The Biology of Arginine Vasopressin in Cardiovascular Disease

by

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DECLARATION

I declare this thesis to be my own work, except where due acknowledgement has been made under the joint supervision of Associate Professor Louise Burrell at the University of Melbourne, Australia and Professor John Connell at the University of Glasgow.

The work here is original research and has not previously been submitted for a higher degree. I am the author of this thesis and have consulted all the references cited.

Signed

Laura V.M. Jordan

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ACE	Angiotensin Converting Enzyme
ACTH	Adrenocorticotropin hormone
AMV-RT	Avian myoblastoma virus-reverse transcriptase
Ang I	Angiotensin I
Ang II	Angiotensin II
AQP-2	Aquaporin 2
AT_1R	Angiotensin type 1 receptor
AT_2R	Angiotensin type 2 receptor
AVP	Arginine vasopressin
BB	Brattleboro
BSA	Bovine serum albumin
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
CCF	Congestive cardiac failure
CD	Collecting duct
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CRF	Chronic renal failure
CVD	Cardiovascular disease
DAG	Diacylglyerol
dDAVP	1-deamino-8-D-arginine vasopressin
DEPC	Diethylpyrocarbonate
DOCA	Deoxycorticosterone acetate
DM	Diabetes mellitus
DM-SHR	Spontaneously hypertensive rat with diabetes mellitus
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSS	Dahl salt sensitive
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylenebis (oxyethylenenitrilo) tetraacetic acid

G protein	Guanine nucleotide binding protein
GBM	Glomerular basement membrane
Gi	Inhibitory G protein
Gs	Stimulatory G protein
HPLC	High performance liquid chromatography
IMCD	Intramedullary collecting duct
IML	Intermedial column
IOP	Intraocular pressure
ISHH	*In situ* hybridisation histochemistry
IP_3	Inositol triphosphate
IV	Intravenous
LV	Left ventricular
mRNA	Messenger RNA
NDI	Nephrogenic diabetes insipidus
NM	Nucleus medianus
NOS	Nitric oxide synthase
NTS	Nucleus tractus solitarius
OVLT	Organum vasculosum of the lamina terminalis
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethanesulfonyl fluoride
PRA	Plasma renin activity
PVN	Paraventricular nucleus
RAS	Renin angiotensin system
rER	Rough endoplasmic reticulum
RMIC	Renal medullary interstitial cell
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RV	Right ventricular
SIADH	Syndrome of inappropriate arginine vasopressin secretion
SBP	Systolic blood pressure

SD	Sprague Dawley
SER	Smooth endoplasmic reticulum
SFO	Subfornical organ
SHR	Spontaneously Hypertensive Rat
SON	Supraoptic nucleus
STZ	Streptozotocin
STZ-DM	Steptozotocin induced diabetes mellitus
TFA	Trifluoroacetic acid
TGF-β1	Transforming growth factor-beta 1
TPA	Tissue plasminogen activator
VACM-1	Vasopressin activating calcium mobilising receptor type 1
VEGF	Vascular Endothelial Growth Factor
$V_{1A}R$	Vasopressin type 1A receptor
$V_{1B}R$	Vasopressin type 1B receptor
V_2 R	Vasopressin type 2 receptor

ABSTRACT

Arginine vasopressin (AVP) is a 9 amino acid, posterior pituitary, peptide hormone that plays a pivotal role in both salt and water homeostasis and blood pressure control. These effects are mediated primarily through 2 main receptor types; the former through the renal tubular V_2 receptor (V_2R) and the later the smooth muscle cell V_{1A} receptor ($V_{1A}R$). AVP has received little attention since its discovery in 1885 and the precise actions of this hormone in both physiology and disease often remains unclear. Recent research has brought to light that AVP does have a number of actions relevant to the progression of cardiovascular diseases (CVDs) and that the AVP receptors are often differentially regulated in a number of these conditions. Fully understanding what these changes are, and how they contribute to CVDs, will open doors for the development of new therapeutic agents to ultimately help reduce morbidity and mortality within our populations.

The aims of this thesis were threefold.

1. To fully characterise *in vitro* 5 novel AVP receptor antagonists (drugs A-E) and from these to select the single most promising agent and undertake further *in vitro* and *in vivo* study.

2. To investigate the possibility of additive cardiovascular benefits of ACE inhibition with $V_{1A}R$ antagonism in an adult, hypertensive rat model.

3. To examine the regulation of AVP and its $V_{1A}Rs$ in a hypertensive rat model with 8 weeks of streptozocin induced diabetes mellitus (STZ-DM).

Within an *in vitro* experimental setting, drugs A-E displaced specific V_{1A} and V_2R antagonists in a dose-dependent manner. Drug A most closely resembled AVP in this regard and was thus selected for further *in vitro* and *in vivo* study. A dose of 10mg/kg was found to be effective acutely with maximal receptor inhibition at 2 hours. Significant inhibition was seen for 4 hours at the $V_{1A}R$ and 8 hours at the V_2R. When administered in a chronic 5 day setting to normotensive rats, systolic blood pressure (SBP) remained unaltered and a diminishing aquaresis was seen.

In experimental, adult, genetic hypertension, 2 weeks of administration of the $V_{1A}R$ antagonist OPC 21268 did not have any antihypertensive benefits while the ACE inhibitor, ramipril, and combination treatment with ramipril and OPC 21268 lowered SBP to the same extent. Likewise, only the ramipril treated animals demonstrated any reductions in cardiac weights. These results imply that $V_{1A}R$ antagonism does not have a role to play in combating established genetic hypertension in the 2 week setting but does not rule out any longer term structural benefits that may come to light in the context of a more prolonged study.

