direct vs indirect regulatory effects by Aldo on these genes was not important and should be addressed in future studies.

In conclusion, our study shows that Aldo and corticosterone induce a common as well as a differential transcriptome through their cognate steroid hormone receptors in CM which provides novel insights into the molecular mechanisms of corticosteroid action in the heart.

Chapter 5 - Steroid Hormone Secretion and Metabolism in Essential Hypertension

5.1 Introduction

Hypertension is a prevalent condition affecting more than 25% of the adult population world wide (Kearney et al., 2005). The RAAS plays a central role in the regulation of blood pressure and primary hyperaldosteronism, a condition characterised by Aldo excess (described in section 1.6.3), has now undoubtedly emerged as the most frequent form of secondary hypertension but its true prevalence among hypertensives and patients with resistant hypertension remains controversial (Calhoun et al., 2002; Douma et al., 2008; Mosso et al., 2003; Rossi et al., 2006). Further support for the pivotal role of the RAAS in the pathogenesis of essential hypertension comes from the Framingham offspring study which suggests that prior to clinical manifestation of hypertension, patients with high-normal Aldo levels are the ones at increased risk to develop hypertension in the future (Vasan et al., 2004). There is biochemical and genetic evidence that a subgroup of patients with essential hypertension have a mild inefficiency of the enzyme 11β-hydroxylase, which catalyses the final enzyme in cortisol synthesis (described in section 1.3.2), which is thought to lead to compensatory HPA activation via ACTH resulting in normal cortisol yet mildly but inappropriately increased Aldo production (Davies et al., 2009).

Similar to mineralocorticoid excess, glucocorticoid excess in Cushing's syndrome results in hypertension (described in section 1.5.3). Several mechanisms may be involved to drive blood pressure In Cushing's syndrome. Besides increased Aldo secretion secondary to ACTH drive in Cushing's disease and forms of ectopic ACTH secretion, 11β-HSD2 may be swamped by the high circulating cortisol levels resulting in illicit activation of the MR, which exhibits similar affinities for Aldo and cortisol *in vitro* (Arriza et al., 1987), in the kidney by glucocorticoids (Stewart et al., 1996; Ulick et al., 1992). Glucocorticoid driven MR activation is also found in patients with the

syndrome of apparent mineralocorticoid excess (AME), which is characterised by a functionally inactive 11β-HSD2 enzyme secondary to mutations in the HSD11B2 gene (described in section 1.7.2.3). Lessons learned from these extreme but very rare forms of hypertension suggest that more subtle changes in 11β-HSD2 activity caused by polymorphism in the HSD11B2 gene may be implicated in the pathogenesis of essential hypertension, particularly in patients who are susceptible to salt (described in section 1.7.2.5). Nevertheless, the exact contribution of glucocorticoid and mineralocorticoid secretion and metabolism to drive blood pressure in essential hypertension remains unclear (Hammer & Stewart, 2006).

We here studied steroid hormone secretion and metabolism by measuring circulating steroid hormones and urinary steroid hormone metabolites in patients with essential hypertension with the aim, firstly, to define the prevalence of primary hyperaldosteronism in unselected patients with essential hypertension and, secondly, to gain more insight into the role of corticosteroid hormones in the pathogenesis of essential hypertension.

5.2 Materials and Methods

5.2.1 Patients

Patients were recruited from GP practices in south Birmingham. Inclusion criteria were white caucasian background, age>18 years, uncomplicated hypertension (blood pressure >150/90 on 3 occasions or on antihypertensive treatment) and current blood pressure <180/110 (if already on treatment). Exclusion criteria comprised a history of diabetes, heart disease, peripheral vascular or cerebrovascular disease and renal or liver disease.

5.2.2 Study protocol

At baseline, patients were assessed by means of taking a clinical history and physical examination; office blood pressure was measured using a validated oscillometric sphygmomanometer (Dinamap® Procare, GE). In addition, a random blood sample was taken for electrolytes, creatinine and urea, random PRA and Aldo. In all patients on antihypertensive medication, antihypertensive agents were stopped sequentially (one drug per week) and blood pressure was monitored weekly. In case of uncontrolled hypertension (office blood pressure > 180/100), patients were excluded from the study and restarted on antihypertensive medication. 3 weeks after cessation of the last antihypertensive drug, patients underwent a 24h ambulatory blood pressure measurement (Meditech® ABPM-04) and collected a 24h urine for analysis of steroid hormone metabolites. Venous blood samples were taken for analysis of electrolytes, creatinine and urea. In addition, PRA and Aldo were measured after 30 min supine rest. After completion of the study, patients were restarted on their antihypertensive medication unless 24h ABP revealed a normal blood pressure (<125/80 mmHg). The protocol was approved by the South Birmingham Local Research Ethics Committee and all patients gave written informed consent prior to enrolment.

5.2.3 Biochemical assays

Plasma Aldo and PRA were measured as described in section 2.2.1.2.1.

5.2.4 Urinary steroid metabolites

Mineralocorticoids and glucocorticoids are metabolised by various enzymes and are excreted as metabolites in the urine. The main urinary metabolites of mineralo- and glucocorticoids including their precursors were measured by GC/MS and global

enzymatic activities were inferred from the ratio of urinary substrate metabolites divided by product metabolites as described in section 2.2.1.1.

5.2.5 Statistical Analysis

Normally distributed data were expressed as means ± SD (unless stated). Non parametric data were expressed as median (interquartile range) and were log-transformed before statistical analysis. For comparisons of two independent variables Student's t-test was used. Correlations were expressed by Pearson's correlation coefficient. Statistical significance was assumed at $p<0.05$.

5.3 Results

Of the 117 patients who fulfilled the inclusion criteria, 4 patients were excluded for developing dangerous hypertension, 5 patients were excluded because of newly developed symptoms (ie shortness of breath or headache), 2 were excluded because of poor comprehension and 2 were lost to follow up, therefore results from 104 patients were analysed. Patient characteristics are given in Table 5-1. 41% of patients were newly diagnosed and on no antihypertensive medication whereas the remaining patients were on one to three antihypertensive agents.

Table 5-1 Patient characteristics at baseline

		Reference Range
Male gender (%)	59 (57)	
Age (years)	56 ± 11	
BMI (kg/m^2)	28.6 ± 4.9	
Na^+ (mmol/l)	139 ± 2.5	(134-145)
K^+ (mmol/l)	4.1 ± 0.35	(3.5 – 5.0)
Creatinine (µmol/l)	81 ± 13	(50 -110)
Urea (mmol/l)	5.6 ± 1.7	(2.5 – 10.7)
eGFR (ml/min/1.73m^2)	73 ± 11	(> 90)
PRA (ng/ml/h)	2.2 (1.2,5.0)	(0.51 – 2.64)
Aldo (pmol/l)	222 (152, 294)	(28 – 445)
ARR (pmol/l/ng/ml/h)	105 (40, 189)	(< 450)
Number of antihypertensive drugs (%)		
nil	43 (41%)	
#1	26 (25%)	
#2	29 (28%)	
#3	6 (6%)	
Antihypertensive class (%)		
ACEi/ARB	40 (38%)	
Diuretic	32 (30%)	
Beta blocker	13 (13%)	
Calcium channel blocker	16 (15%)	

Values are mean ± SD; in brackets: SI units and normal reference range where applicable; ACEi: angiotensin converting enzyme inhibitor; eGFR: estimated glomerular filtration rat; PRA: plasma renin activity; Aldo: aldosterone; ARR: aldosterone renin ratio; ARB: angiotensin receptor blocker.

Three patients (4%) exhibited an increased Aldo / PRA ratio (ARR) > 750 with an absolute Aldo concentration > 400pmol/l suggesting primary hyperaldosteronism (PHA) as the underlying cause. After withdrawal of antihypertensive medication, both systolic and diastolic office blood pressure significantly increased (Table 5-2). Antihypertensives had no effect on sodium or the estimated glomerular filtration rate (eGFR) but potassium levels were significantly lower off medication. Antihypertensive

medication had no significant effect on Aldo levels but PRA was significantly lower and the Aldo renin ratio (ARR) significantly higher after withdrawal of antihypertensive medication (Table 5-2). Stratification of Aldo and PRA by antihypertensive agents revealed that only patients taking ACEi or ARBs had a significantly higher PRA, whereas PRA in patients on other antihypertensive agents including calcium channel blockers (CCB), beta blockers (BB) and diuretics were comparable to treatment naive patients (Figure 5-1a). By contrast, Aldo levels were no different among treatment groups (Figure 5-1b).

Table 5-2 Effect of antihypertensive medication

	on treatment	off treatment
Office blood pressure (mmHg)		
Systolic	145 ± 19.1	154 ± 18.3 (p=0.005)
Diastolic	84 ± 11.0	88 ± 11.0 (p=0.008)
Mean 24h ambulatory blood pressure (mmHg)		
Systolic		144 ± 12.3
Diastolic		88 ± 9.0
Electrolytes & creatinine		
Na^+ (nmol/l)	139 ± 2.6	139 ± 2.3
K^+ (nmol/l)	4.1 ± 0.4	4.0 ± 0.3 (p=0.05)
eGFR (ml/min)	72 ± 10.2	69 ± 14.4
RAAS		
PRA (ng/ml/h)	3.3 (1.3,6.8)	0.95 (0.56,1.71) (p<0.001)
Aldo (pmol/l)	223 (151,315)	244 (138,313)
ARR	62 (32,180)	222 (108,484) (p<0.001)

Values are mean ± SD or median (interquartile range). eGFR: estimated glomerular filtration rat; PRA: plasma renin activity; Aldo: aldosterone; ARR: aldosterone renin ratio. Treatment effects were analysed by t-test for parametric and Mann-Whitney-U test for non-parametric data.

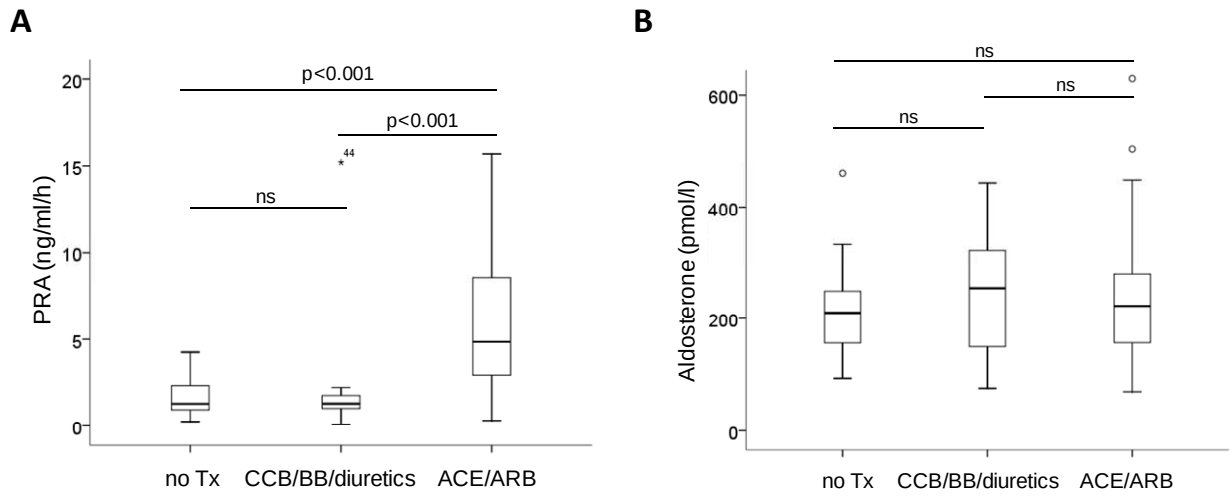

*Figure 5-1 Plasma renin activity (PRA) (**A**) and aldosterone (**B**) in patients with essential hypertension (N=68) stratified by classes of antihypertensive agents (no Tx: no antihypertensive medication (N=17); CCB/BB/diuretics: calcium channel blockers and/or beta-blockers and/or diuretics (N=17); ACEi: angiotensin converting enzyme inhibitor; ARB angiotensin receptor blocker (N=34)). (Treatment groups were compared by Kruskal-Wallis ANOVA).*

Following withdrawal of antihypertensive medication, 24h ABP confirmed hypertension in 96 patients, whereas 8 patients (8%) had a normal mean 24h blood pressure (<125/80 mmHg) suggesting white coat hypertension. Age and kidney function were both negatively correlated with mean 24h diastolic blood pressure (Table 5-3). Plasma Aldo was correlated with 24h urinary excretion of TH-Aldo ($r=0.423$, $p<0.001$), the main urinary Aldo metabolite. PRA showed an inverse association with mean 24h blood pressure, whereas plasma Aldo, the aldosterone-renin ratio (ARR) and 24h urinary TH-Aldo excretion were positively correlated with 24h blood pressure (Table 5-3). By contrast, neither 24h urinary total cortisol metabolite (total F metabolites) excretion nor circulating cortisol was associated with blood pressure (Table 5-3).

Table 5-3 Correlations with blood pressure

	24h ambulatory blood pressure	
	Systolic	Diastolic
Patient characteristics		
Age (years)	0.017 (p=0.864)	-0.254 (p=0.010)
Body weight (kg)	0.071 (p=0.480)	0.084 (p=0.405)
BMI (kg/m2)	0.095 (p=0.344)	0.034 (p=0.738)
eGFR (ml/min)	0.018 (p=0.868)	-0.307 (p=0.003)
Blood parameters		
Na^+ (mmol/l)	-0.025 (p=0.811)	0.009 (p=0.930)
K^+ (mmol/l)	-0.189 (p=0.073)	-0.221 (p=0.036)
PRA (ng/ml/h)	-0.264 (p=0.012)	-0.261 (p=0.013)
Aldo (pmol/l)	0.095 (p=0.371)	0.275 (p=0.009)
ARR	0.304 (p=0.005)	0.397 (p<0.001)
Cortisol (nmol/l)	0.128 (p=0.286)	0.105 (p=0.383)
Urinary steroid hormone metabolites		
TH-Aldo (µg/24h)	0.212 (p=0.038)	0.275 (p=0.007)
Total F metabolites (µg/24h)	0.149 (p=0.145)	0.107 (p=0.298)
DHEA (µg/24h)	0.053 (p=0.613)	0.128 (p=0.218)
Enzymatic activities		
11β-HSD1 (THF+5αTHF / THE)	0.076 (p=0.460)	0.055 (p=0.595)
11β-HSD2 (UFF / UFE)	-0.258 (p=0.011)	-0.247 (p=0.015)
11β-hydroxylase (THS / total F metabolites)	-0.106 (p=0.300)	-0.205 (p=0.044)
5α-reductase (THF / 5αTHF)	-0.075 (p=0.469)	-0.157 (p=0.128)

Values are Pearson correlation coefficients. PRA: plasma renin activity; Aldo: aldosterone; ARR: aldosterone renin ratio; TH-Aldo: tetrahydro-aldosterone; THF: tetrahydro-cortisol; THE: tetrahydro-cortisone; UFF: urinary free cortisol; UFE: urinary free cortisone; THS: tetrahydro-desoxycortisol.

Using ratios of urinary steroid hormone metabolites as a surrogate for global activities of steroidogenic enzymes, there was no indication of an association of 5α-reductase (THF/5α-THF) or 11β-HSD1 activity (THF+5α-THF/THE) with blood pressure. However, 11β-HSD2 activity reflected by the excretion of urinary free cortisol / cortisone (UFF/UFE) and 11β-hydroxylase activity reflected by excretion of (THS / total cortisol metabolites were negatively associated with blood pressure, indicating reduced enzymatic activities with increasing blood pressure (Table 5-3).

5.4 Discussion

In this study we assessed the role of corticosteroid hormone secretion and metabolism in patients with essential hypertension.

Primary hyperaldosteronism is now accepted as the most common form of secondary hypertension, but its true prevalence in hypertensive patients remains unclear. In the largest study to date, the primary hyperaldosteronism prevalence (PAPY) study, 11.2% of patients with hypertension were found to have underlying primary hyperaldosteronism (Rossi et al., 2006). However, this might be an overestimate of the true prevalence among hypertensives as patients were referred from primary care to specialised centers for evaluation of secondary hypertension. Previously, Mosso and colleagues reported a lower primary hyperaldosteronism prevalence of 6.1% in 609 hypertensives recruited from primary care which may be a more precise reflection of its prevalence in unselected hypertensives (Mosso et al., 2003). Here we found 4% of our patients to have a positive ARR of >750 with an Aldo level greater than 400pmol/l suggesting PHA, although confirmatory testing was not done. A likely explanation for the lower prevalence in our study is the fact that about half of our patients (45%) had only mildly elevated blood pressure (JNC-7 grade 1 hypertension: 140-159 / 90-99 mmHg), for which the prevalence of primary hyperaldosteronism in the PAPY study was merely found to be 6.6% (Rossi et al., 2006). In patients with resistant hypertension, who are believed to have the highest percentage of primary hyperaldosteronism, a recent report only found a prevalence of 11.3% although previous reports suggested a much higher prevalence of 20% (Calhoun et al., 2002). Our results are in good agreement with the findings by Mosso and colleagues (Mosso et al., 2003) and suggest that the overall prevalence of primary hyperaldosteronism among unselected hypertensive patients may be in fact closer to 5% than 10%.

After stopping antihypertensive medication, we found that 9% of patients who had high office blood pressure had a normal mean 24h ABP suggesting white coat hypertension. Although, excess cardiovascular risk of these patients remains controversial, a recent meta-analysis did not find an increased risk in patients with white-coat hypertension compared to normotensives (Fagard & Cornelissen, 2007). Guidelines suggest that in the absence of organ damage antihypertensive treatment is not recommended (2003). Given that 9% of patients in our cohort were falsely labeled as hypertensive, 24h ABP measurement which is inexpensive and can conveniently be performed at home, should probably be used more often, particularly in patients with only mildly elevated office blood pressure or if the diagnosis is in doubt in order to prevent unnecessary treatment – a burden for both patients and health care budgets.

We found a significantly up-regulated PRA in patients on ACEi and ARBs compared to patients on calcium channel blockers (CCB), diuretics and or beta blockers (BB), while circulating Aldo was similar suggesting a compensatory activation of the RAAS. Another mechanism that is likely to contribute to the finding that Aldo levels were similar among the different patient groups is the phenomenon of 'aldosterone escape' which describes the normalisation of Aldo levels in patients on long term ACEi or ARB treatment despite an initial suppression when treatment is initiated (Bomback & Klemmer, 2007).

There is no doubt about the pivotal role of Aldo in the pathogenesis and maintenance of hypertension in primary hyperaldosteronism whereas its role in essential hypertension is far less clear. Historic studies in patients with essential hypertension have found conflicting results with some studies reporting higher Aldo levels in patients with essential hypertension (Genest et al., 1975; Ljungman et al., 1982), whereas others have found similar (Wisenbaugh et al., 1972) or even lower levels

(Walker et al., 1979) compared to normotensive controls and as a consequence, plasma Aldo levels have been associated with blood pressure both in a positive (Ljungman et al., 1982) and negative (Walker et al., 1979) fashion. In our cohort plasma Aldo and urinary TH-Aldo excretion were positively correlated with blood pressure suggesting that circulating Aldo levels contribute to elevated blood pressure in patients with essential hypertension. Furthermore, increased 24h urinary excretion of TH-Aldo with blood pressure suggests that relative Aldo excess is secondary to hyperactivity of adrenal mineralocorticoid production. However by contrast, PRA showed a negative association with blood pressure suggesting a salt and volume replete state with increasing blood pressure.

The ARR, the most sensitive marker of mineralocorticoid excess for salt/volume status, is an established screening test for primary hyperaldosteronism and when above a certain cut-off primary hyperaldosteronism is likely but warrants confirmatory testing (Funder et al., 2008). Besides the associations of Aldo and PRA with blood pressure we found the ARR to be most strongly associated with blood pressure suggesting an increasing relative mineralocorticoid excess state (RME) with higher blood pressure levels. In line with this notion we found a negative correlation of blood pressure with circulating potassium levels, a reflection of mineralocorticoid action in epithelial cells of the distal nephron of the kidney. Further support for the gradual mineralocorticoid excess state with increasing blood pressure comes from an interventional study which found the ARR to be highly predictive of the reduction in blood pressure achieved when initiating treatment with the mineralocorticoid receptor antagonist Spiro (Mahmud et al., 2005).

Our findings suggest that the ARR, besides its established role as screening test for the presence of absolute mineralocorticoid excess in primary hyperaldosteronism, is a useful marker of relative mineralocorticoid excess. This finding is supported by two

recent reports (Alvarez-Madrazo et al., 2009; Tomaschitz et al.) which show that the ARR is continuously distributed and over its whole spectrum associated with blood pressure even well below the cut-off used for screening for PHA. This illustrates the importance of ARR on blood pressure modulation not only in hypertensive but also normotensive subjects. On aggregate, the findings of others and us suggest that the role of ARR with regard to blood pressure needs to be expanded from simply being a measure of “absolute” mineralocorticoid excess when screening for PHA, to a more gradual measure of “relative” mineralocorticoid excess (RME). As suggested by the study of Mahmud and colleagues, determination of the relative mineralocorticoid excess of a patient may be highly relevant when deciding on which antihypertensive agent to use (Mahmud et al., 2005).

Given that renin via Ang II is believed the principal regulator of Aldo, the fact that Aldo and PRA correlate divergently with increasing blood pressure raises the question of Aldo regulation. In this respect, a number of reports have suggested that ACTH, which is known to stimulate steroid hormone secretion from of all three layers of the adrenal cortex, might contribute to Aldo secretion in hypertensives. Evidence for this theory comes from the observation that a polymorphism (-344T) in the aldosterone synthase gene (CYP11B2), which has previously been found to be associated with mineralocorticoid production (Davies et al., 1999; Paillard et al., 1999) and blood pressure (Lim et al., 2002), is also associated with a reduced 11β-hydroxylase activity (encoded by the CYP11B1 gene) catalysing the conversion of 11-deoxycortisol to cortisol, the final step in cortisol synthesis (see section 1.3.2). In order to overcome inefficient cortisol synthesis, HPA drive via ACTH is induced resulting in normal cortisol yet mildly but inappropriately elevated Aldo levels (Freel et al., 2008). However, in our study HPA drive (using total cortisol metabolites as a surrogate) was not associated with blood pressure. We therefore conclude that

neither renin nor HPA drive explains Aldo levels in our cohort suggesting that Aldo is regulated by a novel factor which is neither renin nor HPA drive or alternatively, by an intra-adrenal mechanism. Moreover, we did not find evidence for impaired 11β-hydroxylase activity (using the urinary THS/total cortisol metabolites ratio) with higher blood pressure but conversely, found 11β-hydroxylase activity to be increased with higher blood pressure. Similarly, global 11β-HSD2 activity as assessed by the urinary free cortisol / cortisone ratio was also increased rather than reduced suggesting that inefficient cortisol inactivation did not contribute to higher blood pressure in our cohort. Previous studies have given conflicting results with some studies reporting a reduced 11β-HSD2 activity while others did not find such an association suggesting that impaired 11β-HSD2 activity might play a role in a subset of patients but certainly does not appear to be a universal mechanism driving blood pressure in essential hypertension (Ferrari, 2009).

Limitations of this study are its observational nature which does not allow any causative conclusions, the relatively small size of the cohort and a the lack of a matched normotensive control cohort, whereas its strengths are the fact that patient were on no antihypertensive medication and recruited rather than referred from primary care ruling out selection bias.

In summary, we found a low prevalence of suspected primary hyperaldosteronism of 4% in our cohort of unselected patients with high blood pressure. Our results suggest that Aldo plays an important contributory role to high blood pressure defining essential hypertension as a state of relative mineralocorticoid excess.

Chapter 6 - The Effect of Spironolactone on Steroid Hormone Secretion and Metabolism in Chronic Kidney Disease

6.1 Introduction

In chronic kidney disease (CKD) the RAAS becomes progressively activated with decreasing kidney function (Hillege et al., 2000) and Ang II and Aldo have emerged as important drivers of both kidney disease progression and increased cardiovascular mortality (Go et al., 2004; Mann et al., 2002; Tonelli et al., 2006). Consequently, RAAS inhibition by ACEi and ARB remains the mainstay of treatment. However, RAAS inhibition by ACEi or ARBs is only partial and a recent study suggests that addition of the MR antagonist Spiro in CKD patients already treated with an ACEi and ARB, further reduces markers of renal disease progression (Tylicki et al., 2008). Similarly, the use of MR antagonists in heart failure patients in addition to established treatment regimens including ACEi and ARBs has been shown to dramatically improve survival (Pitt et al., 2003b; Pitt et al., 1999).

One important mode of action of MR antagonists in CKD patients is blood pressure reduction through blockade of renal MR in epithelial cells of the distal nephron. The MR has similar affinities for mineralocorticoids and glucocorticoids *in vitro* (Arriza et al., 1987) and is only protected from glucocorticoid activation by the enzyme 11β-HSD2 which inactivates cortisol to cortisone. Reduced activity of 11β-HSD2 results in glucocorticoid mediated MR activation and has been described in selected patients with salt sensitive essential hypertension (Agarwal et al., 2000; Lovati et al., 1999). In CKD patients 11β-HSD2 activity (N'Gankam et al., 2002; Whitworth et al., 1989) and expression (Quinkler et al., 2005) declines with progressively impaired renal function suggesting that in these patients blood pressure and renal damage may be partly driven by glucocorticoid induced MR activation. Therefore this mechanism provides a rationale for the incremental benefit of MR antagonists on lowering blood pressure in CKD patients.

Besides MR blockade, animal and *in vitro* studies also suggest that Spiro and its active metabolites can also inhibit steroidogenic enzymes including 11β-hydroxylase and thereby may impact on corticosteroid hormone synthesis (Cheng et al., 1976; Greiner et al., 1976). However, so far this has never been investigated in humans. In addition, studies in healthy subjects suggest that hippocampal MR may play a role in the modulation of HPA drive (Heuser et al., 2000; Young et al., 1998) but so far this has never been demonstrated in patients on long-term low-dose Spiro treatment.

Here we studied corticosteroid hormone secretion and metabolism in CKD patients with mild-moderate renal impairment at baseline and following treatment with low dose Spiro. We hypothesised that some of the beneficial effects of Spiro may relate to changes in cortisol secretion and/or metabolism.

6.2 Materials and Methods

6.2.1 Study design

In collaboration with Prof Townsend and Dr Edwards (Department of Cardiology, Queen Elizabeth Hospital, UHB Trust, Birmingham) who conducted a clinical trial evaluating the effects of Spiro on left ventricular mass and aortic stiffness in CKD we analysed urinary steroid hormone metabolites by GC/MS from participating patients (Edwards et al., 2009). A sample size of 90 patients assigned equally to the 2 treatment groups was calculated to provide 95% power to detect a change in LV mass of 10 g (SD 12 g) on CMR and 80% power to detect a change in PWV of 0.6 m/s (SD 1.0 m/s) with an alpha error of 0.05 in each case. No power calculations were performed for analysis of steroid hormone secretion and metabolism. This was a single centre, prospective, double-blind, placebo controlled, randomised intervention trial in patients with early stage CKD of diverse aetiologies including

glomerulonephritis (IgA nephropathy, nephrotic disease, focal segmental glomerular sclerosis) (53%), quiescent vasculitis (19%), adult polycystic kidney disease (8%), reflux (8%), calculi (4%) and others (8%) (Henoch-Schoenlein purpura, sickle cell disease, sarcoidosis, nephrectomy). Patients had stage 2 (GFR 60-89 ml/min/1.73m^2) (GFR was calculated using the 4 variable MDRD equation) or stage 3 CKD (GFR 30-59 ml/min/1.73m^2) and evidence of kidney damage for ≥3 months) (Sarnak et al., 2003). All patients were treated with an ACE inhibitor and / or ARB for at least 6 months to maximally tolerated dose and had controlled blood pressure (mean daytime 24 hour ambulatory blood pressure monitoring <130/85 mmHg). 24 patients with CKD secondary to vasculitis were on a stable immunosuppressive treatment with glucocorticoids which was not altered during the study period. Patients were excluded if they had a history of diabetes, or symptomatic ischaemic or non-ischaemic heart disease, peripheral vascular or cerebrovascular disease, renovascular disease, anaemia (<12g/dL) or previous documented hyperkalaemia (>5.5mmol/L).

A Spiro dose of 25mg was chosen in this study as previously low dose Spiro has been shown to be safe and clinical effective in patients with heart failure (Pitt et al., 1999). All patients received a 4 week open label run-in phase of 25mg of Spiro once daily (or alternate days if potassium levels were between 5.5 and 5.9mMol/l), after which patients were randomised to continue treatment with 25 mg Spiro or to placebo for a further 36 weeks. Patients were assessed at baseline (before the run in phase) and at the end of the study (week 40) with a clinical history and examination, 24 hour ambulatory blood pressure monitoring, and collection of a 24h urine sample. Venous blood samples were also collected after 30 minutes supine rest for routine hematology and biochemistry and measurement of PRA, Aldo and Ang II.

During the open-label run-in phase, 1 patient developed serious hyperkalaemia (potassium 6.5mMol/l) and was withdrawn, 6 (5%) patients had potassium levels between 5.5 and 5.9mMol/l and were switched to Spiro on alternate days as per protocol. On blinded treatment, 4 patients had potassium levels between 5.5 and 5.9mMol/l that required a dose reduction to alternate day treatment. After unblinding, two of these 4 patients were found to have received placebo. After randomisation, no patients were withdrawn because of hyperkalemia, and there were no reported side effects, including gynecomastia or menstrual disturbances. The protocol was approved by South Birmingham Local Research Ethics Committee and all patients gave written informed consent.

6.2.2 Blood Pressure

Office brachial blood pressure was recorded with the subject lying supine after 10, 20 and 30 minutes in the non-dominant arm using a validated oscillometric sphygmomanometer (Dinamap® Procare, GE). In addition, all subjects underwent 24 hour ambulatory blood pressure monitoring (Meditech® ABPM-04) at baseline and at week 40.

6.2.3 Biochemical assays

PRA, plasma Ang II and plasma Aldo was measured was measured as described in section 2.2.1.2.2.

6.2.4 Urinary steroid metabolites

Mineralocorticoids and glucocorticoids are metabolised by various enzymes and are excreted as metabolites in the urine. The main urinary metabolites of mineralo- and glucocorticoids including their precursors were measured by GC/MS and global

enzymatic activities were inferred from the ratio of urinary substrate metabolites divided by product metabolites as described in section 2.2.1.1.

6.2.5 Statistical Analysis

Normally distributed data were expressed as means ± SD (unless stated). Non parametric data were expressed as median (interquartile range) and were log-transformed where applicable. Treatment groups were compared using t tests or chi-square tests (at baseline) and repeated measures analysis of variance (for changes over time). Correlations of non-parametric data were assessed by the Spearman's correlation coefficient.

6.3 Results

All 112 patients enrolled in this study had stage 2 or 3 CKD, were all on either ACEi or ARB treatment for more than 6 months and had normal office blood pressure levels (Table 6-1). There were no differences in blood pressure or gender distribution between the two groups. However, body weight and BMI was higher in the Spiro compared to the placebo group and in the Spiro group significantly more patients were treated with beta-blockers and statins compared to the placebo group (Table 6-1). As expected, at baseline patients on beta blockers had a significantly lower PRA (median (interquartile range): 23 mU/l (8, 85) vs 82 mU/l (49, 185), $p<0.001$) and Ang II levels (4.9 pmol/l (3.1, 8.3) vs 9.9 pmol/l (5.0, 23.1), $p<0.05$) compared to patients not taking beta-blockers. However by contrast, the plasma aldosterone concentration (PAC) was slightly but significantly higher in patients on beta-blocker treatment (222 pmol/l (152, 300) vs 166 pmol/l (108, 222), $p<0.05$) suggesting an alternative mechanism of Aldo release in addition to Ang II. Analysis of urinary glucocorticoid and mineralocorticoid steroid hormone metabolites between patients

on and off beta-blockers did not reveal significant differences suggesting that beta-blockers do not have a major influence on steroid hormone production or metabolism. Comparison of patients on ACEi vs ARB at baseline did not reveal any differences in PRA (64 mU/l (37, 133) vs 81 mU/l (25, 151), p=0.676), PAC (169 pmol/l (114, 247) vs 172 pmol/l (97, 238), p=0.627) or 24h urinary excretion of TH-Aldo (18.1 µg/24h (10.5, 26.8) vs 17.5 µg/24h (13.4, 25.4), p=0.984). However as expected, circulating Ang II levels were significantly lower in the ACEi compared to the ARB group (5.3 pmol/l (3.8, 10.5) vs 31.6 pmol/l (12.6, 72.7), p<0.001). No significant differences were found between males and females with regard to PRA, Ang II, PAC or urinary TH-Aldo.

Total body weight and BMI were correlated with 24h total glucocorticoid excretion (r=0.407, p<0.001; r=0.296, p=0.003) but not with 5α-reductase activity as assessed by urinary metabolite ratios (5α-THF/THF). PAC levels were significantly correlated with 24h urinary TH-Aldo excretion (r=-0.214; p=0.036) and furthermore showed a significant negative association with the estimated glomerular filtration rate (eGFR) (r= -0.331, p<0.001). However, 24h urinary TH-Aldo excretion was not associated with eGFR.

Table 6-1 Patient characteristics at baseline.

	Placebo (n=56)	**Spironolactone (n=56)**
Male (%)	33 (59)	32 (57)
Age (years)	53 ± 12	54 ± 12
Body weight (kg)	74 ± 14	81 ± 14†
BMI (kg/m^2)	25.9 ± 3.8	28.0 ± 4.4†
Office blood pressure (mmHg)		
Systolic	130 ± 19	130 ± 16
Diastolic	77 ± 10	77 ± 10
Creatinine (µmol/l)	123 ± 35	132 ± 29
eGFR (ml/min/1.73m^2)	53 ± 12	49 ± 12
	Medication	
ACE Inhibitors (%)	39 (70)	38 (68)
Angiotensin receptor blockers (ARBs) (%)	19 (34)	19 (34)
Beta blockers (%)	8 (14)	15 (27)
Calcium channel blockers (%)	17 (30)	13 (23)
Diuretics (%)	13 (23)	18 (32)
Statins (%)	17 (30)	27 (48)

Values are Mean ± SD; †: p<0.01 spironolactone vs. placebo. Treatment groups were compared by the use of t-tests or chi-square.

Mean diastolic 24h ABP showed a significant correlation with 24h urinary TH-Aldo excretion (Figure 6-1) but not with PAC.

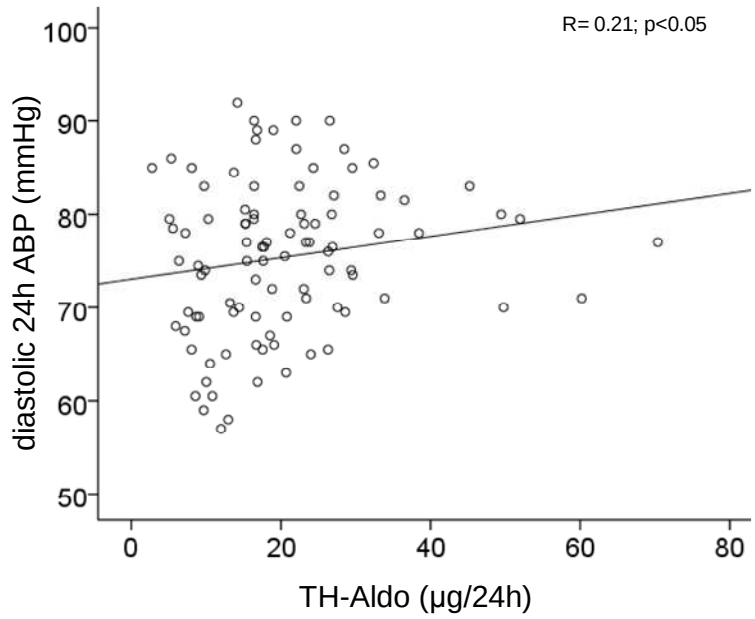

Figure 6-1 Correlation of 24h urinary TH-Aldo excretion with diastolic 24h ABP.