In the setting of 8 weeks of experimental, hypertensive STZ-DM, AVP was elevated with hepatic and renal $V_{1A}Rs$ being up-regulated in association with this. This up-regulation was in terms of receptor numbers only (Bmax), as receptor affinity (Kd) remained unaltered when compared to hypertensive non-diabetic controls. Interestingly, the corresponding receptor mRNA levels were not elevated raising the possibility of a post-transcriptional effect. This is the first study to demonstrate an up-regulation of the AVP receptor system in the context of hypertension and DM.

AVP, via stimulation of its V_{1A} and V_2Rs, elicits important biological actions that have been shown to contribute to the pathophysiology of many forms of CVDs such as hypertension and DM. Therapeutic agents to block the AVP receptor are constantly being developed and assessed. In order to determine its ultimate clinical usefulness Drug A should now undergo further trials in disease models such as cardiac failure and low renin hypertension, a category of hypertension seen mainly in the elderly, African-American and diabetic populations where AVP has been shown to play a more prominent pathological role. It has been shown in this thesis that the AVP V_{1A}R system is up-regulated in DM combined with hypertension. Consequently, AVP V_{1A}R blockade provides an exciting new potential therapeutic target that could play an integral role in any future treatment strategies to help combat the ongoing

epidemic of this cardiovascular disease.

Chapter 1

Literature review

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Literature review

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1.1 INTRODUCTION

Diseases such as hypertension and diabetes mellitus (DM) are among the leading causes of morbidity and mortality in developed countries (1-4). Their prevalence is rapidly escalating and associated spiralling health care costs places huge economic burdens on government resources that ultimately impacts on society (5). A number of therapeutic strategies already exist for the treatment of hypertension and these include the use of diuretics, beta blockers, calcium channel antagonists and angiotensin-converting enzyme (ACE) inhibitors (6, 7). To improve the efficacy of ACE inhibitors in low renin forms of essential hypertension, such as that seen in the diabetic, elderly or African-American populations, it is common clinical practice to co-prescribe a diuretic (8, 9). This is undoubtedly efficacious but does carry with it associated metabolic disturbances that can be problematic, particularly in the elderly (8). ACE inhibitors have also proven to be the single most useful treatment in the diabetic patient, considerably reducing both macrovascular and microvascular complications (10, 11). Hypertension is a common co-morbidity factor in these patients (12-14). Despite the advent of ACE inhibition, mortality and morbidity rates in this disease remain unacceptably high (4, 15) and alternative therapeutic strategies, that may provide additional benefits, are continually being sought.

Two very similar cyclic, 9 amino acid, posterior pituitary hormones exist – arginine vasopressin (AVP) and oxytocin. These substances are synthesised in the hypothalamic nuclei that are connected to the neurohypophysis. Oxytocin acts on the breast to cause milk ejection and on the pregnant myometrium to cause contraction and expulsion of the foetus in labour. In large amounts oxytocin has some AVP-like activity. This thesis has examined the role of AVP and its receptors in experimental models of cardiovascular disease (CVD) and a review of the oxytocin system will not be done here.

In 1895 Oliver and Shafer first discovered the neurohormone arginine vasopressin (AVP) when they intravenously (IV) injected posterior pituitary extracts into dogs and noticed an increase in blood pressure (BP) (16). From this experiment they named the presumptive active substance in the posterior pituitary extracts vasopressin. Subsequent studies revealed that AVP also acted as a potent anti-diuretic (17) and for many years to come the renal water-sparing action of this hormone was the main focus of any research. Only in relatively recent times have the cardiovascular aspects of AVP received attention and research into this has become increasingly prolific since the advent of specific antagonists to AVP receptors (18).

AVP is a 9 amino acid neurohormone that plays a pivotal role in salt and water homeostasis and BP regulation via activation of its receptor subtypes classified as V_1 and V_2. The V_1 receptor (V_1R) is further subclassified into V_{1A} and V_{1B} (or V_3). Interaction of AVP with the V_1R leads to cell membrane phospholipase activation and mobilisation of intracellular calcium to cause vasoconstriction (19, 20) while interaction with the V_2 receptor (V_2R) leads to activation of adenylyl cyclase (AC) and production of cyclic adenosine monophosphate (cAMP) that ultimately causes antidiuresis (21, 22). The V_2R has only conclusively been demonstrated thus far in the kidney (see section 1.3.3.3). The $V_{1A}R$ has a more widespread distribution being found in liver, platelets, blood vessels, testes and brain to name but a few (see section 1.3.2.3) while the V_{1B} receptor ($V_{1B}R$) has been localised in the anterior pituitary, various regions of the rat brain and human adrenal medulla, as well as more recently been found to be abundant within the pancreas (see section 1.3.4.3).

Several lines of evidence indicate that AVP may contribute to the progression of CVD. Firstly, elevated plasma levels of AVP are found in DM (23-25), congestive cardiac failure (CCF) (26) and in some forms of hypertension (27-29). Secondly, AVP has mitogenic and hyperplastic actions that, by analogy to angiotensin II (Ang II), may have deleterious effects when present in chronic excess (30, 31). Thirdly, there is evidence that AVP is synthesised, not only in the hypothalamus of the brain where it acts systemically, but also locally in blood vessels and the heart where it may function as a paracrine/autocrine hormone (32-36).

Over the past decade AVP research has become particularly prolific as orally active, non-peptide, selective V_{1A} and V_2R antagonists have become available. This has allowed various research groups to assess more easily the role of AVP in CVDs. (37-41). Orally effective and selective AVP receptor antagonists, such as OPC 21268 and SR49059, block the pressor effects of AVP at the $V_{1A}R$ (28, 42) while V_2R antagonists such as OPC 31260 (41, 43), OPC 41061 (44) and SR121463A (45) cause aquaresis. Dual V_{1A} and V_2R blockers such as YMO87 (46) have also recently become available and are currently the subject of intensive research while more recently still a selective $V_{1B}R$ non-peptide antagonist, SSR149415, has entered the arena and now represents a unique tool for exploring the functional role of the $V_{1B}R$, a task that has hitherto been extremely difficult (47). Intense research is currently ongoing to develop new, non-peptide V_{1A}, V_2 and in particular dual V_{1A}/V_2R blockers. In contrast to diuretics, AVP receptor antagonists lack undesirable associated metabolic disturbances and may in addition have other beneficial effects such as antihypertrophic and antiproliferative actions. These properties make AVP receptor antagonists an attractive additional therapy to ACE inhibition in the treatment of CVDs.

In the developmental phase of experimental hypertension, in the spontaneously hypertensive rat (SHR), $V_{1A}R$ antagonist, in a similar way to ACE inhibition (48), attenuates the progression of hypertension long after treatment withdrawal (49, 50). Interestingly, treatment with the $V_{1A}R$ antagonist OPC 21268 alone has no effect on the full progression of the adult hypertensive phenotype while the V_2R antagonist OPC 31260 actually exacerbates this progression (28, 43). In developing hypertension, again in the SHR, combination therapy with ACE inhibitor ramipril and OPC 21268 was not additive in its BP lowering effects (49). In an established hypertensive rat model, chronic treatment with OPC 21268 failed to lower BP (50) Thus far in the adult hypertensive model combination therapy with both ACE antagonism and $V_{1A}R$ blockade has not been investigated.

With regard to DM, regulation of the AVP system has thus far been little studied. The regulation of V_{1A} and V_2Rs has been looked at in a 2 week, normotensive rat with streptozotocin–induced DM and this demonstrated an increase in plasma AVP with associated down-regulation of the $V_{1A}Rs$ in both liver and kidney. The V_2R numbers remained unchanged (51). Little is known of AVP receptor regulation with more long term DM or in DM associated with hypertension.

The aims of this thesis therefore were: 1) to characterise *in vitro* a new range of non-peptide, orally active, AVP receptor antagonists and further characterise *in vitro* and *in vivo* the most "promising" of the mixed antagonists in rat. 2) To look for any additive or synergistic benefits of ACE inhibition and $V_{1A}R$ antagonism in the adult hypertensive rat.

3) To study the regulation of the AVP $V_{1A}R$ system in an 8 week, hypertensive, rat model of DM.

1.2 ARGININE VASOPRESSIN (AVP)

1.2.1 Structure and biosynthesis

1.2.1.1 Gene

The AVP gene, in humans, is assigned to chromosome 20 and contains 3 exons separated by 2 introns. The exons contain the genetic code for the functional domains of the large polypeptide precursor of AVP- preprovasopressin (52). Exon A encodes the actual hormone along with a 5' untranslated region and the signal sequence. Exon B encodes most of the conserved part of the carrier neurophysin and exon C the remaining C-terminal variable neurophysin sequence, a glycopeptide and the 3' untranslated region (52) (Figure 1.1). The rat and human AVP precursor genes are highly conserved in structure and the three functional domains of the rat precursor – AVP, neurophysin and glycoprotein- are encoded, as with the human precursor, on three distinct exons. The rat gene also includes a consensus sequence found at exon-intron junction. This is a modified RNA polymerase II binding site CATAAAT located 29bp upstream of the start point of transcription and a polyadenylation site in the 3' untranslated region (52).

1.2.1.2 Protein

The post-translational processing of neuropeptide polyprotein precursors is an important feature of neuropeptide expression. The biosynthetic pathway for AVP, a 9 amino acid hormone, involves the proteolytic cleavage of its precursor preprovasopressin (53, 54). This precursor contains 3 peptide units- AVP and a signal peptide located at the N-terminus, the carrier neurophysin and a C-terminal glycopeptide. The hormone precursor

undergoes a number of maturation steps with these processes occurring in the secretory vesicles during axonal transport. There are a number of consensus signals that are involved in modification and proteolytic processing. Separating AVP from the neurophysin are a pair of basic amino acids, that serve as a signal for proteolytic cleavage, and a glycine residue that is essential for the amidation of the AVP C-terminal amino acid (55). The neurophysin is connected to the C-terminal glycoprotein by a single basic residue. The glycopeptide contains a typical glycosylation site Asn-X-Thr although its physiological significance is unknown. Given that its sequence is highly conserved across species and that it is cleaved and secreted into the circulation along with AVP and neurophysin, it seems likely that the glycopeptide may confer some biological role (53, 54). Post-translational modification begins with the transfer of the initial protein product from hypothalamic rough endoplasmic reticulum (rER) to Golgi apparatus and then further proteolytic maturation steps occur continuously during axonal transport with both cleaved and uncleaved products appearing in the posterior pituitary after a few hours (56). The Brattleboro (BB) rat represents an autosomally recessive form of nephrogenic diabetes insipidus (NDI) (57). These animals lack, not only circulating AVP, but also its corresponding neurophysin and the glycopeptide moiety. This disease state results from a frame shift mutation in the AVP gene that yields a mutated precursor protein unable to be transported to the Golgi apparatus for further processing (57).