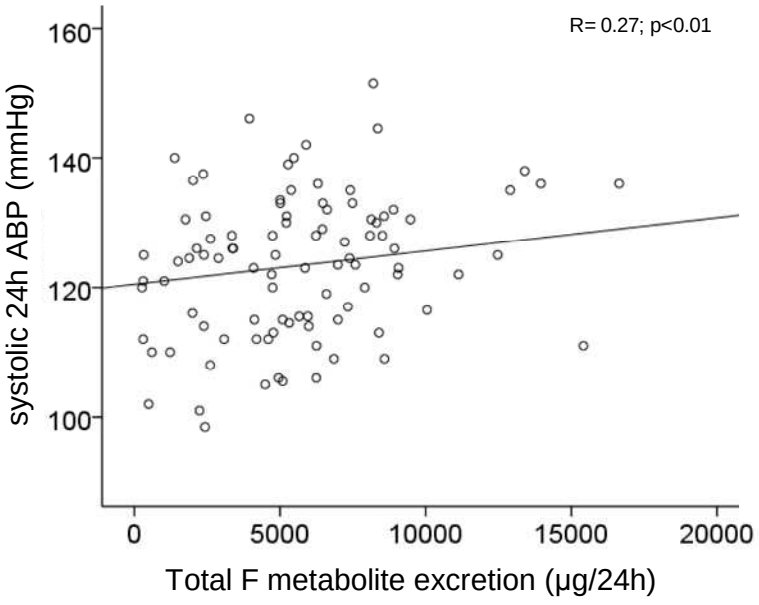

Figure 6-2 Correlation of 24h urinary total cortisol (F) metabolite excretion with systolic 24h ABP.

Moreover, after adjusting for age, BMI and eGFR, systolic 24h ABP was significantly associated with 24h urinary total cortisol metabolite excretion at baseline (Figure 6-2).

Table 6-2 Changes following placebo / spironolactone treatment.

	Placebo		**Spironolactone**	
	Week 0	Week 40	Week 0	Week 40
24 ABP (mmHg)				
Systolic	125 ± 12	124 ± 11	124 ± 11	119 ± 11†
Diastolic	77 ± 9	76 ± 7	76 ± 8	73 ± 8†
Na+ (mmol/l)	139 (138,141)	140 (139,142)	140 (139,142)	140 (138,141)
K+ (mmol/l)	4.3 (4.1,4.6)	4.3 (4.2,4.5)	4.4 (4.1,4.7)	4.5 (4.3,5.0)
Creatinine (mmol/l)	123 ± 35	126 ± 35	132 ± 29	140 ± 32††
eGFR (mmol/min/1.73m^2)	53 ± 12.3	52 ± 12.1	49 ± 12	46 ± 15.9
ACR (mg/mmol)	8.2 ± 48.4	9.5 ± 34.9	17.8 ± 48.6	5.4 ± 34.9†
	Renin Angiotensin Aldosterone System			
PRA (mU/l)	83 (34,138)	68 (42,148)	75 (36,146)	130 (72,260)††
Angiotensin II (pmol/l)	9.0 (4.6,17.0)	7.9 (4.5,20.1)	7.5 (4.5,18.2)	13.6 (6.2, 22.4)††
PAC (pmol/l)	186 (114,252)	166 (114,219)	133 (83,230)	360 (233, 557)††
	24h urinary steroid hormone metabolite excretion			
Total F (µg/24h)	5218 (2510,7370)	4938 (2992,7327)	6108 (3928,8663)	6024 (4040,8218)
5β-TH-DOC (µg/24h)	10.1 (7.0, 18.0)	12.0 (7.1,19.9)	14.4 (8.2,20.8)	10.4 (6.8,16.6)††
5α-TH-DOC (µg/24h)	3.1 (1.7,4.4)	3.1 (1.5,5.3)	2.8 (2.1,5.7)	2.7 (1.6,5.5)
TH-Aldo (µg/24h)	16.8 (13.3,26.5)	18.3 (12.8, 23.3)	18.9 (10.6, 26.3)	27.3 (17.4, 42.3)††
	Steroid hormone metabolite ratios			
5α-reductase (THF/5α-THF)	1.12 (0.82,2.13)	1.16 (0.83, 2.06)	1.26 (0.91,1.74)	1.10 (0.80,1.76)††
11β-HSD1 (5αTHF+THF/THE)	1.1 (0.8,1.3)	1.1 (0.83,1.37)	1.0 (0.7, 1.4)	1.1 (0.8, 1.5)
11β-HSD2 (F/E)	0.69 (0.59,0.87)	0.68 (0.55,0.94)	0.62 (0.51,0.84)	0.69 (0.54,0.83)
11β-hydroxylase (total F metabolites/THS)	98 (66, 137)	86 (50, 121)	105 (80, 151)	110 (79, 145)

Values are median (interquartile range). ACR: albumin creatinine ratio; TH-DOC: tetrahydro-DOC. Normally distributed values are presented as mean ± SD otherwise as mean (inter quartile range). To compare changes in the two groups repeated measures analysis of variance with the time point (week 0, week 40) as the 'within subjects' factor and the group (spironolactone and placebo) as the 'between subjects' factor were used. † p<0.01, †† p<0.01 spironolactone vs. placebo at week 40 (changes over time were analysed by repeated measures analysis of variance).

No correlation was found for systolic (r=0.156, p=0.1) or diastolic blood pressure (r=0.105, p=0.271), TH-Aldo (r=-0.112, p=0.284), eGFR (r=-0.097, p=0.313) and the ARR. Moreover, neither urinary (THF + 5α-THF / THE) ratio) reflecting global 11β-HSD1 activity, nor urinary free cortisol / cortisone (UFF/UFE) reflecting 11β-HSD type 2 activity were associated with blood pressure or eGFR (Table 6-2). However, PRA was positively associated with the urinary THF+5αTHF/THE ratio (Figure 6-3).

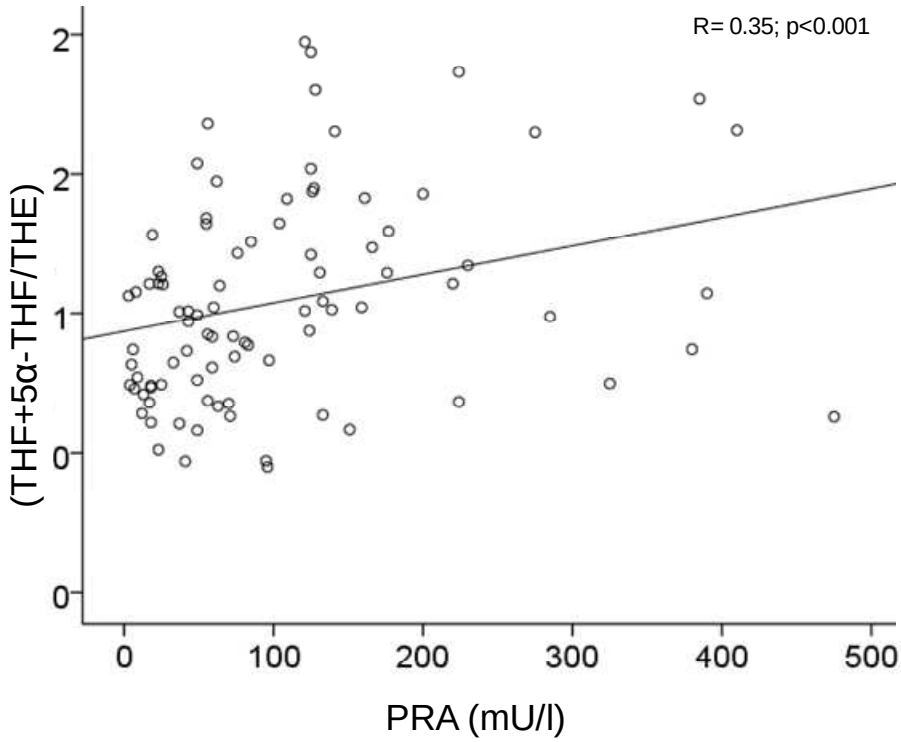

Figure 6-3 Correlation of PRA with the urinary THF+5αTHF/THE ratio reflecting global 11β-HSD1 activity.

24 patients were on stable glucocorticoid treatment for their underlying kidney disease throughout the study period. However, inclusion or exclusion of glucocorticoid treated patients did not have a significant effect on the analysis of steroid hormone metabolites (total glucocorticoid excretion, TH-Aldo, enzymatic activities) between the placebo and Spiro group. Treatment with Spiro compared to

placebo significantly reduced both systolic and diastolic 24h ABP despite a significant increase in circulating PRA, Ang II and Aldo levels (Table 6-2). Induction of the RAAS was reflected by a significant increase in 24h urinary TH-Aldo excretion, consistent with an increased aldosterone synthase activity (Table 6-2). By contrast, Spiro had no effect on global 11β-hydroxylase, 11β-HSD type 1 and 2 activity or total cortisol metabolite excretion as assessed by 24h urinary steroid hormone excretion.

6.4 Discussion

In this study we assessed corticosteroid hormone secretion and metabolism in CKD patients on stable treatment with ACEi or ARB before and after addition of Spiro by means of urinary steroid hormone metabolite analysis which allows assessment of cumulative 24h steroid hormone production and, secondly, an estimation of steroid hormone enzyme activities.

As expected, 24h urinary TH-Aldo excretion correlated with PAC although this association was weak. A possible explanation for this weak association could be that the 24h urine collection for TH-Aldo was performed on a different day than blood sampling for circulating Aldo was done. Additionally, Aldo levels are stimulated by ACTH during stress such as a venous puncture which is likely to vary between individuals. Circulating Aldo concentrations have previously been shown to correlate with blood pressure in black hypertensive (Kidambi et al., 2007) and the polycystic ovarian syndrome (Cascella et al., 2006) patients. However, we did not find a correlation of PAC with systolic or diastolic 24h ABP. In this study lack of association might be explained by the interfering effects of ACEi and ARB on the RAAS and variable stress induced PAC fluctuations during blood sampling. Nevertheless, cumulative 24h urinary TH-Aldo excretion reflecting 24h Aldo production was indeed positively correlated with 24h blood pressure as well as PAC. It was recently reported

that Aldo levels in CKD patients treated with an ACEi are lower compared to ARB treated patients (Haddad et al., 2007). However, in our study we did not find any significant differences in PAC or urinary TH-Aldo excretion, suggesting that ACEi and ARBs have a similar effect on Aldo secretion.

Total urinary 24h cortisol metabolite excretion is a well established measure to assess daily cortisol production rate and thus reflects activity of the HPA axis. In agreement with previous studies, we found total glucocorticoid metabolite excretion to be correlated with total body weight and BMI (Andrew et al., 2002). Furthermore, 24h glucocorticoid secretion rate was positively correlated with 24h systolic blood pressure after correction of potential confounders such as age, BMI, and eGFR, suggesting that increased total glucocorticoid production as a result of increased HPA activity is implicated in blood pressure regulation in CKD patients. Although the vast majority of patients with Cushing's syndrome exhibit hypertension (Hammer & Stewart, 2006), increased 24h cortisol production within the physiologic range has to our knowledge not been associated with blood pressure so far. Increased HPA-drive may therefore be a novel risk factor in CKD patients by driving blood pressure levels. These findings are in line with a recent report in which high yet within the normal range serum cortisol levels were found to be associated with an adverse outcome in heart failure patients (Guder et al., 2007). Further clinical studies are urgently needed to better define and understand the mechanisms of high-normal as opposed to low-normal cortisol levels on blood pressure and ultimately cardiovascular risk in these patients.

Previous studies on glucocorticoid metabolism in CKD patients suggest that 11β-HSD2 activity (N'Gankam et al., 2002; Whitworth et al., 1989) and expression (Quinkler et al., 2005) declines with reduced GFR which in turn may lead to increased MR activation by glucocorticoids leading to increased sodium retention and

blood pressure. In our study we did not find a correlation between renal function and the UFF / UFE ratio reflecting renal 11β-HSD2 activity. This is most likely explained by the relatively mild impairment in renal function in our cohort, whereas cohorts in previous studies included patients with more severe renal impairment as well as patients on haemodialysis.

Following Spiro treatment all components of the RAAS were significantly up-regulated including PRA, Ang II and PAC levels. Consistent with a RAAS activating effect, total 24h urinary TH-Aldo excretion was also significantly increased in the Spiro but not the placebo group. Our findings are in good agreement with a recent report on the neurohormonal effects of Spiro in patients with CHF and on stable ACEi treatment, which showed increased Ang II and Aldo levels following Spiro treatment (Rousseau et al., 2002). These findings suggest that MR blockade by Spiro results in a compensatory stimulation of the RAAS despite tonic inhibition by ACEi and ARBs. However, the degree of compensation inflicted by Spiro is not complete as both diastolic and systolic 24h ABP were reduced compared to baseline.

Previous reports suggested that Spiro and its metabolites exert direct inhibitory effects on steroidogenic enzymes (Cheng et al., 1976; Greiner et al., 1976). Here, using urinary steroid hormone metabolites as a surrogate, Spiro did not exert an obvious direct inhibitory effect on 11β-hydroxylase. The most likely explanation for this finding may be that the Spiro dose used in this study was too low to exert a measurable effect on 11β-hydroxylase compared to previous studies.

However, by contrast, we found an increased aldosterone synthase activity following Spiro treatment. AS is the final enzyme involved in Aldo production by converting DOC to Aldo in the *zona glomerulosa* and is regulated by circulating Ang II which was also significantly increased following Spiro. This finding is consistent with *in vitro*

studies which show increased expression of AS following Ang II treatment (Bird et al., 1993).

Compared to baseline, Spiro treatment did not alter total urinary 24h cortisol metabolite excretion in this study. The main conclusion that can be drawn from this observation is that MR blockade does not seem to interfere with the negative feedback mechanism of cortisol on the HPA axis. Although the hypothalamus mainly expresses the GR but not the MR (Pryce, 2008), the hippocampus readily expresses both receptors and is implicated in HPA axis modulation (de Kloet et al., 1998; Jacobson & Sapolsky, 1991). It is therefore conceivable that the negative feedback of cortisol is not solely mediated through the GR, particularly as the MR *in vitro* shows a 10 fold higher affinity for cortisol than the GR (Rupprecht et al., 1993). A number of studies have addressed the role of the MR in mediating negative cortisol feedback. Most studies have shown a short term stimulatory effect on the HPA axis following canrenoate infusion (Arvat et al., 2001; Dodt et al., 1993; Grottoli et al., 2002) or Spiro treatment (Heuser et al., 2000; Young et al., 1998). However, a recent study assessing the effects of the GR antagonist RU486 and Spiro alone or in combination on the HPA axis (Mattsson et al., 2009) showed that RU28486 and Spiro by themselves had no effect but in combination showed a significant compensatory activation of the HPA axis. It is difficult to compare the findings of these studies with our own results for a number of reasons. First of all we assessed HPA axis activity indirectly by means of urinary steroid metabolite excretion. Moreover, treatment duration and doses of MR antagonists were considerably different. Our study does not support a major effect of low dose Spiro on HPA axis modulation but equally does not rule out more subtle changes.

Indirect analysis of global 11β-HSD type 1 and 2 activity by means of urinary steroid hormone metabolite ratios did not reveal any significant differences following Spiro

treatment. It has been suggested that the UFF/UFE ratio more precisely reflects renal 11β-HSD2 activity than the (THF + 5α-THF) / THE ratio which is a better surrogate marker for global 11β-HSD1 activity (Palermo et al., 1996; Stewart et al., 1988). In the kidney the enzyme 11β-HSD2 protects the MR from illicit cortisol binding and thereby ensures Aldo specificity. Loss of function mutations are the underlying cause of severe hypertension and hypokalaemia in AME patients, whereas polymorphisms in HSD11B2 encoding for 11β-HSD2 have been associated with salt-sensitive essential hypertension (Hammer & Stewart, 2006). Spiro did not result in downregulation of 11β-HSD type 2 activity, which would allow cortisol to activate the MR and thereby lead to sodium and fluid retention, suggesting that only RAAS activation but not modulation of 11β-HSD2 in the kidney is involved in compensating MR antagonism.

Interestingly, global 11β-HSD1 activity was correlated with PRA in this study. We are not aware of a direct interaction of 11β-HSD1 activity and PRA but one could speculate that 11β-HSD1 activity and PRA are regulated by a common factor.

In summary, total 24h production of Aldo and cortisol are positively associated with blood pressure in CKD patients suggesting that adrenal hyperactivity may contribute to the hypertension and increased cardiovascular risk. Addition of Spiro resulted in compensatory RAAS activation but did not affect glucocorticoid production or metabolism.

Chapter 7 – Final Conclusions and Future Directions

The rationale for our interest to characterise and study corticosteroid hormone action in the CVS is based upon the adverse effects of corticosteroid hormones on cardiac structure and function which is highlighted in states of hormone excess such as Cushing's syndrome (Baykan et al., 2008; Muiesan et al., 2003) and primary hyperaldosteronism (Matsumura et al., 2006; Muiesan et al., 2008; Rossi et al., 1996). Furthermore, even in the absence of overt steroid excess corticosteroid hormones appear to play an adverse modulatory role in heart disease (Beygui et al., 2006; Guder et al., 2007; Palmer et al., 2008; Swedberg et al., 1990; Vantrimpont et al., 1998) and pharmacological blockade of the MR provides cardio protection (Fraccarollo et al., 2003; Fraccarollo et al., 2005; Rocha et al., 2000) and thereby reduces morbidity and mortality in heart failure patients (Pitt et al., 1996; Pitt et al., 2003a).

7.1 Experimental studies

We have characterised relative expression levels of key components involved in corticosteroid hormone signalling including GR, MR, 11β-HSD1 and 2 in isolated adult rat CM and cardiac MyoFb in culture. All components involved in corticosteroid signalling were readily expressed in adult CM and cardiac MyoFb with the exception of 11β-HSD2. Compared to liver, expression of 11β-HSD1 in CM was low whereas its abundance in cardiac MyoFb was high. These findings were underlined by enzymatic studies, which predominantly showed 11β-HSD oxoreductase as opposed to dehydrogenase activity. Moreover, in the heart 11β-HSD oxoreductase activity was dependent on the presence of H6PDH. Functional studies of 11β-HSD1 showed facilitated transcription of glucocorticoid target genes and an inhibitory effect on cardiac MyoFb proliferation.

It was recently shown that global 11β-HSD1 knock out mice exhibit increased neovascularisation and improved cardiac function following MI (McSweeney et al., 2010; Small et al., 2005) suggesting that glucocorticoids play a modulatory role in cardiac remodelling following MI. However, these studies do not provide an answer as to which cell type is responsible for the observed effects. Our results indicate that 11β-HSD1 is highly expressed and functionally active in MyoFb and thereby impacts on proliferation. McSweeney *et al* reported thicker infarct scars following MI in 11β-HSD1 knock out compared to WT mice. It is therefore intriguing to speculate that reduced cellular glucocorticoid levels in 11β-HSD1 knock out mice results in increased MyoFb proliferation and formation of thicker scars. Furthermore, it will be interesting to investigate if pharmacological inhibition 11β-HSD1 has similar effects which may provide a novel therapeutic strategy to improve outcome in MI patients. Besides MI, glucocorticoids may play a role in CHF as circulating glucocorticoid levels in these patients predict mortality (Guder et al., 2007). It will therefore be interesting to address if a reduction in local glucocorticoid regeneration by 11β-HSD1 will also impact on the course of CHF.