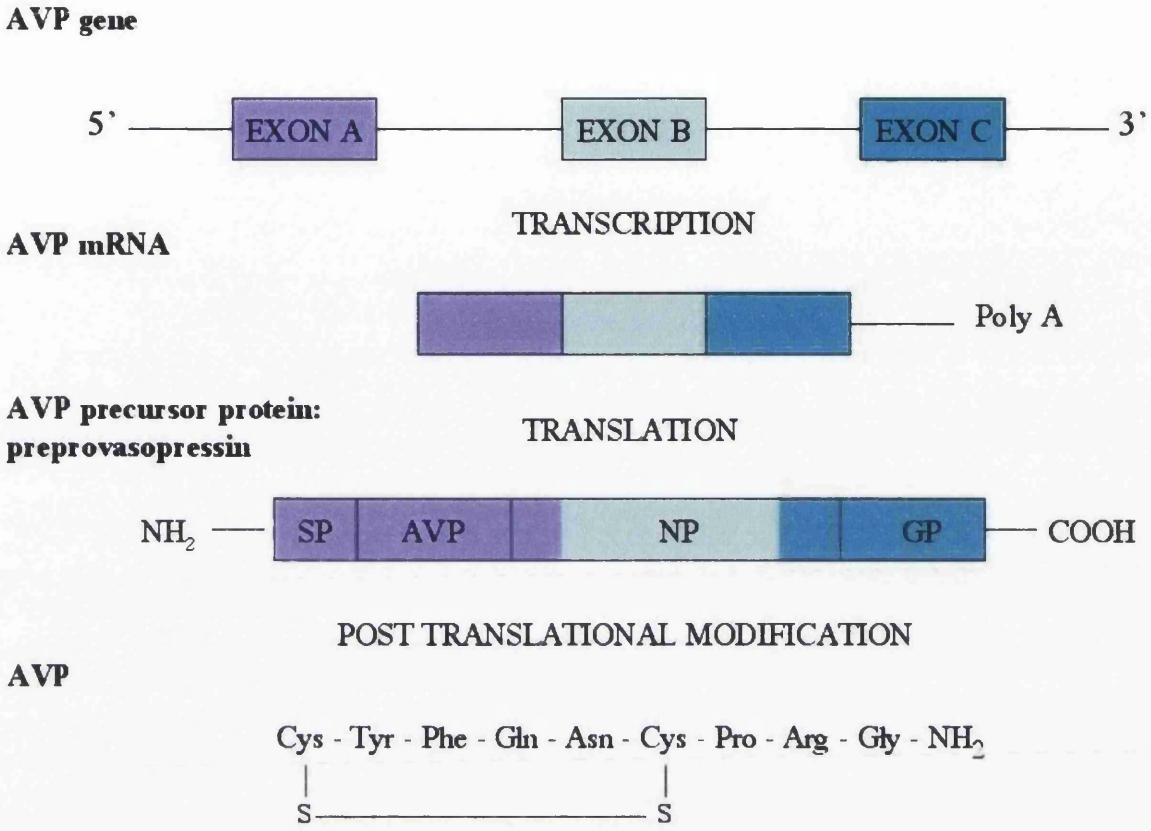

Figure 1.1. A representation of the structural organisation of the AVP gene, precursor and AVP peptide. Modified from (55)

Abbreviations: SP, signal peptide. NP, neuropeptide. GP, glycoprotein

1.2.2 Storage and release

1.2.2.1 Central nervous system (CNS)

AVP is synthesised, transported within and secreted from two distinct classes of neurones found in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. Firstly, the magno-cellular AVP-containing neurones of the hypothalamic PVN and SON form the hypothalamic-hypophyseal pathway and this projects to the posterior pituitary where AVP is then stored and secreted into the general circulation (58). Secondly, the hypothalamic parvocellular neurones project to the median eminence where AVP and oxytocin are released into the portal vessel system to modulate anterior pituitary function (58). In addition, parvocellular AVP-containing neurones found within the hypothalamus have extrahypothalamic projections to centres in the medulla such as the nucleus tractus solitarius, the dorsal vagal complex, the locus coerulus and the intermedial column of the spinal cord, while axonal vasopressinergic projection to the forebrain and lateral septum may be involved in AVP's role in memory and behaviour (58-60). There are also ascending catecholaminergic pathways from medullary centres that terminate in the PVN and SON. For example, noradrenergic axons from the ventral lateral medulla synapse with both magnocellular and parvocellular neurones of the PVN and SON. Those of the dorsal motor nucleus of the vagus and the locus coerulus, on the other hand, synapse predominantly on parvocellular neurones of the PVN (60-62). These ascending and descending pathways provide the basis for a possible feedback loop that enables AVP to influence autonomic and cardiovascular function as well as provide a structural basis for catecholaminergic effect on AVP release.

1.2.2.2 Peripheral

Traditionally AVP was thought of as a neurohypophyseal hormone. Today, although AVP is predominantly thought to be synthesised within the hypothalamus, there is increasing evidence also for its production in peripheral tissues such as the aorta (35, 63), heart (36), coronary vasculature (36), platelets (64), ovary (32, 65), testis (66, 67), pancreas (68, 69), trachea (70), adrenal (71), thymus (72) and sympathetic ganglia (73, 74) of different species. The physiological significance of peripheral, locally produced AVP, is not clear although there is evidence to suggest that AVP may act locally to modulate hypertrophy and hyperplasia of cardiomyocytes (75) and smooth muscle cells as well as influence coronary vascular tone (36) and the actions of local growth factors such as transforming growth factor β (TGFβ) (30, 31).