Our results in human cardiac biopsies suggest that cardiac hypertrophy is associated with increased glucocorticoid hormone signalling as we found increased expression levels of GR in cardiac biopsies from hypertrophied hearts. Future studies in CM specific GR knock out mice will clarify if increased GR expression plays a causative role or rather is a consequence of hypertrophy. In case of the former, it will be interesting to see if a reduction of local glucocorticoid hormone levels by pharmacological inhibition or inactivation of the 11β-HSD1 gene will result in amelioration of hypertrophy. Finally, animal models of CM specific GR and MR knock will be key to dissect the contribution of GR and MR not only in hypertrophy but also ischaemic heart disease and CHF.

To better understand differential effects on gene transcription by corticosteroid hormones we performed a microarray screening experiment. Our results show that transcriptional events were rather ligand than receptor dependent and that presence of GR and MR are required for a maximal biologic effect of corticosterone suggesting formation of GR-MR heterodimers. Moreover, we found a high degree of overlap in corticosterone and Aldo signalling via the GR and MR, respectively, but at the same time discovered several genes that were selectively regulated by Aldo and have previously been shown to play a role in cardiac disease. These genes may provide novel insights into the molecular mechanisms by which Aldo exerts its adverse effects on cardiac remodelling. Although these genes provide promising novel Aldo targets, future studies are required to validate and further characterise regulation of these genes and their downstream effects by Aldo.

7.2 Clinical studies

We studied the role of corticosteroid hormone action in patients with essential hypertension and the effects of Spiro upon corticosteroid hormone secretion and metabolism in patients with CKD. Our results suggest that mineralocorticoids but not glucocorticoids play a role in blood pressure modulation in essential hypertension as we found an increasing relative mineralocorticoid excess state with increasing blood pressure. However, the lack of a well matched control cohort did not allow us to compare our findings in hypertensives with those of healthy, normotensive controls which would substantiate our findings. Mahumud *et al* have shown that the ARR predicts the fall in blood pressure in patients with long standing, uncontrolled hypertension in response to treatment with Spiro (Mahmud et al., 2005). It would be interesting to see in future studies if these findings also apply to unselected patients with essential hypertension. If the findings by Mahmud *et al* hold true in such a

cohort, MR antagonists may become first line antihypertensive agents for all patients with essential hypertension who have an elevated ARR. It is conceivable that this will lead to better blood pressure control and as a consequence a lower cardiovascular risk in these patients. Moreover, our clinical study addressing the effects of Spiro in CKD patients shows that MR blockade results in reduced blood pressure levels despite compensatory up-regulation of the RAAS but did not affect glucocorticoid secretion or metabolism. These findings are reassuring as an increased in the HPA drive secondary to treatment with MR antagonists may have adverse metabolic effects.

The future studies described in this chapter will help to further determine the modulatory role and the underlying molecular mechanisms of corticosteroid hormone action in the CVS with the overall aim to improve outcome of cardiovascular disease in the future.

References

1997. Randomised, placebo-controlled trial of carvedilol in patients with congestive heart failure due to ischaemic heart disease. Australia/New Zealand Heart Failure Research Collaborative Group. Lancet 349, 375-380.

2003. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. J.Hypertens. 21, 1011-1053.

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H., Krieger, M., 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 271, 518-520.

Agarwal, A.K., Giacchetti, G., Lavery, G., Nikkila, H., Palermo, M., Ricketts, M., McTernan, C., Bianchi, G., Manunta, P., Strazzullo, P., Mantero, F., White, P.C., Stewart, P.M., 2000. CA-Repeat polymorphism in intron 1 of HSD11B2 : effects on gene expression and salt sensitivity. Hypertension 36, 187-194.

Agarwal, A.K., Mune, T., Monder, C., White, P.C., 1994. NAD(+)-dependent isoform of 11 beta-hydroxysteroid dehydrogenase. Cloning and characterization of cDNA from sheep kidney. J.Biol.Chem. 269, 25959-25962.

Agarwal, A.K., Rogerson, F.M., Mune, T., White, P.C., 1995. Gene structure and chromosomal localization of the human HSD11K gene encoding the kidney (type 2) isozyme of 11 beta-hydroxysteroid dehydrogenase. Genomics 29, 195-199.

Ahmed, A., Saksena, S., Sherlock, M., Olliff, S.P., Elias, E., Stewart, P.M., 2008. Induction of hepatic 11beta-hydroxysteroid dehydrogenase type 1 in patients with alcoholic liver disease. Clin Endocrinol (Oxf) 68, 898-903.

Albiston, A.L., Obeyesekere, V.R., Smith, R.E., Krozowski, Z.S., 1994. Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. Mol.Cell Endocrinol. 105, R11-R17.

Alikhani-Koupaei, R., Fouladkou, F., Fustier, P., Cenni, B., Sharma, A.M., Deter, H.C., Frey, B.M., Frey, F.J., 2007. Identification of polymorphisms in the human 11beta-hydroxysteroid dehydrogenase type 2 gene promoter: functional characterization and relevance for salt sensitivity. FASEB J. 21, 3618-3628.

Alvarez-Madrazo, S., Padmanabhan, S., Mayosi, B.M., Watkins, H., Avery, P., Wallace, A.M., Fraser, R., Davies, E., Keavney, B., Connell, J.M., 2009. Familial and phenotypic associations of the aldosterone Renin ratio. J Clin Endocrinol Metab 94, 4324-4333.

Andrew, R., Gale, C.R., Walker, B.R., Seckl, J.R., Martyn, C.N., 2002. Glucocorticoid metabolism and the Metabolic Syndrome: associations in an elderly cohort. Exp.Clin.Endocrinol.Diabetes 110, 284-290.

Arlt, W., Stewart, P.M., 2005. Adrenal corticosteroid biosynthesis, metabolism, and action. Endocrinol.Metab Clin.North Am. 34, 293-313, viii.

Arnaldi, G., Mancini, T., Polenta, B., Boscaro, M., 2004. Cardiovascular risk in Cushing's syndrome. Pituitary. 7, 253-256.

Arner, P., 2005. Human fat cell lipolysis: biochemistry, regulation and clinical role. Best.Pract.Res.Clin.Endocrinol.Metab 19, 471-482.

Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E., Evans, R.M., 1987. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237, 268-275.

Arvat, E., Maccagno, B., Giordano, R., Pellegrino, M., Broglio, F., Gianotti, L., Maccario, M., Camanni, F., Ghigo, E., 2001. Mineralocorticoid receptor blockade by canrenoate increases both spontaneous and stimulated adrenal function in humans. J.Clin.Endocrinol.Metab 86, 3176-3181.

Barnett, C.A., Pritchett, E.L., 1988. Detection of corticosteroid type I binding sites in heart. Mol.Cell Endocrinol. 56, 191-198.

Barreto-Chaves, M.L., Heimann, A., Krieger, J.E., 2000. Stimulatory effect of dexamethasone on angiotensin-converting enzyme in neonatal rat cardiac myocytes. Braz.J.Med.Biol.Res. 33, 661-664.

Baykan, M., Erem, C., Gedikli, O., Hacihasanoglu, A., Erdogan, T., Kocak, M., Kaplan, S., Kiris, A., Orem, C., Celik, S., 2008. Assessment of left ventricular diastolic function and Tei index by tissue Doppler imaging in patients with Cushing's Syndrome. Echocardiography. 25, 182-190.

Beggah, A.T., Escoubet, B., Puttini, S., Cailmail, S., Delage, V., Ouvrard-Pascaud, A., Bocchi, B., Peuchmaur, M., Delcayre, C., Farman, N., Jaisser, F., 2002. Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. Proc.Natl.Acad.Sci.U.S.A 99, 7160-7165.

Benitah, J.P., Perrier, E., Gomez, A.M., Vassort, G., 2001. Effects of aldosterone on transient outward K+ current density in rat ventricular myocytes. J.Physiol 537, 151-160.

Benitah, J.P., Vassort, G., 1999. Aldosterone upregulates Ca(2+) current in adult rat cardiomyocytes. Circ.Res. 85, 1139-1145.

Benjamin, E.J., Rice, K.M., Arking, D.E., Pfeufer, A., van Noord, C., Smith, A.V., Schnabel, R.B., Bis, J.C., Boerwinkle, E., Sinner, M.F., Dehghan, A., Lubitz, S.A., D'Agostino, R.B., Sr., Lumley, T., Ehret, G.B., Heeringa, J., Aspelund, T., Newton-Cheh, C., Larson, M.G., Marciante, K.D., Soliman, E.Z., Rivadeneira, F., Wang, T.J., Eiriksdottir, G., Levy, D., Psaty, B.M., Li, M., Chamberlain, A.M., Hofman, A., Vasan, R.S., Harris, T.B., Rotter, J.I., Kao, W.H., Agarwal, S.K., Stricker, B.H., Wang, K., Launer, L.J., Smith, N.L., Chakravarti, A., Uitterlinden, A.G., Wolf, P.A., Sotoodehnia, N., Kottgen, A., van Duijn, C.M., Meitinger, T., Mueller, M., Perz, S., Steinbeck, G., Wichmann, H.E., Lunetta, K.L., Heckbert, S.R., Gudnason, V., Alonso, A., Kaab, S., Ellinor, P.T., Witteman, J.C., 2009. Variants in ZFHX3 are associated with atrial fibrillation in individuals of European ancestry. Nat Genet 41, 879-881.

Bens, M., Chassin, C., Vandewalle, A., 2006. Regulation of NaCl transport in the renal collecting duct: lessons from cultured cells. Pflugers Arch. 453, 133-146.

Beretta-Piccoli, C., Davies, D.L., Brown, J.J., Ferriss, B., Fraser, R., Lasaridis, A., Lever, A.F., Morton, J.J., Robertson, J.I., Semple, P.F., 1983. Relation of

blood pressure with body and plasma electrolytes in Conn's syndrome. J.Hypertens. 1, 197-205.

Berger, S., Bleich, M., Schmid, W., Cole, T.J., Peters, J., Watanabe, H., Kriz, W., Warth, R., Greger, R., Schutz, G., 1998. Mineralocorticoid receptor knockout mice: pathophysiology of Na+ metabolism. Proc.Natl.Acad.Sci.U.S.A 95, 9424-9429.

Berk, B.C., Fujiwara, K., Lehoux, S., 2007. ECM remodeling in hypertensive heart disease. J.Clin.Invest 117, 568-575.

Beygui, F., Collet, J.P., Benoliel, J.J., Vignolles, N., Dumaine, R., Barthelemy, O., Montalescot, G., 2006. High plasma aldosterone levels on admission are associated with death in patients presenting with acute ST-elevation myocardial infarction. Circulation 114, 2604-2610.

Bird, I.M., Hanley, N.A., Word, R.A., Mathis, J.M., McCarthy, J.L., Mason, J.I., Rainey, W.E., 1993. Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion. Endocrinology 133, 1555-1561.

Boixel, C., Gavillet, B., Rougier, J.S., Abriel, H., 2006. Aldosterone increases voltage-gated sodium current in ventricular myocytes. Am.J.Physiol Heart Circ.Physiol 290, H2257-H2266.

Boldyreff, B., Wehling, M., 2003. Rapid aldosterone actions: from the membrane to signaling cascades to gene transcription and physiological effects. J.Steroid Biochem.Mol.Biol. 85, 375-381.

Bomback, A.S., Klemmer, P.J., 2007. The incidence and implications of aldosterone breakthrough. Nat.Clin.Pract.Nephrol. 3, 486-492.

Borghi, C., Boschi, S., Ambrosioni, E., Melandri, G., Branzi, A., Magnani, B., 1993. Evidence of a partial escape of renin-angiotensin-aldosterone blockade in patients with acute myocardial infarction treated with ACE inhibitors. J.Clin.Pharmacol. 33, 40-45.

Brereton, P.S., van Driel, R.R., Suhaimi, F., Koyama, K., Dilley, R., Krozowski, Z., 2001. Light and electron microscopy localization of the 11beta-hydroxysteroid dehydrogenase type I enzyme in the rat. Endocrinology 142, 1644-1651.

Bridgham, J.T., Carroll, S.M., Thornton, J.W., 2006. Evolution of hormone-receptor complexity by molecular exploitation. Science 312, 97-101.

Brien, T.G., 1981. Human corticosteroid binding globulin. Clin.Endocrinol.(Oxf) 14, 193-212.

Brilla, C.G., Matsubara, L.S., Weber, K.T., 1993. Anti-aldosterone treatment and the prevention of myocardial fibrosis in primary and secondary hyperaldosteronism. J.Mol.Cell Cardiol. 25, 563-575.

Brilla, C.G., Pick, R., Tan, L.B., Janicki, J.S., Weber, K.T., 1990. Remodeling of the rat right and left ventricles in experimental hypertension. Circ.Res. 67, 1355-1364.

Brilla, C.G., Weber, K.T., 1992. Mineralocorticoid excess, dietary sodium, and myocardial fibrosis. J.Lab Clin.Med. 120, 893-901.

Brilla, C.G., Zhou, G., Matsubara, L., Weber, K.T., 1994. Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. J.Mol.Cell Cardiol. 26, 809-820.

Brown, R.D., Ambler, S.K., Mitchell, M.D., Long, C.S., 2005. The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. Annu.Rev.Pharmacol.Toxicol. 45, 657-687.

Brown, R.W., Chapman, K.E., Edwards, C.R., Seckl, J.R., 1993. Human placental 11 beta-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. Endocrinology 132, 2614-2621.

Brown, R.W., Chapman, K.E., Murad, P., Edwards, C.R., Seckl, J.R., 1996a. Purification of 11 beta-hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity labelling technique. Biochem.J. 313 (Pt 3), 997-1005.

Brown, R.W., Diaz, R., Robson, A.C., Kotelevtsev, Y.V., Mullins, J.J., Kaufman, M.H., Seckl, J.R., 1996b. The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. Endocrinology 137, 794-797.

Bulkley, B.H., Roberts, W.C., 1974. Steroid therapy during acute myocardial infarction. A cause of delayed healing and of ventricular aneurysm. Am J Med 56, 244-250.

Burniston, J.G., Saini, A., Tan, L.B., Goldspink, D.F., 2005. Aldosterone induces myocyte apoptosis in the heart and skeletal muscles of rats in vivo. J.Mol.Cell Cardiol. 39, 395-399.

Calhoun, D.A., Nishizaka, M.K., Zaman, M.A., Thakkar, R.B., Weissmann, P., 2002. Hyperaldosteronism among black and white subjects with resistant hypertension. Hypertension 40, 892-896.

Camelliti, P., Borg, T.K., Kohl, P., 2005. Structural and functional characterisation of cardiac fibroblasts. Cardiovasc.Res. 65, 40-51.

Card, W.I., Mitchell, W., Strong, J.A., Taylor, N.R., Tompsett, S.L., Wilson, J.M., 1953. Effects of liquorice and its derivatives on salt and water metabolism. Lancet 1, 663-668.

Carvajal, C.A., Romero, D.G., Mosso, L.M., Gonzalez, A.A., Campino, C., Montero, J., Fardella, C.E., 2005. Biochemical and genetic characterization of 11 beta-hydroxysteroid dehydrogenase type 2 in low-renin essential hypertensives. J.Hypertens. 23, 71-77.

Cascella, T., Palomba, S., Tauchmanova, L., Manguso, F., Di Biase, S., Labella, D., Giallauria, F., Vigorito, C., Colao, A., Lombardi, G., Orio, F., 2006. Serum aldosterone concentration and cardiovascular risk in women with polycystic ovarian syndrome. J.Clin.Endocrinol.Metab 91, 4395-4400.

Centenera, M.M., Harris, J.M., Tilley, W.D., Butler, L.M., 2008. The contribution of different androgen receptor domains to receptor dimerization and signaling. Mol Endocrinol 22, 2373-2382.

Chapman, N., Dobson, J., Wilson, S., Dahlof, B., Sever, P.S., Wedel, H., Poulter, N.R., 2007. Effect of spironolactone on blood pressure in subjects with resistant hypertension. Hypertension 49, 839-845.

Cheng, S.C., Suzuki, K., Sadee, W., Harding, B.W., 1976. Effects of spironolactone, canrenone and canrenoate-K on cytochrome P450, and 11beta- and 18-hydroxylation in bovine and human adrenal cortical mitochondria. Endocrinology 99, 1097-1106.

Choi, J.W., Herr, D.R., Noguchi, K., Yung, Y.C., Lee, C.W., Mutoh, T., Lin, M.E., Teo, S.T., Park, K.E., Mosley, A.N., Chun, J., LPA receptors: subtypes and biological actions. Annu Rev Pharmacol Toxicol 50, 157-186.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal.Biochem. 162, 156-159.

Chun, T.Y., Bloem, L.J., Pratt, J.H., 2003. Aldosterone inhibits inducible nitric oxide synthase in neonatal rat cardiomyocytes. Endocrinology 144, 1712-1717.

Cohn, J.N., Ferrari, R., Sharpe, N., 2000. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J.Am.Coll.Cardiol. 35, 569-582.

Coirini, H., Magarinos, A.M., De Nicola, A.F., Rainbow, T.C., McEwen, B.S., 1985. Further studies of brain aldosterone binding sites employing new mineralocorticoid and glucocorticoid receptor markers in vitro. Brain Res 361, 212-216.

Cole, T.J., 1995. Cloning of the mouse 11 beta-hydroxysteroid dehydrogenase type 2 gene: tissue specific expression and localization in distal convoluted tubules and collecting ducts of the kidney. Endocrinology 136, 4693-4696.

Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., Schutz, G., 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. 9, 1608-1621.

Coppage, W.S., Jr., Island, D.P., Cooner, A.E., Liddle, G.W., 1962. The metabolism of aldosterone in normal subjects and in patients with hepatic cirrhosis. J.Clin.Invest 41, 1672-1680.

Davies, E., Holloway, C.D., Ingram, M.C., Inglis, G.C., Friel, E.C., Morrison, C., Anderson, N.H., Fraser, R., Connell, J.M., 1999. Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2. Hypertension 33, 703-707.

Davies, E., Mackenzie, S.M., Freel, E.M., Alvarez-Madrazo, S., Fraser, R., Connell, J.M., 2009. Altered corticosteroid biosynthesis in essential hypertension: A digenic phenomenon. Mol Cell Endocrinol 300, 185-191.