1.2.2.3 Osmotic control of release of AVP

In 1947 E.B.Verney originally proposed the concept of osmoreceptors from studies that assessed the influence of body fluid hypertonicity on antidiuresis. He demonstrated that infusion of hypertonic and non sodium-containing solutions into the carotid arteries of dogs caused an antidiuresis. He also concluded that osmoreceptors influenced the release of an antidiuretic hormone from the posterior pituitary gland since intracarotid infusion of a posterior pituitary extract mimicked the antidiuresis observed when using hypertonic solution (17).

Lesion and electrophysiological studies have since provided strong evidence regarding the localisation of these osmoreceptors (76). Three anatomically defined areas of the lamina terminalis are implicated in water balance control. The organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) lie outside the blood brain barrier

while the nucleus medianus (NM) lies within it. Where the SFO is primarily concerned with Ang II-mediated thirst and AVP release, the OVLT is involved in osmotically stimulated AVP secretion (77). The NM acts to integrate signals from the SFO and OVLT and peripheral cardiovascular pathways and to provide stimulatory inputs to the magnocellular and lateral hypothalamic neurones for AVP release and thirst (78, 79).

1.2.2.4 Non-osmotic control of release of AVP

Non-osmotic factors such as hypovolaemia and hypotension can also stimulate AVP release from the posterior pituitary via two baroregulatory systems. Low-pressure volume mechanoreceptors found in the left atrium detect changes in blood volume. High-pressure volume mechanoreceptors located in the carotid sinus and aortic arch detect and respond to changes in BP (80). Consequently, increased BP and volume lead to increased firing of baroreceptors and decreased AVP release. In man, high pressure baroreceptors are thought to play a more important role than low pressure baroreceptors as only a 5% decrease in arterial pressure compared to a 10-15% reduction in blood volume is required to influence AVP release (80, 81).

Other factors known to affect AVP release from the hypothalamus include Ang II (82, 83), atrial natriuretic factor, satiety (84) and environmental factors (85, 86) such as temperature and humidity (87).

1.3 AVP RECEPTORS

1.3.1 General overview

AVP mediates its physiological effects through G-protein-coupled membrane-bound receptors that have been divided into two broad subtypes based on their second messenger systems. (88). The classic antidiuretic effect of AVP is mediated through renal V_2Rs with cAMP production, via AC, as a second messenger while vasopressor action is mediated through V_1Rs, with phophatidylinositol phosphate hydrolysis as a second messenger. The anterior pituitary AVP receptor has been subclassified as a V_{1B}R on the basis of different profiles of [^{3}H]AVP binding compared with classical hepatic/vascular V_{1A}Rs (89, 90). Since the molecular cloning of the V_{1B}R some have adopted the classification of V_3 to describe this receptor and this is based on the fact that the V_{1B}R has no greater sequence homology with the V_{1A}R as compared with other members of the AVP/oxytocin receptor family. As yet no international consensus has been reached regarding a new classification or nomenclature for this receptor and henceforth any reference to it within this thesis will use the V_{1A}, V_{1B} and V_2 classification.

1.3.2 V_{1A} receptors (V_{1A}Rs)

1.3.2.1 Molecular biology

In 1992, Morel *et al* first described the molecular cloning of a cDNA encoding rat V_{1A}R isolated from rat liver, a particularly significant event in the history of AVP research (91). A number of groups have, since then, described the cloning, expression and distribution of rat, human and sheep V_{1A}R genes (91-93). From these nucleotide sequences of this cloned cDNA the receptor protein amino acid sequences have been deduced.

The translated $V_{1A}R$ protein is G-protein coupled and has the characteristic extracellular amino-terminus, an intracellular carboxyl-terminus and spans the cell membrane 7 times in between (Figure 1.2). This feature was deduced from their amino acid sequences by the relative positions and sizes of hydrophobic and hydrophilic clusters of amino acids. The amino acid sequence homology between human, rat and sheep $V_{1A}Rs$ is approximately 70-80% (93). There is approximately 40% preservation of amino acid sequence between the rat AVP $V_{1A}R$ and the rat V_2R and almost half of the non-conserved amino acids are located in the extracellular amino-terminus and intra-cellular third loop. All receptors of the AVP/oxytocin subfamily have conservation of amino acids located in the first 2 extracellular loops, a conservation that is not seen in other receptor subfamilies. The conserved sequence found in the first extracellular loop is FXGPDXLCRXVK while that in the second, DCWAXFXXPWG (94). It has been postulated that these shared amino acids, being unique, form part of a ligand recognition site (95). Moreover, it has been shown that a disulfide bond between cysteines (Cys^{124} and Cys^{205}) in the first 2 extracellular loops is essential to the integrity of the binding site (91). In a site-directed mutagenesis study the effects of single amino acid substitutions on AVP binding to the $V_{1A}R$ were examined in transfected COS-7 cells. Following a number of such substitutions, the conversion of Asp^{97} in the second transmembrane domain caused by far the greatest reduction in affinity for AVP (3800-fold decrease) and thus suggests that this particular amino acid has a key role to play in receptor recognition (96).

In terms of G-protein activation by the $V_{1A}R$, an RTVK sequence at the distal membrane interface of the third intracellular loop is thought to play an important role here (96).

The sequence NPWIY, found on the seventh transmembrane domain, is also conserved amongst the AVP/oxytocin family and is thought to be responsible for receptor-mediated endocytosis (77). The third cytoplasmic loop and the cytoplasmic tail contain a number of serine and threonine residues that are potential sites of regulation by protein phosphorylation while two adjacent cysteine residues in the cytoplasmic tail are potential sites for palmitoylation. This binding of fatty acid to amino acid serves the purpose of anchoring the cytoplasmic tail to the inner face of the plasma membrane (97).

1.3.2.2 Second messengers

Many biological signals are processed by the binding of chemicals to cell surface receptors. Signals are switched from these receptors to intracellular language via guanine nucleotide binding regulatory proteins (G-proteins) that are present in all eukaryotic cells and thus G-proteins serve as interfaces between receptor and biological responses. The $V_{1A}R$ is linked to a heterotrimeric G-protein (98). The stimulatory subunit (G_S) of this is activated following the binding of AVP and leads to activation of phospholipase C (PLC). This activated PLC causes hydrolysis of membrane lipid phophatidylinositol-4,5-bisphosphate to produce 2 second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on intracellular IP_3 receptors located on the smooth endoplasmic reticulum (sER). These receptors form a calcium selective ion channel and, following binding of IP_3, this IP_3 receptor ion channel opens to release intracellular stores of calcium from the sER into the cytosol. DAG formed from activated PLC activates protein kinase C (PKC) (19, 20, 99)(Figure 1.2). Stimulation of $V_{1A}Rs$ in smooth muscle cells or Chinese hamster ovary cells has been shown to stimulate other signal transduction pathways involving phospholipase A_2, C and D (100, 101). Activated $V_{1A}Rs$ have also been shown to stimulate tyrosine phosphorylation in fibroblast-like Swiss 3T3 cells (102).

Activated V_{1A}Rs have been shown to induce protein synthesis in neonatal cardiomyocytes via increased stimulation of the mitogen-activated protein kinase signal transduction pathway and intracellular calcium (75, 103).

1.3.2.3 Localisation and distribution

In vitro autoradiographic techniques have demonstrated that the V_{1A}R is widely distributed throughout the body.

In the rat brain V_{1A}Rs have been found in many regions such as the lateral septum, bed nucleus of the stria terminalis, arcuate nucleus, area postrema, choroid plexus as well as within the blood vessels (104-108). *In situ* hybridisation histochemistry (ISHH) studies have subsequently confirmed and extended the above distribution for the V_{1A}R by demonstrating V_{1A}R mRNA transcripts throughout the brain (109, 110).

In the heart, V_{1A}Rs have been shown to be associated with the cardiomyocytes (111-113) and with the coronary vasculature (36, 114).

In the kidney, V_{1A}Rs are found in the outer part of the inner medulla associated with the vascular structures, in the inner stripe of the outer medulla associated with the vasa recta (42, 106) and within nephron segments (115). Although localisation studies have failed to show V_{1A}R mRNA in glomeruli of normal adult rats, V_{1A}R mRNA is present in glomerular mesangial cells in developing rat kidney (116). V_{1A}Rs are known to be present on the luminal membrane of the collecting tubule principle cells (117-119).

In liver, $V_{1A}R$ protein and mRNA is associated with the hepatocytes and vascular epithelial cells, as well as with human hepatic stellate cells (106, 109, 120-125).

$V_{1A}Rs$ have also been localised to vascular smooth muscle cells, gonads and sympathetic ganglia (106), platelets (126, 127), peripheral mononuclear cells (128), thymus, spleen (129), adrenal (130, 131), uterus (132), as well as lung (133).