De Bosscher, K., Van Craenenbroeck, K., Meijer, O.C., Haegeman, G., 2008. Selective transrepression versus transactivation mechanisms by glucocorticoid receptor modulators in stress and immune systems. Eur.J.Pharmacol. 583, 290-302.

de Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., Joels, M., 1998. Brain corticosteroid receptor balance in health and disease. Endocr.Rev. 19, 269-301.

de Vries, W.B., van der Leij, F.R., Bakker, J.M., Kamphuis, P.J., van Oosterhout, M.F., Schipper, M.E., Smid, G.B., Bartelds, B., van Bel, F., 2002. Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr.Res. 52, 900-906.

Deroo, B.J., Archer, T.K., 2001. Glucocorticoid receptor-mediated chromatin remodeling in vivo. Oncogene 20, 3039-3046.

Dickstein, K., Cohen-Solal, A., Filippatos, G., McMurray, J.J., Ponikowski, P., Poole-Wilson, P.A., Stromberg, A., van Veldhuisen, D.J., Atar, D., Hoes, A.W., Keren, A., Mebazaa, A., Nieminen, M., Priori, S.G., Swedberg, K., Vahanian, A., Camm, J., De Caterina, R., Dean, V., Funck-Brentano, C., Hellemans, I., Kristensen, S.D., McGregor, K., Sechtem, U., Silber, S., Tendera, M., Widimsky, P., Zamorano, J.L., 2008. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur.Heart J. 29, 2388-2442.

Dodt, C., Kern, W., Fehm, H.L., Born, J., 1993. Antimineralocorticoid canrenoate enhances secretory activity of the hypothalamus-pituitary-adrenocortical (HPA) axis in humans. Neuroendocrinology 58, 570-574.

Doi, T., Sakoda, T., Akagami, T., Naka, T., Mori, Y., Tsujino, T., Masuyama, T., Ohyanagi, M., 2008. Aldosterone induces interleukin-18 through endothelin-1, angiotensin II, Rho/Rho-kinase, and PPARs in cardiomyocytes. Am.J.Physiol Heart Circ.Physiol 295, H1279-H1287.

Douma, S., Petidis, K., Doumas, M., Papaefthimiou, P., Triantafyllou, A., Kartali, N., Papadopoulos, N., Vogiatzis, K., Zamboulis, C., 2008. Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. Lancet 371, 1921-1926.

Draper, N., Walker, E.A., Bujalska, I.J., Tomlinson, J.W., Chalder, S.M., Arlt, W., Lavery, G.G., Bedendo, O., Ray, D.W., Laing, I., Malunowicz, E., White, P.C., Hewison, M., Mason, P.J., Connell, J.M., Shackleton, C.H., Stewart, P.M., 2003. Mutations in the genes encoding 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. Nat.Genet. 34, 434-439.

Duperrex, H., Kenouch, S., Gaeggeler, H.P., Seckl, J.R., Edwards, C.R., Farman, N., Rossier, B.C., 1993. Rat liver 11 beta-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid encodes oxoreductase activity in a mineralocorticoid-responsive toad bladder cell line. Endocrinology 132, 612-619.

Duprez, D.A., Bauwens, F.R., De Buyzere, M.L., De Backer, T.L., Kaufman, J.M., Van Hoecke, J., Vermeulen, A., Clement, D.L., 1993. Influence of arterial blood pressure and aldosterone on left ventricular hypertrophy in moderate essential hypertension. Am.J.Cardiol. 71, 17A-20A.

Durant, S., Duval, D., Homo-Delarche, F., 1986. Factors involved in the control of fibroblast proliferation by glucocorticoids: a review. Endocr Rev 7, 254-269.

Edwards, C.R., Stewart, P.M., Burt, D., Brett, L., McIntyre, M.A., Sutanto, W.S., de Kloet, E.R., Monder, C., 1988. Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. Lancet 2, 986-989.

Edwards, N.C., Steeds, R.P., Stewart, P.M., Ferro, C.J., Townend, J.N., 2009. Effect of spironolactone on left ventricular mass and aortic stiffness in early-stage chronic kidney disease: a randomized controlled trial. J.Am.Coll.Cardiol. 54, 505-512.

El-Helou, V., Proulx, C., Gosselin, H., Clement, R., Mimee, A., Villeneuve, L., Calderone, A., 2008. Dexamethasone treatment of post-MI rats attenuates sympathetic innervation of the infarct region. J Appl Physiol 104, 150-156.

Esteban, N.V., Loughlin, T., Yergey, A.L., Zawadzki, J.K., Booth, J.D., Winterer, J.C., Loriaux, D.L., 1991. Daily cortisol production rate in man determined by stable isotope dilution/mass spectrometry. J.Clin.Endocrinol.Metab 72, 39-45.

Etxabe, J., Vazquez, J.A., 1994. Morbidity and mortality in Cushing's disease: an epidemiological approach. Clin.Endocrinol.(Oxf) 40, 479-484.

Fagard, R.H., Cornelissen, V.A., 2007. Incidence of cardiovascular events in white-coat, masked and sustained hypertension versus true normotension: a meta-analysis. J Hypertens 25, 2193-2198.

Farman, N., Rafestin-Oblin, M.E., 2001. Multiple aspects of mineralocorticoid selectivity. Am J Physiol Renal Physiol 280, F181-192.

Fejes-Toth, G., Naray-Fejes-Toth, A., 2007. Early aldosterone-regulated genes in cardiomyocytes: clues to cardiac remodeling? Endocrinology 148, 1502-1510.

Ferrari, P., 2009. The role of 11beta-hydroxysteroid dehydrogenase type 2 in human hypertension. Biochim Biophys Acta.

Filipeanu, C.M., Zhou, F., Claycomb, W.C., Wu, G., 2004. Regulation of the cell surface expression and function of angiotensin II type 1 receptor by Rab1-mediated endoplasmic reticulum-to-Golgi transport in cardiac myocytes. J Biol Chem 279, 41077-41084.

Fraccarollo, D., Galuppo, P., Hildemann, S., Christ, M., Ertl, G., Bauersachs, J., 2003. Additive improvement of left ventricular remodeling and neurohormonal activation by aldosterone receptor blockade with eplerenone and ACE inhibition in rats with myocardial infarction. J.Am.Coll.Cardiol. 42, 1666-1673.

Fraccarollo, D., Galuppo, P., Schmidt, I., Ertl, G., Bauersachs, J., 2005. Additive amelioration of left ventricular remodeling and molecular alterations by combined aldosterone and angiotensin receptor blockade after myocardial infarction. Cardiovasc.Res. 67, 97-105.

Fraccarollo, D., Galuppo, P., Schraut, S., Kneitz, S., van Rooijen, N., Ertl, G., Bauersachs, J., 2008. Immediate mineralocorticoid receptor blockade improves myocardial infarct healing by modulation of the inflammatory response. Hypertension 51, 905-914.

Freel, E.M., Ingram, M., Friel, E.C., Fraser, R., Brown, M., Samani, N.J., Caulfield, M., Munroe, P., Farrall, M., Webster, J., Clayton, D., Dominiczak, A.F., Davies, E., Connell, J.M., 2007. Phenotypic consequences of variation across the aldosterone synthase and 11-beta hydroxylase locus in a hypertensive cohort: data from the MRC BRIGHT Study. Clin Endocrinol (Oxf) 67, 832-838.

Freel, E.M., Ingram, M., Wallace, A.M., White, A., Fraser, R., Davies, E., Connell, J.M., 2008. Effect of variation in CYP11B1 and CYP11B2 on corticosteroid phenotype and hypothalamic-pituitary-adrenal axis activity in hypertensive and normotensive subjects. Clin Endocrinol (Oxf) 68, 700-706.

Fullerton, M.J., Funder, J.W., 1994. Aldosterone and cardiac fibrosis: in vitro studies. Cardiovasc.Res. 28, 1863-1867.

Funder, J.W., Carey, R.M., Fardella, C., Gomez-Sanchez, C.E., Mantero, F., Stowasser, M., Young, W.F., Jr., Montori, V.M., 2008. Case detection,

diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 93, 3266-3281.

Funder, J.W., Pearce, P.T., Smith, R., Smith, A.I., 1988. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 242, 583-585.

Garg, R., Yusuf, S., 1995. Overview of randomized trials of angiotensin-converting enzyme inhibitors on mortality and morbidity in patients with heart failure. Collaborative Group on ACE Inhibitor Trials. JAMA 273, 1450-1456.

Garnier, A., Bendall, J.K., Fuchs, S., Escoubet, B., Rochais, F., Hoerter, J., Nehme, J., Ambroisine, M.L., De Angelis, N., Morineau, G., d'Estienne, P., Fischmeister, R., Heymes, C., Pinet, F., Delcayre, C., 2004. Cardiac specific increase in aldosterone production induces coronary dysfunction in aldosterone synthase-transgenic mice. Circulation 110, 1819-1825.

Ge, R., Huang, Y., Liang, G., Li, X., 11beta-hydroxysteroid dehydrogenase type 1 inhibitors as promising therapeutic drugs for diabetes: status and development. Curr Med Chem 17, 412-422.

Genest, J., Nowaczynski, W., Boucher, R., Kuchel, O., Rojo-Ortega, J.M., 1975. Aldosterone and renin in essential hypertension. Can Med Assoc J 113, 421-431.

Gillis, S., Crabtree, G.R., Smith, K.A., 1979. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. J.Immunol. 123, 1624-1631.

Go, A.S., Chertow, G.M., Fan, D., McCulloch, C.E., Hsu, C.Y., 2004. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N.Engl.J.Med. 351, 1296-1305.

Gomez-Sanchez, E.P., Ahmad, N., Romero, D.G., Gomez-Sanchez, C.E., 2004. Origin of aldosterone in the rat heart. Endocrinology 145, 4796-4802.

Gomez-Sanchez, E.P., Romero, D.G., de Rodriguez, A.F., Warden, M.P., Krozowski, Z., Gomez-Sanchez, C.E., 2008. Hexose-6-phosphate dehydrogenase and 11beta-hydroxysteroid dehydrogenase-1 tissue distribution in the rat. Endocrinology 149, 525-533.

Gomez Sanchez, E.P., 1995. Mineralocorticoid modulation of central control of blood pressure. Steroids 60, 69-72.

Greiner, J.W., Kramer, R.E., Jarrell, J., Colby, H.D., 1976. Mechanism of action of spironolactone on adrenocortical function in guinea pigs. J.Pharmacol.Exp.Ther. 198, 709-715.

Grottoli, S., Giordano, R., Maccagno, B., Pellegrino, M., Ghigo, E., Arvat, E., 2002. The stimulatory effect of canrenoate, a mineralocorticoid antagonist, on the activity of the hypothalamus-pituitary-adrenal axis is abolished by alprazolam, a benzodiazepine, in humans. J.Clin.Endocrinol.Metab 87, 4616-4620.

Guder, G., Bauersachs, J., Frantz, S., Weismann, D., Allolio, B., Ertl, G., Angermann, C.E., Stork, S., 2007. Complementary and incremental mortality risk prediction by cortisol and aldosterone in chronic heart failure. Circulation 115, 1754-1761.

Gupta, M., Sueblinvong, V., Raman, J., Jeevanandam, V., Gupta, M.P., 2003. Single-stranded DNA-binding proteins PURalpha and PURbeta bind to a purine-rich negative regulatory element of the alpha-myosin heavy chain gene and control

transcriptional and translational regulation of the gene expression. Implications in the repression of alpha-myosin heavy chain during heart failure. J Biol Chem 278, 44935-44948.

Gupta, M., Zak, R., Libermann, T.A., Gupta, M.P., 1998. Tissue-restricted expression of the cardiac alpha-myosin heavy chain gene is controlled by a downstream repressor element containing a palindrome of two ets-binding sites. Mol Cell Biol 18, 7243-7258.

Haddad, N., Rajan, J., Nagaraja, H.N., Agarwal, A.K., Hebert, L.A., 2007. Usual ACE inhibitor therapy in CKD patients is associated with lower plasma aldosterone levels than usual angiotensin receptor blocker therapy. Kidney Blood Press Res. 30, 299-305.

Hadoke, P.W., Iqbal, J., Walker, B.R., 2009. Therapeutic manipulation of glucocorticoid metabolism in cardiovascular disease. Br J Pharmacol 156, 689-712.

Haley, S.A., Zhao, T., Zou, L., Klysik, J.E., Padbury, J.F., Kochilas, L.K., 2008. Forced expression of the cell cycle inhibitor p57Kip2 in cardiomyocytes attenuates ischemia-reperfusion injury in the mouse heart. BMC Physiol 8, 4.

Hammer, F., Stewart, P.M., 2006. Cortisol metabolism in hypertension. Best.Pract.Res.Clin.Endocrinol.Metab 20, 337-353.

Harada, E., Yoshimura, M., Yasue, H., Nakagawa, O., Nakagawa, M., Harada, M., Mizuno, Y., Nakayama, M., Shimasaki, Y., Ito, T., Nakamura, S., Kuwahara, K., Saito, Y., Nakao, K., Ogawa, H., 2001. Aldosterone induces angiotensin-converting-enzyme gene expression in cultured neonatal rat cardiocytes. Circulation 104, 137-139.

Hardy, R.S., Filer, A., Cooper, M.S., Parsonage, G., Raza, K., Hardie, D.L., Rabbitt, E.H., Stewart, P.M., Buckley, C.D., Hewison, M., 2006. Differential expression, function and response to inflammatory stimuli of 11beta-hydroxysteroid dehydrogenase type 1 in human fibroblasts: a mechanism for tissue-specific regulation of inflammation. Arthritis Res Ther 8, R108.

Hatakeyama, H., Inaba, S., Miyamori, I., 1999. 11beta-hydroxysteroid dehydrogenase in cultured human vascular cells. Possible role in the development of hypertension. Hypertension 33, 1179-1184.

Heuser, I., Deuschle, M., Weber, B., Stalla, G.K., Holsboer, F., 2000. Increased activity of the hypothalamus-pituitary-adrenal system after treatment with the mineralocorticoid receptor antagonist spironolactone. Psychoneuroendocrinology 25, 513-518.

Hilal-Dandan, R., Means, C.K., Gustafsson, A.B., Morissette, M.R., Adams, J.W., Brunton, L.L., Heller Brown, J., 2004. Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho. J Mol Cell Cardiol 36, 481-493.

Hillege, H.L., Girbes, A.R., de Kam, P.J., Boomsma, F., de Zeeuw, D., Charlesworth, A., Hampton, J.R., van Veldhuisen, D.J., 2000. Renal function, neurohormonal activation, and survival in patients with chronic heart failure. Circulation 102, 203-210.

Ikeda, U., Hyman, R., Smith, T.W., Medford, R.M., 1991. Aldosterone-mediated regulation of Na+, K(+)-ATPase gene expression in adult and neonatal rat cardiocytes. J.Biol.Chem. 266, 12058-12066.

Iravanian, S., Dudley, S.C., Jr., 2008. The renin-angiotensin-aldosterone system (RAAS) and cardiac arrhythmias. Heart Rhythm 5, S12-17.

Iwai, A., Fujii, Y., Kawakami, S., Takazawa, R., Kageyama, Y., Yoshida, M.A., Kihara, K., 2004. Down-regulation of vascular endothelial growth factor in renal cell carcinoma cells by glucocorticoids. Mol.Cell Endocrinol. 226, 11-17.

Jacobson, L., Sapolsky, R., 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr.Rev. 12, 118-134.

Jessup, M., Brozena, S., 2003. Heart failure. N.Engl.J.Med. 348, 2007-2018.

Johar, S., Cave, A.C., Narayanapanicker, A., Grieve, D.J., Shah, A.M., 2006. Aldosterone mediates angiotensin II-induced interstitial cardiac fibrosis via a Nox2-containing NADPH oxidase. FASEB J. 20, 1546-1548.

Juenger, J., Schellberg, D., Kraemer, S., Haunstetter, A., Zugck, C., Herzog, W., Haass, M., 2002. Health related quality of life in patients with congestive heart failure: comparison with other chronic diseases and relation to functional variables. Heart 87, 235-241.

Jugdutt, B.I., 2003. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? Circulation 108, 1395-1403.

Kalinyak, J.E., Dorin, R.I., Hoffman, A.R., Perlman, A.J., 1987. Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. J.Biol.Chem. 262, 10441-10444.

Kamynina, E., Staub, O., 2002. Concerted action of ENaC, Nedd4-2, and Sgk1 in transepithelial Na(+) transport. Am.J.Physiol Renal Physiol 283, F377-F387.

Karagiannis, A., Tziomalos, K., Papageorgiou, A., Kakafika, A.I., Pagourelias, E.D., Anagnostis, P., Athyros, V.G., Mikhailidis, D.P., 2008. Spironolactone versus eplerenone for the treatment of idiopathic hyperaldosteronism. Expert.Opin.Pharmacother. 9, 509-515.

Kayes-Wandover, K.M., White, P.C., 2000. Steroidogenic enzyme gene expression in the human heart. J.Clin.Endocrinol.Metab 85, 2519-2525.

Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K., He, J., 2005. Global burden of hypertension: analysis of worldwide data. Lancet 365, 217-223.

Kellner, M., Peiter, A., Hafner, M., Feuring, M., Christ, M., Wehling, M., Falkenstein, E., Losel, R., 2003. Early aldosterone up-regulated genes: new pathways for renal disease? Kidney Int 64, 1199-1207.

Kidambi, S., Kotchen, J.M., Grim, C.E., Raff, H., Mao, J., Singh, R.J., Kotchen, T.A., 2007. Association of adrenal steroids with hypertension and the metabolic syndrome in blacks. Hypertension 49, 704-711.

Kiryluk, K., Isom, R., 2007. Thiazolidinediones and fluid retention. Kidney Int. 72, 762-768.

Kotelevtsev, Y., Brown, R.W., Fleming, S., Kenyon, C., Edwards, C.R., Seckl, J.R., Mullins, J.J., 1999. Hypertension in mice lacking 11beta-hydroxysteroid dehydrogenase type 2. J.Clin.Invest 103, 683-689.

Kotelevtsev, Y., Holmes, M.C., Burchell, A., Houston, P.M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C.R., Seckl, J.R., Mullins, J.J., 1997. 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated

glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proc.Natl.Acad.Sci.U.S.A 94, 14924-14929.

Kraemer, F.B., 2007. Adrenal cholesterol utilization. Mol.Cell Endocrinol. 265-266, 42-45.

Kuster, G.M., Kotlyar, E., Rude, M.K., Siwik, D.A., Liao, R., Colucci, W.S., Sam, F., 2005. Mineralocorticoid receptor inhibition ameliorates the transition to myocardial failure and decreases oxidative stress and inflammation in mice with chronic pressure overload. Circulation 111, 420-427.

La Mear, N.S., MacGilvray, S.S., Myers, T.F., 1997. Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol.Neonate 72, 175-180.