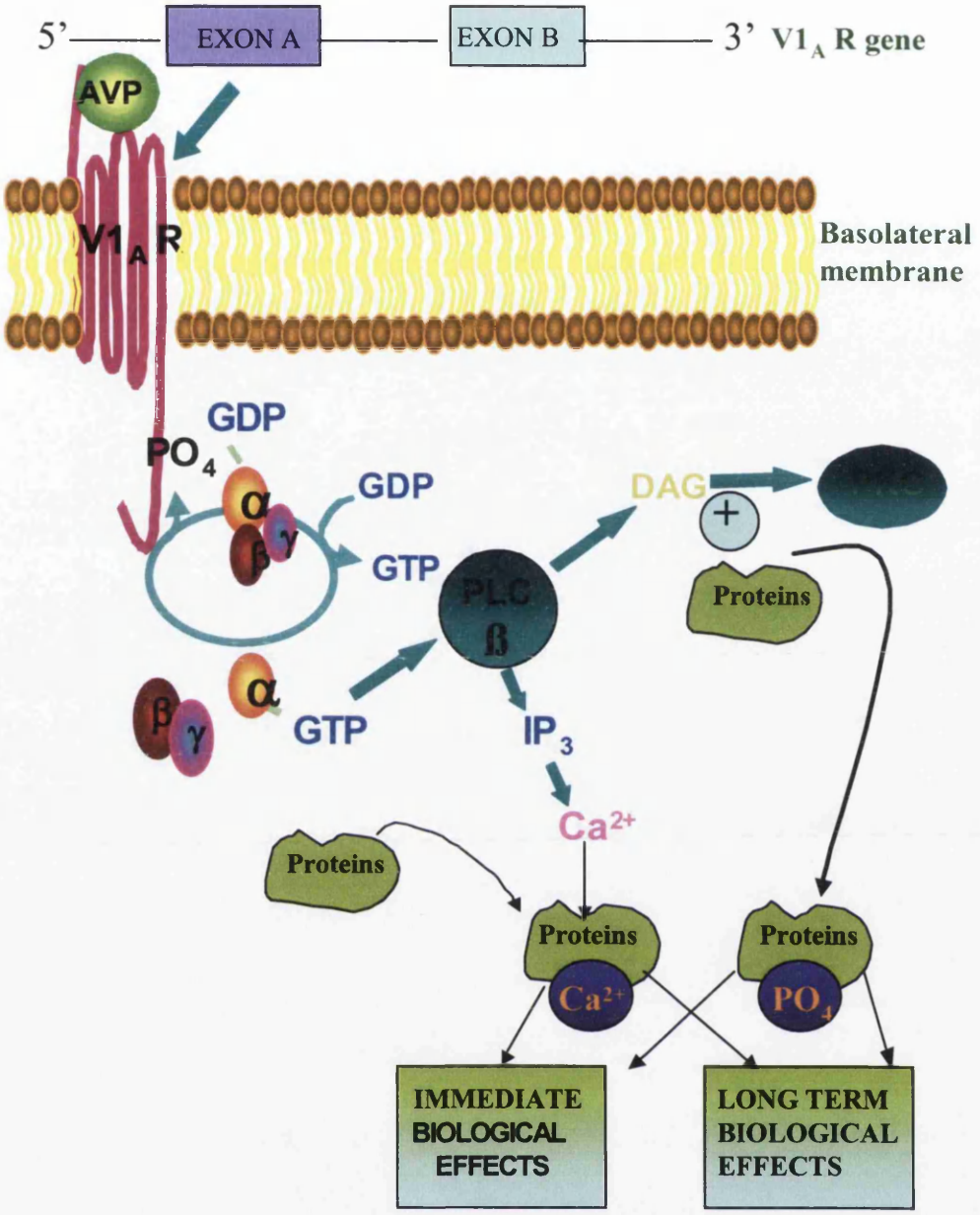

Figure 1.2. A representation of the $V_{1A}R$ system and its interaction with second messengers.

Abbreviations: PO_4, phosphate. Ca^{2+}, calcium. GDP, guanidine diphosphate. GTP, guanidine triphosphate.

The expression of mRNA for V_{1A}Rs is developmentally regulated and tissue specific. For instance, by reverse transcriptase polymerase chain reaction (RT-PCR), V_{1A}R mRNA was detected in newborn rat heart but not in the adult rat heart (134). By contrast, V_{1A}R mRNA was detected in adult lung but not in that of the newborn while the expression in liver from newborn rats compared to that in the adult has been shown to be lower (134). In embryonic (day 16-19) kidney tissue, V_{1A}R mRNA predominates in the developing cortex but by 1 day old this has switched to the vascular elements, cells in the developing medullary collecting ducts (CD) and mesangial cells of glomeruli (110).

1.3.2.4 Biological actions of AVP at the V_{1A}R

The principle physiological effects and sites of action for AVP are summarized in figure 1.3.

Renal effects: Although blood flow to the inner renal medulla comprises less than 1% of the total renal blood flow, changes in flow to this region can have a major effect on sodium and water homeostasis and on the long-term control of arterial BP (135). Using videomicroscopy and laser-Doppler flowmetry techniques it has been shown that AVP has unique effects to modulate renal urinary concentrating ability through its vasoconstrictor effects on the medullary microcirculation to decrease renal medullary blood flow in anaesthetised rats (135-137). Infusion of a V_{1A}R selective agonist [Phe^2,Ile^3,Orn^8]AVP into the renal medulla selectively reduced blood flow in the outer medulla by 15% and to the inner medulla by 35% and resulted in a sustained hypertension. This suggests that the observed increase in BP may be related to changes in medullary blood flow (138-140). Stimulation of V_2Rs by medullary interstitial infusion of the V_2R agonist 1-desamino-8-D-AVP or infusion of AVP in rats, pretreated with a V_{1A}R antagonist, increased medullary

blood flow by 16% and 27% respectively (141, 142). These studies suggest that, in terms of renal medullary blood flow, AVP has 2 opposing actions resulting from stimulation of V_{1A} and V_2Rs. It seems likely therefore, that as a result of this balance, AVP itself is unable to induce systemic hypertension or sodium retention (142).

The role of AVP to modulate glomerular function is unclear. Although glomerular mesangial cell V_{1A}Rs have been described *in vitro* (143) their presence *in vivo,* in adult rat, has not been clearly identified. *In vitro* studies have suggested that AVP may modulate growth of glomerular mesangial cells in a similar way to vascular smooth muscle cells (144, 145). In the SHR, infusion of low and high dose AVP, at subpressor levels, for 15 days, led to dose-dependent proliferative changes that were associated with increased expression of TGFβ1 and collagen type-1. This suggests that AVP may contribute to glomerular proliferation and that AVP may exert its effects, in part, via induction of TGF-β1 (31). Chronic and selective blockade of the V_{1A}R with OPC 21268 did not lower BP in salt-loaded SHR but did lead to a suppression of glomerular platelet derived growth factor B-chain and proliferative cell nuclear antigen (30). Taken together, data from these studies suggest that AVP, via the V_{1A}R, may play a role in modulating glomerular function particularly in hypertensive states where renal proliferative injury is not uncommon.

Cardiac effects: There is increasing evidence to suggest that the heart is a target organ for AVP. *In vitro* studies show that AVP mediates negative inotropic effects in the dog heart (146) and modulates nitric oxide synthase activity in cardiac myocytes (147). Recently cardiac AVP mRNA has been detected in isolated, perfused, pressure overloaded rat hearts and localised mainly to endothelial cells and vascular smooth muscle cells of arterioles and perivascular tissue (36). The induction of cardiac AVP in this pressure overload model

subsequently led to coronary vasoconstriction and impaired relaxation and these effects were prevented by $V_{1A}R$ blockade (36). A study in the isolated perfused rat heart in which coronary artery blood flow is kept constant has shown that AVP, via activation of cardiomyocyte $V_{1A}Rs$, produces a positive inotropic effect at low concentrations and a negative inotropic effect at higher concentrations (148). It has been suggested that AVP may play a role in the regulation of cardiomyocyte contractility via activation of the $V_{1A}R$ and a subsequent increase in intracellular calcium (149). In the rabbit heart, AVP has been shown to induce protein synthesis and this is mediated via the calcium dependent $V_{1A}R$ (150).