Lakshmi, V., Monder, C., 1988. Purification and characterization of the corticosteroid 11 beta-dehydrogenase component of the rat liver 11 beta-hydroxysteroid dehydrogenase complex. Endocrinology 123, 2390-2398.

Lalevee, N., Rebsamen, M.C., Barrere-Lemaire, S., Perrier, E., Nargeot, J., Benitah, J.P., Rossier, M.F., 2005. Aldosterone increases T-type calcium channel expression and in vitro beating frequency in neonatal rat cardiomyocytes. Cardiovasc.Res. 67, 216-224.

Lang, F., Klingel, K., Wagner, C.A., Stegen, C., Warntges, S., Friedrich, B., Lanzendorfer, M., Melzig, J., Moschen, I., Steuer, S., Waldegger, S., Sauter, M., Paulmichl, M., Gerke, V., Risler, T., Gamba, G., Capasso, G., Kandolf, R., Hebert, S.C., Massry, S.G., Broer, S., 2000. Deranged transcriptional regulation of cell-volume-sensitive kinase hSGK in diabetic nephropathy. Proc.Natl.Acad.Sci.U.S.A 97, 8157-8162.

Lavery, G.G., Walker, E.A., Draper, N., Jeyasuria, P., Marcos, J., Shackleton, C.H., Parker, K.L., White, P.C., Stewart, P.M., 2006. Hexose-6-phosphate dehydrogenase knock-out mice lack 11 beta-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation. J.Biol.Chem. 281, 6546-6551.

Lavery, G.G., Walker, E.A., Tiganescu, A., Ride, J.P., Shackleton, C.H., Tomlinson, J.W., Connell, J.M., Ray, D.W., Biason-Lauber, A., Malunowicz, E.M., Arlt, W., Stewart, P.M., 2008. Steroid biomarkers and genetic studies reveal inactivating mutations in hexose-6-phosphate dehydrogenase in patients with cortisone reductase deficiency. J.Clin.Endocrinol.Metab 93, 3827-3832.

Leask, A., 2007. TGFbeta, cardiac fibroblasts, and the fibrotic response. Cardiovasc Res 74, 207-212.

Lee, A.F., MacFadyen, R.J., Struthers, A.D., 1999. Neurohormonal reactivation in heart failure patients on chronic ACE inhibitor therapy: a longitudinal study. Eur.J.Heart Fail. 1, 401-406.

Lee, Y.S., Kim, J.A., Kim, K.L., Jang, H.S., Kim, J.M., Lee, J.Y., Shin, I.S., Lee, J.S., Suh, W., Choi, J.H., Jeon, E.S., Byun, J., Kim, D.K., 2004. Aldosterone upregulates connective tissue growth factor gene expression via p38 MAPK pathway and mineralocorticoid receptor in ventricular myocytes. J.Korean Med.Sci. 19, 805-811.

Lewis, C.J., Gong, H., Brown, M.J., Harding, S.E., 2004. Overexpression of beta 1-adrenoceptors in adult rat ventricular myocytes enhances CGP 12177A cardiostimulation: implications for 'putative' beta 4-adrenoceptor pharmacology. Br.J.Pharmacol. 141, 813-824.

Li, J., Fu, J., Toumazou, C., Yoon, H.G., Wong, J., 2006. A role of the amino-terminal (N) and carboxyl-terminal (C) interaction in binding of androgen receptor to chromatin. Mol Endocrinol 20, 776-785.

Lim, P.O., Macdonald, T.M., Holloway, C., Friel, E., Anderson, N.H., Dow, E., Jung, R.T., Davies, E., Fraser, R., Connell, J.M., 2002. Variation at the aldosterone synthase (CYP11B2) locus contributes to hypertension in subjects with a raised aldosterone-to-renin ratio. J Clin Endocrinol Metab 87, 4398-4402.

Lister, K., Autelitano, D.J., Jenkins, A., Hannan, R.D., Sheppard, K.E., 2006. Cross talk between corticosteroids and alpha-adrenergic signalling augments cardiomyocyte hypertrophy: a possible role for SGK1. Cardiovasc.Res. 70, 555-565.

Liu, W., Wang, J., Sauter, N.K., Pearce, D., 1995. Steroid receptor heterodimerization demonstrated in vitro and in vivo. Proc Natl Acad Sci U S A 92, 12480-12484.

Liu, X., Ramjiganesh, T., Chen, Y.H., Chung, S.W., Hall, S.R., Schissel, S.L., Padera, R.F., Jr., Liao, R., Ackerman, K.G., Kajstura, J., Leri, A., Anversa, P., Yet, S.F., Layne, M.D., Perrella, M.A., 2009a. Disruption of striated preferentially expressed gene locus leads to dilated cardiomyopathy in mice. Circulation 119, 261-268.

Liu, X., Ren, Z., Zhan, R., Wang, X., Zhang, Z., Leng, X., Yang, Z., Qian, L., 2009b. Prohibitin protects against oxidative stress-induced cell injury in cultured neonatal cardiomyocyte. Cell Stress Chaperones 14, 311-319.

Ljungman, S., Aurell, M., Hartford, M., Wikstrand, J., Berglund, G., 1982. Blood pressure in relation to the renin-angiotensin-aldosterone system. Acta Med Scand 211, 351-360.

Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T.B., Flegal, K., Ford, E., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M., Meigs, J., Mozaffarian, D., Nichol, G., O'Donnell, C., Roger, V., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Wong, N., Wylie-Rosett, J., Hong, Y., 2009. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 119, e21-181.

Lombes, M., Alfaidy, N., Eugene, E., Lessana, A., Farman, N., Bonvalet, J.P., 1995. Prerequisite for cardiac aldosterone action. Mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase in the human heart. Circulation 92, 175-182.

Lombes, M., Kenouch, S., Souque, A., Farman, N., Rafestin-Oblin, M.E., 1994. The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 beta-hydroxysteroid dehydrogenase. Endocrinology 135, 834-840.

Lombes, M., Oblin, M.E., Gasc, J.M., Baulieu, E.E., Farman, N., Bonvalet, J.P., 1992. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. Circ.Res. 71, 503-510.

Lonard, D.M., O'Malley B, W., 2007. Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. Mol Cell 27, 691-700.

Lonard, D.M., O'Malley, B.W., 2006. The expanding cosmos of nuclear receptor coactivators. Cell 125, 411-414.

Lopez-Andres, N., Inigo, C., Gallego, I., Diez, J., Fortuno, M.A., 2008. Aldosterone induces cardiotrophin-1 expression in HL-1 adult cardiomyocytes. Endocrinology 149, 4970-4978.

Losel, R., Schultz, A., Wehling, M., 2004. A quick glance at rapid aldosterone action. Mol.Cell Endocrinol. 217, 137-141.

Lovati, E., Ferrari, P., Dick, B., Jostarndt, K., Frey, B.M., Frey, F.J., Schorr, U., Sharma, A.M., 1999. Molecular basis of human salt sensitivity: the role of the 11beta-hydroxysteroid dehydrogenase type 2. J.Clin.Endocrinol.Metab 84, 3745-3749.

Low, S.C., Chapman, K.E., Edwards, C.R., Seckl, J.R., 1994. 'Liver-type' 11 beta-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. J.Mol.Endocrinol. 13, 167-174.

Lumbers, E.R., Boyce, A.C., Joulianos, G., Kumarasamy, V., Barner, E., Segar, J.L., Burrell, J.H., 2005. Effects of cortisol on cardiac myocytes and on expression of cardiac genes in fetal sheep. Am J Physiol Regul Integr Comp Physiol 288, R567-574.

MacDermott, R.P., Stacey, M.C., 1981. Further characterization of the human autologous mixed leukocyte reaction (MLR). J.Immunol. 126, 729-734.

Macdonald, J.E., Kennedy, N., Struthers, A.D., 2004. Effects of spironolactone on endothelial function, vascular angiotensin converting enzyme activity, and other prognostic markers in patients with mild heart failure already taking optimal treatment. Heart 90, 765-770.

MacFadyen, R.J., Lee, A.F., Morton, J.J., Pringle, S.D., Struthers, A.D., 1999. How often are angiotensin II and aldosterone concentrations raised during chronic ACE inhibitor treatment in cardiac failure? Heart 82, 57-61.

Machein, M.R., Kullmer, J., Ronicke, V., Machein, U., Krieg, M., Damert, A., Breier, G., Risau, W., Plate, K.H., 1999. Differential downregulation of vascular endothelial growth factor by dexamethasone in normoxic and hypoxic rat glioma cells. Neuropathol.Appl.Neurobiol. 25, 104-112.

Mahmud, A., Mahgoub, M., Hall, M., Feely, J., 2005. Does aldosterone-to-renin ratio predict the antihypertensive effect of the aldosterone antagonist spironolactone? Am J Hypertens 18, 1631-1635.

Maillet, M., Lynch, J.M., Sanna, B., York, A.J., Zheng, Y., Molkentin, J.D., 2009. Cdc42 is an antihypertrophic molecular switch in the mouse heart. J Clin Invest 119, 3079-3088.

Mann, J.F., Gerstein, H.C., Pogue, J., Lonn, E., Yusuf, S., 2002. Cardiovascular risk in patients with early renal insufficiency: implications for the use of ACE inhibitors. Am.J.Cardiovasc.Drugs 2, 157-162.

Mano, A., Tatsumi, T., Shiraishi, J., Keira, N., Nomura, T., Takeda, M., Nishikawa, S., Yamanaka, S., Matoba, S., Kobara, M., Tanaka, H., Shirayama, T., Takamatsu, T., Nozawa, Y., Matsubara, H., 2004. Aldosterone directly induces myocyte apoptosis through calcineurin-dependent pathways. Circulation 110, 317-323.

Mariniello, B., Ronconi, V., Sardu, C., Pagliericcio, A., Galletti, F., Strazzullo, P., Palermo, M., Boscaro, M., Stewart, P.M., Mantero, F., Giacchetti, G., 2005. Analysis of the 11beta-hydroxysteroid dehydrogenase type 2 gene (HSD11B2) in human essential hypertension. Am.J.Hypertens. 18, 1091-1098.

Marino, M., Galluzzo, P., Ascenzi, P., 2006. Estrogen signaling multiple pathways to impact gene transcription. Curr.Genomics 7, 497-508.

Mason, J.I., Rainey, W.E., 1987. Steroidogenesis in the human fetal adrenal: a role for cholesterol synthesized de novo. J.Clin.Endocrinol.Metab 64, 140-147.

Massie, B.M., Shah, N.B., 1996. The heart failure epidemic: magnitude of the problem and potential mitigating approaches. Curr.Opin.Cardiol. 11, 221-226.

Masur, S.K., Dewal, H.S., Dinh, T.T., Erenburg, I., Petridou, S., 1996. Myofibroblasts differentiate from fibroblasts when plated at low density. Proc Natl Acad Sci U S A 93, 4219-4223.

Masuzaki, H., Paterson, J., Shinyama, H., Morton, N.M., Mullins, J.J., Seckl, J.R., Flier, J.S., 2001. A transgenic model of visceral obesity and the metabolic syndrome. Science 294, 2166-2170.

Masuzaki, H., Yamamoto, H., Kenyon, C.J., Elmquist, J.K., Morton, N.M., Paterson, J.M., Shinyama, H., Sharp, M.G., Fleming, S., Mullins, J.J., Seckl, J.R., Flier, J.S., 2003. Transgenic amplification of glucocorticoid action in adipose tissue causes high blood pressure in mice. J.Clin.Invest 112, 83-90.

Matsumura, K., Fujii, K., Oniki, H., Oka, M., Iida, M., 2006. Role of aldosterone in left ventricular hypertrophy in hypertension. Am.J.Hypertens. 19, 13-18.

Mattsson, C., Reynolds, R.M., Simonyte, K., Olsson, T., Walker, B.R., 2009. Combined Receptor Antagonist Stimulation of the HPA axis test identifies impaired negative feedback sensitivity to cortisol in obese men. J.Clin.Endocrinol.Metab.

Mazancova, K., Kopecky, M., Miksik, I., Pacha, J., 2005. 11beta-Hydroxysteroid dehydrogenase in the heart of normotensive and hypertensive rats. J.Steroid Biochem.Mol.Biol. 94, 273-277.

McKelvie, R.S., Yusuf, S., Pericak, D., Avezum, A., Burns, R.J., Probstfield, J., Tsuyuki, R.T., White, M., Rouleau, J., Latini, R., Maggioni, A., Young, J., Pogue, J., 1999. Comparison of candesartan, enalapril, and their combination in congestive heart failure: randomized evaluation of strategies for left ventricular dysfunction (RESOLVD) pilot study. The RESOLVD Pilot Study Investigators. Circulation 100, 1056-1064.

McMurray, J.J., Pfeffer, M.A., 2005. Heart failure. Lancet 365, 1877-1889.

McSweeney, S.J., Hadoke, P.W., Kozak, A.M., Small, G.R., Khaled, H., Walker, B.R., Gray, G.A., 2010. Improved heart function follows enhanced inflammatory cell recruitment and angiogenesis in 11{beta}HSD1-deficient mice post-MI. Cardiovasc Res 88, 159-167.

Michea, L., Villagran, A., Urzua, A., Kuntsmann, S., Venegas, P., Carrasco, L., Gonzalez, M., Marusic, E.T., 2008. Mineralocorticoid receptor antagonism attenuates cardiac hypertrophy and prevents oxidative stress in uremic rats. Hypertension 52, 295-300.

Mihailidou, A.S., Loan Le, T.Y., Mardini, M., Funder, J.W., 2009. Glucocorticoids activate cardiac mineralocorticoid receptors during experimental myocardial infarction. Hypertension 54, 1306-1312.

Millar, J.A., Leckie, B.J., Morton, J.J., Jordan, J., Tree, M., 1980. A microassay for active and total renin concentration in human plasma based on antibody trapping. Clin.Chim.Acta 101, 5-15.

Milliez, P., Girerd, X., Plouin, P.F., Blacher, J., Safar, M.E., Mourad, J.J., 2005. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. J.Am.Coll.Cardiol. 45, 1243-1248.

Mizuno, Y., Yoshimura, M., Yasue, H., Sakamoto, T., Ogawa, H., Kugiyama, K., Harada, E., Nakayama, M., Nakamura, S., Ito, T., Shimasaki, Y., Saito, Y., Nakao, K., 2001. Aldosterone production is activated in failing ventricle in humans. Circulation 103, 72-77.

Molnar, G.A., Lindschau, C., Dubrovska, G., Mertens, P.R., Kirsch, T., Quinkler, M., Gollasch, M., Wresche, S., Luft, F.C., Muller, D.N., Fiebeler, A., 2008. Glucocorticoid-related signaling effects in vascular smooth muscle cells. Hypertension 51, 1372-1378.

Monder, C., Lakshmi, V., 1988. Corticosteroid 11 beta-hydroxysteroid dehydrogenase activities in vertebrate liver. Steroids 52, 515-528.

Monder, C., Stewart, P.M., Lakshmi, V., Valentino, R., Burt, D., Edwards, C.R., 1989. Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: in vivo and in vitro studies. Endocrinology 125, 1046-1053.

Moore, K.P., Wong, F., Gines, P., Bernardi, M., Ochs, A., Salerno, F., Angeli, P., Porayko, M., Moreau, R., Garcia-Tsao, G., Jimenez, W., Planas, R., Arroyo, V., 2003. The management of ascites in cirrhosis: report on the consensus conference of the International Ascites Club. Hepatology 38, 258-266.

Moore, R.Y., Eichler, V.B., 1972. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res. 42, 201-206.

Morgan, S.A., Sherlock, M., Gathercole, L.L., Lavery, G.G., Lenaghan, C., Bujalska, I.J., Laber, D., Yu, A., Convey, G., Mayers, R., Hegyi, K., Sethi, J.K., Stewart, P.M., Smith, D.M., Tomlinson, J.W., 2009. 11beta-hydroxysteroid dehydrogenase type 1 regulates glucocorticoid-induced insulin resistance in skeletal muscle. Diabetes 58, 2506-2515.

Morton, J.J., Webb, D.J., 1985. Measurement of plasma angiotensin II. Clin.Sci.(Lond) 68, 483-484.

Mosso, L., Carvajal, C., Gonzalez, A., Barraza, A., Avila, F., Montero, J., Huete, A., Gederlini, A., Fardella, C.E., 2003. Primary aldosteronism and hypertensive disease. Hypertension 42, 161-165.

Mottram, P.M., Haluska, B., Leano, R., Cowley, D., Stowasser, M., Marwick, T.H., 2004. Effect of aldosterone antagonism on myocardial dysfunction in hypertensive patients with diastolic heart failure. Circulation 110, 558-565.

Muiesan, M.L., Lupia, M., Salvetti, M., Grigoletto, C., Sonino, N., Boscaro, M., Rosei, E.A., Mantero, F., Fallo, F., 2003. Left ventricular structural and functional characteristics in Cushing's syndrome. J.Am.Coll.Cardiol. 41, 2275-2279.

Muiesan, M.L., Salvetti, M., Paini, A., Agabiti-Rosei, C., Monteduro, C., Galbassini, G., Belotti, E., Aggiusti, C., Rizzoni, D., Castellano, M., Agabiti-Rosei, E., 2008. Inappropriate left ventricular mass in patients with primary aldosteronism. Hypertension 52, 529-534.

Muller, O., Pradervand, S., Berger, S., Centeno, G., Milet, A., Nicod, P., Pedrazzini, T., Tronche, F., Schutz, G., Chien, K., Rossier, B.C., Firsov, D., 2007.

Identification of corticosteroid-regulated genes in cardiomyocytes by serial analysis of gene expression. Genomics 89, 370-377.

Muraguchi, T., Kawawa, A., Kubota, S., 2010. Prohibitin protects against hypoxia-induced H9c2 cardiomyocyte cell death. Biomed Res 31, 113-122.

Murai-Takeda, A., Shibata, H., Kurihara, I., Kobayashi, S., Yokota, K., Suda, N., Mitsuishi, Y., Jo, R., Kitagawa, H., Kato, S., Saruta, T., Itoh, H., 2010. NF-YC functions as a corepressor of agonist-bound mineralocorticoid receptor. J Biol Chem 285, 8084-8093.

Muto, T., Ueda, N., Opthof, T., Ohkusa, T., Nagata, K., Suzuki, S., Tsuji, Y., Horiba, M., Lee, J.K., Honjo, H., Kamiya, K., Kodama, I., Yasui, K., 2007. Aldosterone modulates I(f) current through gene expression in cultured neonatal rat ventricular myocytes. Am.J.Physiol Heart Circ.Physiol 293, H2710-H2718.

N'Gankam, V., Uehlinger, D., Dick, B., Frey, B.M., Frey, F.J., 2002. Increased cortisol metabolites and reduced activity of 11beta-hydroxysteroid dehydrogenase in patients on hemodialysis. Kidney Int. 61, 1859-1866.

Nagai, T., Tanaka-Ishikawa, M., Aikawa, R., Ishihara, H., Zhu, W., Yazaki, Y., Nagai, R., Komuro, I., 2003. Cdc42 plays a critical role in assembly of sarcomere units in series of cardiac myocytes. Biochem Biophys Res Commun 305, 806-810.

Nagase, H., Woessner, J.F., Jr., 1999. Matrix metalloproteinases. J.Biol.Chem. 274, 21491-21494.

Nagata, K., Obata, K., Xu, J., Ichihara, S., Noda, A., Kimata, H., Kato, T., Izawa, H., Murohara, T., Yokota, M., 2006. Mineralocorticoid receptor antagonism attenuates cardiac hypertrophy and failure in low-aldosterone hypertensive rats. Hypertension 47, 656-664.

Neubauer, S., 2007. The failing heart--an engine out of fuel. N.Engl.J.Med. 356, 1140-1151.

Oakley, R.H., Jewell, C.M., Yudt, M.R., Bofetiado, D.M., Cidlowski, J.A., 1999. The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. J.Biol.Chem. 274, 27857-27866.

Odermatt, A., Arnold, P., Stauffer, A., Frey, B.M., Frey, F.J., 1999. The N-terminal anchor sequences of 11beta-hydroxysteroid dehydrogenases determine their orientation in the endoplasmic reticulum membrane. J.Biol.Chem. 274, 28762-28770.

Ohba, T., Watanabe, H., Murakami, M., Sato, T., Ono, K., Ito, H., 2009. Essential role of STIM1 in the development of cardiomyocyte hypertrophy. Biochem Biophys Res Commun 389, 172-176.

Ohtani, T., Mano, T., Hikoso, S., Sakata, Y., Nishio, M., Takeda, Y., Otsu, K., Miwa, T., Masuyama, T., Hori, M., Yamamoto, K., 2009. Cardiac steroidogenesis and glucocorticoid in the development of cardiac hypertrophy during the progression to heart failure. J.Hypertens. 27, 1074-1083.

Okoshi, M.P., Yan, X., Okoshi, K., Nakayama, M., Schuldt, A.J., O'Connell, T.D., Simpson, P.C., Lorell, B.H., 2004. Aldosterone directly stimulates cardiac myocyte hypertrophy. J.Card Fail. 10, 511-518.

Onyimba, C.U., Vijapurapu, N., Curnow, S.J., Khosla, P., Stewart, P.M., Murray, P.I., Walker, E.A., Rauz, S., 2006. Characterisation of the prereceptor regulation of

glucocorticoids in the anterior segment of the rabbit eye. J Endocrinol 190, 483-493.

Opie, L.H., Commerford, P.J., Gersh, B.J., Pfeffer, M.A., 2006. Controversies in ventricular remodelling. Lancet 367, 356-367.

Ouvrard-Pascaud, A., Sainte-Marie, Y., Benitah, J.P., Perrier, R., Soukaseum, C., Cat, A.N., Royer, A., Le Quang, K., Charpentier, F., Demolombe, S., Mechta-Grigoriou, F., Beggah, A.T., Maison-Blanche, P., Oblin, M.E., Delcayre, C., Fishman, G.I., Farman, N., Escoubet, B., Jaisser, F., 2005. Conditional mineralocorticoid receptor expression in the heart leads to life-threatening arrhythmias. Circulation 111, 3025-3033.

Ozols, J., 1995. Lumenal orientation and post-translational modifications of the liver microsomal 11 beta-hydroxysteroid dehydrogenase. J.Biol.Chem. 270, 10360.

Packer, M., Bristow, M.R., Cohn, J.N., Colucci, W.S., Fowler, M.B., Gilbert, E.M., Shusterman, N.H., 1996. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group. N.Engl.J.Med. 334, 1349-1355.

Paillard, F., Chansel, D., Brand, E., Benetos, A., Thomas, F., Czekalski, S., Ardaillou, R., Soubrier, F., 1999. Genotype-phenotype relationships for the renin-angiotensin-aldosterone system in a normal population. Hypertension 34, 423-429.

Palermo, M., Shackleton, C.H., Mantero, F., Stewart, P.M., 1996. Urinary free cortisone and the assessment of 11 beta-hydroxysteroid dehydrogenase activity in man. Clin.Endocrinol.(Oxf) 45, 605-611.

Palmer, B.R., Pilbrow, A.P., Frampton, C.M., Yandle, T.G., Skelton, L., Nicholls, M.G., Richards, A.M., 2008. Plasma aldosterone levels during hospitalization are predictive of survival post-myocardial infarction. Eur.Heart J.

Paterson, J.M., Morton, N.M., Fievet, C., Kenyon, C.J., Holmes, M.C., Staels, B., Seckl, J.R., Mullins, J.J., 2004. Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proc.Natl.Acad.Sci.U.S.A 101, 7088-7093.

Pearce, P., Funder, J.W., 1987. High affinity aldosterone binding sites (type I receptors) in rat heart. Clin.Exp.Pharmacol.Physiol 14, 859-866.

Pirone, D.M., Fukuhara, S., Gutkind, J.S., Burbelo, P.D., 2000. SPECs, small binding proteins for Cdc42. J Biol Chem 275, 22650-22656.

Pitt, B., Chang, P., Grossman, W., Dunlay, M., Timmermans, P.B., 1996. Rationale, background, and design of the randomized angiotensin receptor antagonist--angiotensin-converting enzyme inhibitor study (RAAS). Am.J.Cardiol. 78, 1129-1131.

Pitt, B., Reichek, N., Willenbrock, R., Zannad, F., Phillips, R.A., Roniker, B., Kleiman, J., Krause, S., Burns, D., Williams, G.H., 2003a. Effects of eplerenone, enalapril, and eplerenone/enalapril in patients with essential hypertension and left ventricular hypertrophy: the 4E-left ventricular hypertrophy study. Circulation 108, 1831-1838.

Pitt, B., Remme, W., Zannad, F., Neaton, J., Martinez, F., Roniker, B., Bittman, R., Hurley, S., Kleiman, J., Gatlin, M., 2003b. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N.Engl.J.Med. 348, 1309-1321.

Pitt, B., Zannad, F., Remme, W.J., Cody, R., Castaigne, A., Perez, A., Palensky, J., Wittes, J., 1999. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N.Engl.J.Med. 341, 709-717.

Plotz, C.M., Knowlton, A.I., Ragan, C., 1952. The natural history of Cushing's syndrome. Am J Med 13, 597-614.

Porter, K.E., Turner, N.A., 2009. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol.Ther. 123, 255-278.

Pryce, C.R., 2008. Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: inter-species and intra-species differences. Brain Res.Rev. 57, 596-605.

Pujols, L., Mullol, J., Perez, M., Roca-Ferrer, J., Juan, M., Xaubet, A., Cidlowski, J.A., Picado, C., 2001. Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. Am.J.Respir.Cell Mol.Biol. 24, 49-57.

Qin, W., Rudolph, A.E., Bond, B.R., Rocha, R., Blomme, E.A., Goellner, J.J., Funder, J.W., McMahon, E.G., 2003. Transgenic model of aldosterone-driven cardiac hypertrophy and heart failure. Circ.Res. 93, 69-76.

Quinkler, M., Zehnder, D., Lepenies, J., Petrelli, M.D., Moore, J.S., Hughes, S.V., Cockwell, P., Hewison, M., Stewart, P.M., 2005. Expression of renal 11beta-hydroxysteroid dehydrogenase type 2 is decreased in patients with impaired renal function. Eur.J.Endocrinol. 153, 291-299.

Rebsamen, M.C., Perrier, E., Gerber-Wicht, C., Benitah, J.P., Lang, U., 2004. Direct and indirect effects of aldosterone on cyclooxygenase-2 and interleukin-6 expression in rat cardiac cells in culture and after myocardial infarction. Endocrinology 145, 3135-3142.

Reichardt, H.M., Tuckermann, J.P., Gottlicher, M., Vujic, M., Weih, F., Angel, P., Herrlich, P., Schutz, G., 2001. Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J. 20, 7168-7173.

Rickard, A.J., Funder, J.W., Fuller, P.J., Young, M.J., 2006. The role of the glucocorticoid receptor in mineralocorticoid/salt-mediated cardiac fibrosis. Endocrinology 147, 5901-5906.

Rickard, A.J., Funder, J.W., Morgan, J., Fuller, P.J., Young, M.J., 2007. Does glucocorticoid receptor blockade exacerbate tissue damage after mineralocorticoid/salt administration? Endocrinology 148, 4829-4835.

Ricketts, M.L., Verhaeg, J.M., Bujalska, I., Howie, A.J., Rainey, W.E., Stewart, P.M., 1998. Immunohistochemical localization of type 1 11beta-hydroxysteroid dehydrogenase in human tissues. J.Clin.Endocrinol.Metab 83, 1325-1335.

Rocha, R., Martin-Berger, C.L., Yang, P., Scherrer, R., Delyani, J., McMahon, E., 2002a. Selective aldosterone blockade prevents angiotensin II/salt-induced vascular inflammation in the rat heart. Endocrinology 143, 4828-4836.

Rocha, R., Rudolph, A.E., Frierdich, G.E., Nachowiak, D.A., Kekec, B.K., Blomme, E.A., McMahon, E.G., Delyani, J.A., 2002b. Aldosterone induces a vascular inflammatory phenotype in the rat heart. Am.J.Physiol Heart Circ.Physiol 283, H1802-H1810.

Rocha, R., Stier, C.T., Jr., Kifor, I., Ochoa-Maya, M.R., Rennke, H.G., Williams, G.H., Adler, G.K., 2000. Aldosterone: a mediator of myocardial necrosis and renal arteriopathy. Endocrinology 141, 3871-3878.

Rogerson, F.M., Fuller, P.J., 2003. Interdomain interactions in the mineralocorticoid receptor. Mol Cell Endocrinol 200, 45-55.

Roig, E., Perez-Villa, F., Morales, M., Jimenez, W., Orus, J., Heras, M., Sanz, G., 2000. Clinical implications of increased plasma angiotensin II despite ACE inhibitor therapy in patients with congestive heart failure. Eur.Heart J. 21, 53-57.

Roland, B.L., Funder, J.W., 1996. Localization of 11beta-hydroxysteroid dehydrogenase type 2 in rat tissues: in situ studies. Endocrinology 137, 1123-1128.

Roland, B.L., Li, K.X., Funder, J.W., 1995. Hybridization histochemical localization of 11 beta-hydroxysteroid dehydrogenase type 2 in rat brain. Endocrinology 136, 4697-4700.

Rombouts, K., Wielant, A., Hellemans, K., Schuppan, D., Geerts, A., 2001. Influence of aldosterone on collagen synthesis and proliferation of rat cardiac fibroblasts. Br J Pharmacol 134, 224-232.

Romito, A., Lonardo, E., Roma, G., Minchiotti, G., Ballabio, A., Cobellis, G., 2010. Lack of sik1 in mouse embryonic stem cells impairs cardiomyogenesis by down-regulating the cyclin-dependent kinase inhibitor p57kip2. PLoS One 5, e9029.

Rone, M.B., Fan, J., Papadopoulos, V., 2009. Cholesterol transport in steroid biosynthesis: Role of protein-protein interactions and implications in disease states. Biochim.Biophys.Acta.

Rooney, D.P., Neely, R.D., Cullen, C., Ennis, C.N., Sheridan, B., Atkinson, A.B., Trimble, E.R., Bell, P.M., 1993. The effect of cortisol on glucose/glucose-6-phosphate cycle activity and insulin action. J.Clin.Endocrinol.Metab 77, 1180-1183.

Rossi, G.P., Bernini, G., Caliumi, C., Desideri, G., Fabris, B., Ferri, C., Ganzaroli, C., Giacchetti, G., Letizia, C., Maccario, M., Mallamaci, F., Mannelli, M., Mattarello, M.J., Moretti, A., Palumbo, G., Parenti, G., Porteri, E., Semplicini, A., Rizzoni, D., Rossi, E., Boscaro, M., Pessina, A.C., Mantero, F., 2006. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. J.Am.Coll.Cardiol. 48, 2293-2300.

Rossi, G.P., Di, B.V., Ganzaroli, C., Sacchetto, A., Cesari, M., Bertini, A., Giorgi, D., Scognamiglio, R., Mariani, M., Pessina, A.C., 2002. Excess aldosterone is associated with alterations of myocardial texture in primary aldosteronism. Hypertension 40, 23-27.

Rossi, G.P., Sacchetto, A., Visentin, P., Canali, C., Graniero, G.R., Palatini, P., Pessina, A.C., 1996. Changes in left ventricular anatomy and function in hypertension and primary aldosteronism. Hypertension 27, 1039-1045.

Rousseau, M.F., Gurne, O., Duprez, D., Van Mieghem, W., Robert, A., Ahn, S., Galanti, L., Ketelslegers, J.M., 2002. Beneficial neurohormonal profile of spironolactone in severe congestive heart failure: results from the RALES neurohormonal substudy. J.Am.Coll.Cardiol. 40, 1596-1601.

Rude, M.K., Duhaney, T.A., Kuster, G.M., Judge, S., Heo, J., Colucci, W.S., Siwik, D.A., Sam, F., 2005. Aldosterone stimulates matrix metalloproteinases and reactive oxygen species in adult rat ventricular cardiomyocytes. Hypertension 46, 555-561.

Rupprecht, R., Arriza, J.L., Spengler, D., Reul, J.M., Evans, R.M., Holsboer, F., Damm, K., 1993. Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. Mol.Endocrinol. 7, 597-603.

Sabbah, H.N., Sharov, V.G., Lesch, M., Goldstein, S., 1995. Progression of heart failure: a role for interstitial fibrosis. Mol.Cell Biochem. 147, 29-34.

Sai, S., Esteves, C.L., Kelly, V., Michailidou, Z., Anderson, K., Coll, A.P., Nakagawa, Y., Ohzeki, T., Seckl, J.R., Chapman, K.E., 2008. Glucocorticoid regulation of the promoter of 11beta-hydroxysteroid dehydrogenase type 1 is indirect and requires CCAAT/enhancer-binding protein-beta. Mol Endocrinol 22, 2049-2060.

Sainte-Marie, Y., Nguyen Dinh, C.A., Perrier, R., Mangin, L., Soukaseum, C., Peuchmaur, M., Tronche, F., Farman, N., Escoubet, B., Benitah, J.P., Jaisser, F., 2007. Conditional glucocorticoid receptor expression in the heart induces atrio-ventricular block. FASEB J. 21, 3133-3141.

Salem, M., Tainsh, R.E., Jr., Bromberg, J., Loriaux, D.L., Chernow, B., 1994. Perioperative glucocorticoid coverage. A reassessment 42 years after emergence of a problem. Ann.Surg. 219, 416-425.

Sarnak, M.J., Levey, A.S., Schoolwerth, A.C., Coresh, J., Culleton, B., Hamm, L.L., McCullough, P.A., Kasiske, B.L., Kelepouris, E., Klag, M.J., Parfrey, P., Pfeffer, M., Raij, L., Spinosa, D.J., Wilson, P.W., 2003. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation 108, 2154-2169.

Sato, A., Saruta, T., 2001. Aldosterone escape during angiotensin-converting enzyme inhibitor therapy in essential hypertensive patients with left ventricular hypertrophy. J.Int.Med.Res. 29, 13-21.

Satoh, M., Nakamura, M., Saitoh, H., Satoh, H., Akatsu, T., Iwasaka, J., Masuda, T., Hiramori, K., 2002. Aldosterone synthase (CYP11B2) expression and myocardial fibrosis in the failing human heart. Clin.Sci.(Lond) 102, 381-386.

Savory, J.G., Prefontaine, G.G., Lamprecht, C., Liao, M., Walther, R.F., Lefebvre, Y.A., Hache, R.J., 2001. Glucocorticoid receptor homodimers and glucocorticoid-mineralocorticoid receptor heterodimers form in the cytoplasm through alternative dimerization interfaces. Mol Cell Biol 21, 781-793.

Schunkert, H., Dzau, V.J., Tang, S.S., Hirsch, A.T., Apstein, C.S., Lorell, B.H., 1990. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. J Clin Invest 86, 1913-1920.

Seckl, J.R., Holmes, M.C., 2007. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. Nat.Clin.Pract.Endocrinol.Metab 3, 479-488.

Selye, H., 1941. The anesthetic effect of steroid hormones. pp. 116-121.

Shackleton, C.H., Rodriguez, J., Arteaga, E., Lopez, J.M., Winter, J.S., 1985. Congenital 11 beta-hydroxysteroid dehydrogenase deficiency associated with juvenile hypertension: corticosteroid metabolite profiles of four patients and their families. Clin.Endocrinol.(Oxf) 22, 701-712.

Shams, M., Kilby, M.D., Somerset, D.A., Howie, A.J., Gupta, A., Wood, P.J., Afnan, M., Stewart, P.M., 1998. 11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction. Hum.Reprod. 13, 799-804.

Sheppard, K.E., Autelitano, D.J., 2002. 11Beta-hydroxysteroid dehydrogenase 1 transforms 11-dehydrocorticosterone into transcriptionally active glucocorticoid in neonatal rat heart. Endocrinology 143, 198-204.

Shizukuda, Y., Miura, T., Ishimoto, R., Itoya, M., Iimura, O., 1991. Effect of prednisolone on myocardial infarct healing: characteristics and comparison with indomethacin. Can J Cardiol 7, 447-454.

Sholter, D.E., Armstrong, P.W., 2000. Adverse effects of corticosteroids on the cardiovascular system. Can J Cardiol 16, 505-511.

Silvestre, J.S., Heymes, C., Oubenaissa, A., Robert, V., Aupetit-Faisant, B., Carayon, A., Swynghedauw, B., Delcayre, C., 1999. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. Circulation 99, 2694-2701.

Silvestre, J.S., Robert, V., Heymes, C., Aupetit-Faisant, B., Mouas, C., Moalic, J.M., Swynghedauw, B., Delcayre, C., 1998. Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. J.Biol.Chem. 273, 4883-4891.

Sjostrom, M., Stenstrom, K., Eneling, K., Zwiller, J., Katz, A.I., Takemori, H., Bertorello, A.M., 2007. SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process. Proc Natl Acad Sci U S A 104, 16922-16927.

Sklan, E.H., Serrano, R.L., Einav, S., Pfeffer, S.R., Lambright, D.G., Glenn, J.S., 2007. TBC1D20 is a Rab1 GTPase-activating protein that mediates hepatitis C virus replication. J Biol Chem 282, 36354-36361.

Slight, S.H., Ganjam, V.K., Gomez-Sanchez, C.E., Zhou, M.Y., Weber, K.T., 1996. High affinity NAD(+)-dependent 11 beta-hydroxysteroid dehydrogenase in the human heart. J.Mol.Cell Cardiol. 28, 781-787.

Small, G.R., Hadoke, P.W., Sharif, I., Dover, A.R., Armour, D., Kenyon, C.J., Gray, G.A., Walker, B.R., 2005. Preventing local regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1 enhances angiogenesis. Proc.Natl.Acad.Sci.U.S.A 102, 12165-12170.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3, Article3.

Snyder, P.M., 2005. Minireview: regulation of epithelial Na+ channel trafficking. Endocrinology 146, 5079-5085.

So, A., Duffy, D.L., Gordon, R.D., Jeske, Y.W., Lin-Su, K., New, M.I., Stowasser, M., 2005. Familial hyperaldosteronism type II is linked to the chromosome 7p22 region but also shows predicted heterogeneity. J Hypertens 23, 1477-1484.

Souverein, P.C., Berard, A., Van Staa, T.P., Cooper, C., Egberts, A.C., Leufkens, H.G., Walker, B.R., 2004. Use of oral glucocorticoids and risk of cardiovascular and cerebrovascular disease in a population based case-control study. Heart 90, 859-865.

Stallcup, M.R., Kim, J.H., Teyssier, C., Lee, Y.H., Ma, H., Chen, D., 2003. The roles of protein-protein interactions and protein methylation in transcriptional activation by nuclear receptors and their coactivators. J Steroid Biochem Mol Biol 85, 139-145.

Stewart, P.M., Corrie, J.E., Shackleton, C.H., Edwards, C.R., 1988. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. J.Clin.Invest 82, 340-349.

Stewart, P.M., Krozowski, Z.S., Gupta, A., Milford, D.V., Howie, A.J., Sheppard, M.C., Whorwood, C.B., 1996. Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11 beta-hydroxysteroid dehydrogenase type 2 gene. Lancet 347, 88-91.

Stewart, P.M., Murry, B.A., Mason, J.I., 1994. Human kidney 11 beta-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform. J.Clin.Endocrinol.Metab 79, 480-484.

Stewart, P.M., Tomlinson, J.W., 2009. Selective inhibitors of 11beta-hydroxysteroid dehydrogenase type 1 for patients with metabolic syndrome: is the target liver, fat, or both? Diabetes 58, 14-15.

Stewart, R.A., Kerr, A.J., Cowan, B.R., Young, A.A., Occleshaw, C., Richards, A.M., Edwards, C., Whalley, G.A., Freidlander, D., Williams, M., Doughty, R.N., Zeng, I., White, H.D., 2008. A randomized trial of the aldosterone-receptor antagonist eplerenone in asymptomatic moderate-severe aortic stenosis. Am.Heart J. 156, 348-355.

Stewart, S., Jenkins, A., Buchan, S., McGuire, A., Capewell, S., McMurray, J.J., 2002. The current cost of heart failure to the National Health Service in the UK. Eur.J.Heart Fail. 4, 361-371.

Stocco, D.M., 2002. Clinical disorders associated with abnormal cholesterol transport: mutations in the steroidogenic acute regulatory protein. Mol.Cell Endocrinol. 191, 19-25.

Stockand, J.D., Meszaros, J.G., 2003. Aldosterone stimulates proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling. Am.J.Physiol Heart Circ.Physiol 284, H176-H184.

Studer, R., Reinecke, H., Muller, B., Holtz, J., Just, H., Drexler, H., 1994. Increased angiotensin-I converting enzyme gene expression in the failing human heart. Quantification by competitive RNA polymerase chain reaction. J.Clin.Invest 94, 301-310.

Sun, K., Myatt, L., 2003. Enhancement of glucocorticoid-induced 11beta-hydroxysteroid dehydrogenase type 1 expression by proinflammatory cytokines in cultured human amnion fibroblasts. Endocrinology 144, 5568-5577.

Sun, Y., 2009. Myocardial repair/remodelling following infarction: roles of local factors. Cardiovasc.Res. 81, 482-490.

Sun, Y., Weber, K.T., 2000. Infarct scar: a dynamic tissue. Cardiovasc.Res. 46, 250-256.

Swedberg, K., Eneroth, P., Kjekshus, J., Snapinn, S., 1990. Effects of enalapril and neuroendocrine activation on prognosis in severe congestive heart failure (follow-up of the CONSENSUS trial). CONSENSUS Trial Study Group. Am.J.Cardiol. 66, 40D-44D.

Swynghedauw, B., 1999. Molecular mechanisms of myocardial remodeling. Physiol Rev. 79, 215-262.

Takeda, Y., Yoneda, T., Demura, M., Miyamori, I., Mabuchi, H., 2000. Cardiac aldosterone production in genetically hypertensive rats. Hypertension 36, 495-500.

Tannin, G.M., Agarwal, A.K., Monder, C., New, M.I., White, P.C., 1991. The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. J.Biol.Chem. 266, 16653-16658.

Tian, X.L., Pinto, Y.M., Costerousse, O., Franz, W.M., Lippoldt, A., Hoffmann, S., Unger, T., Paul, M., 2004. Over-expression of angiotensin converting enzyme-1 augments cardiac hypertrophy in transgenic rats. Hum Mol Genet 13, 1441-1450.

Tomaschitz, A., Maerz, W., Pilz, S., Ritz, E., Scharnagl, H., Renner, W., Boehm, B.O., Fahrleitner-Pammer, A., Weihrauch, G., Dobnig, H., 2010. Aldosterone/renin ratio determines peripheral and central blood pressure values over a broad range. J Am Coll Cardiol 55, 2171-2180.

Tomlinson, J.W., Walker, E.A., Bujalska, I.J., Draper, N., Lavery, G.G., Cooper, M.S., Hewison, M., Stewart, P.M., 2004. 11beta-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. Endocr.Rev. 25, 831-866.

Tonelli, M., Wiebe, N., Culleton, B., House, A., Rabbat, C., Fok, M., McAlister, F., Garg, A.X., 2006. Chronic kidney disease and mortality risk: a systematic review. J.Am.Soc.Nephrol. 17, 2034-2047.

Towbin, H., Staehelin, T., Gordon, J., 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology 24, 145-149.

Trapp, T., Rupprecht, R., Castren, M., Reul, J.M., Holsboer, F., 1994. Heterodimerization between mineralocorticoid and glucocorticoid receptor: a new principle of glucocorticoid action in the CNS. Neuron 13, 1457-1462.

Turchin, A., Guo, C.Z., Adler, G.K., Ricchiuti, V., Kohane, I.S., Williams, G.H., 2006. Effect of acute aldosterone administration on gene expression profile in the heart. Endocrinology 147, 3183-3189.

Turner, B.B., Moses, L.F., 1986. Sex differences in the distribution and regulation of glucocorticoid receptors in cardiac tissues of rats. J.Mol.Cell Cardiol. 18, 331-333.

Tylicki, L., Rutkowski, P., Renke, M., Larczynski, W., Aleksandrowicz, E., Lysiak-Szydlowska, W., Rutkowski, B., 2008. Triple pharmacological blockade of the renin-angiotensin-aldosterone system in nondiabetic CKD: an open-label crossover randomized controlled trial. Am.J.Kidney Dis. 52, 486-493.

Ulick, S., Wang, J.Z., Blumenfeld, J.D., Pickering, T.G., 1992. Cortisol inactivation overload: a mechanism of mineralocorticoid hypertension in the ectopic adrenocorticotropin syndrome. J.Clin.Endocrinol.Metab 74, 963-967.

Vallon, V., Wyatt, A.W., Klingel, K., Huang, D.Y., Hussain, A., Berchtold, S., Friedrich, B., Grahammer, F., Belaiba, R.S., Gorlach, A., Wulff, P., Daut, J., Dalton, N.D., Ross, J., Jr., Flogel, U., Schrader, J., Osswald, H., Kandolf, R., Kuhl, D., Lang, F., 2006. SGK1-dependent cardiac CTGF formation and fibrosis following DOCA treatment. J.Mol.Med. 84, 396-404.

van Raalte, D.H., Ouwens, D.M., Diamant, M., 2009. Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options? Eur.J.Clin.Invest 39, 81-93.

Vantrimpont, P., Rouleau, J.L., Ciampi, A., Harel, F., de Champlain, J., Bichet, D., Moye, L.A., Pfeffer, M., 1998. Two-year time course and significance of neurohumoral activation in the Survival and Ventricular Enlargement (SAVE) Study. Eur.Heart J. 19, 1552-1563.

Vasan, R.S., Evans, J.C., Larson, M.G., Wilson, P.W., Meigs, J.B., Rifai, N., Benjamin, E.J., Levy, D., 2004. Serum aldosterone and the incidence of hypertension in nonhypertensive persons. N.Engl.J.Med. 351, 33-41.

Venkateswaran, R.V., Steeds, R.P., Quinn, D.W., Nightingale, P., Wilson, I.C., Mascaro, J.G., Thompson, R.D., Townend, J.N., Bonser, R.S., 2009. The haemodynamic effects of adjunctive hormone therapy in potential heart donors: a prospective randomized double-blind factorially designed controlled trial. Eur Heart J 30, 1771-1780.

Visse, R., Nagase, H., 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ.Res. 92, 827-839.

Waerntges, S., Klingel, K., Weigert, C., Fillon, S., Buck, M., Schleicher, E., Rodemann, H.P., Knabbe, C., Kandolf, R., Lang, F., 2002. Excessive transcription of the human serum and glucocorticoid dependent kinase hSGK1 in lung fibrosis. Cell Physiol Biochem. 12, 135-142.

Walker, B.R., Campbell, J.C., Williams, B.C., Edwards, C.R., 1992. Tissue-specific distribution of the NAD(+)-dependent isoform of 11 beta-hydroxysteroid dehydrogenase. Endocrinology 131, 970-972.

Walker, B.R., Stewart, P.M., Shackleton, C.H., Padfield, P.L., Edwards, C.R., 1993. Deficient inactivation of cortisol by 11 beta-hydroxysteroid dehydrogenase in essential hypertension. Clin.Endocrinol.(Oxf) 39, 221-227.

Walker, B.R., Yau, J.L., Brett, L.P., Seckl, J.R., Monder, C., Williams, B.C., Edwards, C.R., 1991. 11 beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. Endocrinology 129, 3305-3312.

Walker, E.A., Ahmed, A., Lavery, G.G., Tomlinson, J.W., Kim, S.Y., Cooper, M.S., Ride, J.P., Hughes, B.A., Shackleton, C.H., McKiernan, P., Elias, E., Chou, J.Y., Stewart, P.M., 2007. 11beta-Hydroxysteroid Dehydrogenase Type 1 Regulation by Intracellular Glucose 6-Phosphate Provides Evidence for a Novel Link between Glucose Metabolism and Hypothalamo-Pituitary-Adrenal Axis Function. J Biol Chem 282, 27030-27036.

Walker, W.G., Whelton, P.K., Saito, H., Russell, R.P., Hermann, J., 1979. Relation between blood pressure and renin, renin substrate, angiotensin II, aldosterone and urinary sodium and potassium in 574 ambulatory subjects. Hypertension 1, 287-291.

Warth, A., Eckle, T., Kohler, D., Faigle, M., Zug, S., Klingel, K., Eltzschig, H.K., Wolburg, H., 2007. Upregulation of the water channel aquaporin-4 as a potential cause of postischemic cell swelling in a murine model of myocardial infarction. Cardiology 107, 402-410.

Weber, K.T., 2001. Aldosterone in congestive heart failure. N.Engl.J.Med. 345, 1689-1697.

Weber, K.T., Pick, R., Jalil, J.E., Janicki, J.S., Carroll, E.P., 1989. Patterns of myocardial fibrosis. J.Mol.Cell Cardiol. 21 Suppl 5, 121-131.

Weber, K.T., Villarreal, D., 1993. Aldosterone and antialdosterone therapy in congestive heart failure. Am.J.Cardiol. 71, 3A-11A.

Wehling, M., Schultz, A., Losel, R., 2006. Nongenomic actions of estrogens: exciting opportunities for pharmacology. Maturitas 54, 321-326.

Wei, L., MacDonald, T.M., Walker, B.R., 2004. Taking glucocorticoids by prescription is associated with subsequent cardiovascular disease. Ann.Intern.Med. 141, 764-770.

Whitehurst, R.M., Jr., Zhang, M., Bhattacharjee, A., Li, M., 1999. Dexamethasone-induced hypertrophy in rat neonatal cardiac myocytes involves an elevated L-type Ca(2+)current. J.Mol.Cell Cardiol. 31, 1551-1558.

Whitworth, J.A., Stewart, P.M., Burt, D., Atherden, S.M., Edwards, C.R., 1989. The kidney is the major site of cortisone production in man. Clin.Endocrinol.(Oxf) 31, 355-361.

Whorwood, C.B., Sheppard, M.C., Stewart, P.M., 1993. Licorice inhibits 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid levels and potentiates glucocorticoid hormone action. Endocrinology 132, 2287-2292.

Wilson, R.C., Nimkarn, S., New, M.I., 2001. Apparent mineralocorticoid excess. Trends Endocrinol.Metab 12, 104-111.

Wisenbaugh, P.E., Garst, J.B., Hull, C., Freedman, R.J., Matthews, D.N., Hadady, M., 1972. Renin, aldosterone, sodium and hypertension. Am J Med 52, 175-186.

Wu, G., Yussman, M.G., Barrett, T.J., Hahn, H.S., Osinska, H., Hilliard, G.M., Wang, X., Toyokawa, T., Yatani, A., Lynch, R.A., Robbins, J., Dorn, G.W., 2nd, 2001. Increased myocardial Rab GTPase expression: a consequence and cause of cardiomyopathy. Circ Res 89, 1130-1137.

Wu, L., Einstein, M., Geissler, W.M., Chan, H.K., Elliston, K.O., Andersson, S., 1993. Expression cloning and characterization of human 17 beta-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20 alpha-hydroxysteroid dehydrogenase activity. J.Biol.Chem. 268, 12964-12969.

Xu, J., Li, Q., 2003. Review of the in vivo functions of the p160 steroid receptor coactivator family. Mol Endocrinol 17, 1681-1692.

Yamamoto, N., Yasue, H., Mizuno, Y., Yoshimura, M., Fujii, H., Nakayama, M., Harada, E., Nakamura, S., Ito, T., Ogawa, H., 2002. Aldosterone is produced

from ventricles in patients with essential hypertension. Hypertension 39, 958-962.

Yamamuro, M., Yoshimura, M., Nakayama, M., Abe, K., Sumida, H., Sugiyama, S., Saito, Y., Nakao, K., Yasue, H., Ogawa, H., 2008. Aldosterone, but not angiotensin II, reduces angiotensin converting enzyme 2 gene expression levels in cultured neonatal rat cardiomyocytes. Circ.J. 72, 1346-1350.

Yang, J., Young, M.J., 2009. The mineralocorticoid receptor and its coregulators. J Mol Endocrinol 43, 53-64.

Yang, Z., Zhu, P., Guo, C., Zhu, X., Sun, K., 2009. Expression of 11beta-hydroxysteroid dehydrogenase type 1 in human fetal lung and regulation of its expression by interleukin-1beta and cortisol. J Clin Endocrinol Metab 94, 306-313.

Yoshikawa, N., Nagasaki, M., Sano, M., Tokudome, S., Ueno, K., Shimizu, N., Imoto, S., Miyano, S., Suematsu, M., Fukuda, K., Morimoto, C., Tanaka, H., 2009. Ligand-based gene expression profiling reveals novel roles of glucocorticoid receptor in cardiac metabolism. Am J Physiol Endocrinol Metab 296, E1363-1373.

Yoshimura, M., Nakamura, S., Ito, T., Nakayama, M., Harada, E., Mizuno, Y., Sakamoto, T., Yamamuro, M., Saito, Y., Nakao, K., Yasue, H., Ogawa, H., 2002. Expression of aldosterone synthase gene in failing human heart: quantitative analysis using modified real-time polymerase chain reaction. J.Clin.Endocrinol.Metab 87, 3936-3940.

Young, E.A., Lopez, J.F., Murphy-Weinberg, V., Watson, S.J., Akil, H., 1998. The role of mineralocorticoid receptors in hypothalamic-pituitary-adrenal axis regulation in humans. J.Clin.Endocrinol.Metab 83, 3339-3345.

Young, M., Head, G., Funder, J., 1995. Determinants of cardiac fibrosis in experimental hypermineralocorticoid states. Am.J.Physiol 269, E657-E662.

Young, M.J., Clyne, C.D., Cole, T.J., Funder, J.W., 2001. Cardiac steroidogenesis in the normal and failing heart. J.Clin.Endocrinol.Metab 86, 5121-5126.

Young, M.J., Morgan, J., Brolin, K., Fuller, P.J., Funder, J.W., 2010. Activation of mineralocorticoid receptors by exogenous glucocorticoids and the development of cardiovascular inflammatory responses in adrenalectomized rats. Endocrinology 151, 2622-2628.

Young, W.F., 2007. Primary aldosteronism: renaissance of a syndrome. Clin.Endocrinol.(Oxf) 66, 607-618.

Zhou, G., Kandala, J.C., Tyagi, S.C., Katwa, L.C., Weber, K.T., 1996. Effects of angiotensin II and aldosterone on collagen gene expression and protein turnover in cardiac fibroblasts. Mol.Cell Biochem. 154, 171-178.

Zhou, M.Y., Gomez-Sanchez, E.P., Cox, D.L., Cosby, D., Gomez-Sanchez, C.E., 1995. Cloning, expression, and tissue distribution of the rat nicotinamide adenine dinucleotide-dependent 11 beta-hydroxysteroid dehydrogenase. Endocrinology 136, 3729-3734.

Publications

Investigating hypertension in a young person.

Hammer F, Stewart PM.

BMJ. 2009 Apr 6;338:b1043.

The effect of spironolactone upon corticosteroid hormone metabolism in patients with early stage chronic kidney disease.

Hammer F, Edwards NC, Hughes BA, Steeds RP, Ferro CJ, Townend JN, Stewart PM.

Clin Endocrinol (Oxf). 2010 Nov;73(5):566-72.