Central effects: Although AVP is one of the most potent vasoconstrictors of vascular smooth muscle known, supra-physiological concentrations of AVP are required to cause any BP increase (151). This is secondary to a central effect of AVP that enhances those cardiovascular reflexes that buffer any such BP rise. Studies examining the effect of AVP on arterial baroreflex function have shown that constant infusion of AVP results in a dose-dependent reduction in heart rate and sympathetic nerve activity in response to an increase in arterial pressure and that this ultimately leads to a leftward shift of the baroreflex curve (152, 153). Furthermore, several lines of evidence point to the $V_{1A}R$ as that responsible for these AVP effects on baroreflex action. In the rabbit (154) and baboon (155), administration of a $V_{1A}R$ antagonist blocks the effect of AVP on baroreflex function. The effect of AVP infusion and that of selective V_1 and V_2R antagonists and agonists on baroreflex control of heart rate, during changes in arterial pressure, has also been evaluated in conscious rabbits (153). These authors found that a selective V_1R antagonist blocked the characteristic shift in baroreflex function to lower pressures caused by AVP. Furthermore they were able to mimic the action of AVP in this regard using a selective

V_1R agonist, while a selective V_2R agonist had no effect. These central effects of circulating AVP on the baroreflexes appear to be mediated primarily through the area postrema. This region has a number of projections to centres in the brain involved in cardiovascular regulation such as the NTS. The NTS is rich in AVP $V_{1A}Rs$ and is devoid of a blood-brain barrier and thus is ideally suited to respond to and modulate the cardiovascular actions of circulating hormones. For instance, microinjections of small amounts of AVP given directly onto the area postrema caused a dose-dependent decrease in renal sympathetic nerve activity and enhanced baroreflex sympatho-inhibition during infusions of adrenaline (156).

Hepatic effects: In rodent liver, AVP acts through hepatic $V_{1A}Rs$ to stimulate glycogenolysis, gluconeogenesis and raise blood glucose (157-159), effects that have been demonstrated in other animals (159) and also in man (158). AVP has also been shown to stimulate urea synthesis within the liver and this is thought to contribute to providing more urea to the kidney for improving urinary concentrating capacity (160). Although not tested *in vivo* AVP, through $V_{1A}R$-mediated contraction and proliferation of human hepatic stellate cells, may also play a role in modulating sinusoidal resistance (161).

Platelet effects: Other AVP actions mediated through the $V_{1A}R$ include the promotion of platelet adhesion, aggregation and secretory activity (162, 163). In humans, immunoreactive plasma AVP concentrations have been shown to be higher in platelet rich plasma compared to platelet poor plasma in normal individuals, as well as those with hypertension and CCF (164, 165). The physiological significance of this platelet AVP is, at present, not clear. It has been proposed however that the platelet may act as a carrier of AVP, presumably via binding to its $V_{1A}R$. As platelets are in close apposition to the

vascular wall they may provide a mechanism for delivery of a higher concentration of AVP to local vascular sites (64).

Adrenal effects: V_{1A}Rs have been found in the zona glomerulosa and fasciculata of the human adrenal gland (166). AVP, through its effects at the V_{1A}R, is as potent as Ang II in stimulating the release of aldosterone from glomerulosa cells *in vitro* and stimulates cortisol release from fasciculata cells (166).

Mitogenic effects: AVP, via activation of the V_{1A}R subtype, is increasingly recognised as a cellular growth factor and this effect has been implicated in a number of normal and pathophysiological responses (167, 168). In vascular smooth muscle cells, AVP induces hyperplasia and hypertrophy (169) as well as stimulating the expression of early growth response genes (170, 171).

Uterine effects: In both rats and humans, the myometrium is sensitive to AVP and oxytocin. In rats, the myometrium is more responsive to oxytocin yet the binding affinities of the 2 peptides to myometrial receptors are roughly identical (172). However in the human uterus the non-pregnant myometrium responds more strongly to AVP, a situation that is reversed as term approaches (173). It is unclear at this stage through which receptor type oxytocin and AVP are acting as ^{3}[H]oxytocin can displace both AVP and oxytocin from uterine membranes (174, 175). A study using OPC 21268 suggests that the V_{1A}R is responsible as this selective V_{1A}R antagonist inhibited both oxytocin- and AVP-induced contractions of myometrial strips from rats and full-term pregnant women and did so in a dose-dependent and reversible manner (172).

Eye effects: Little is known of the AVP system within the eye and information currently available in the literature is often conflicting. AVP has been demonstrated within the mammalian retina, anterior uvea and iris (176-179) and at physiological levels can stimulate active sodium transport into the eye to raise intraocular pressure (IOP) (176). This would suggest that AVP receptor blockade represents a good target in the treatment of diseases associated with raised IOP such as glaucoma and diabetic retinopathy. In cultured human retinal pigment epithelial cells AVP has been shown to stimulate the production of IP_3 and calcium and this effect is inhibited by prior treatment with a $V_{1A}R$ blocker (180). In the same model, another group, while demonstrating an increase in the IP_3 second messenger system, found this to be activated by the addition of a V_2R agonist with only limited activation with a V_1R agonist (181). In the monkey, a tritiated V_1R antagonist was found to bind with high density in the region of the iris (182). Recently a study using a rabbit model with raised IOP demonstrated lowered pressures after treatment with the V_2R selective antagonist SR 121463 and this drug had a good local and systemic safety profile within this model (183).

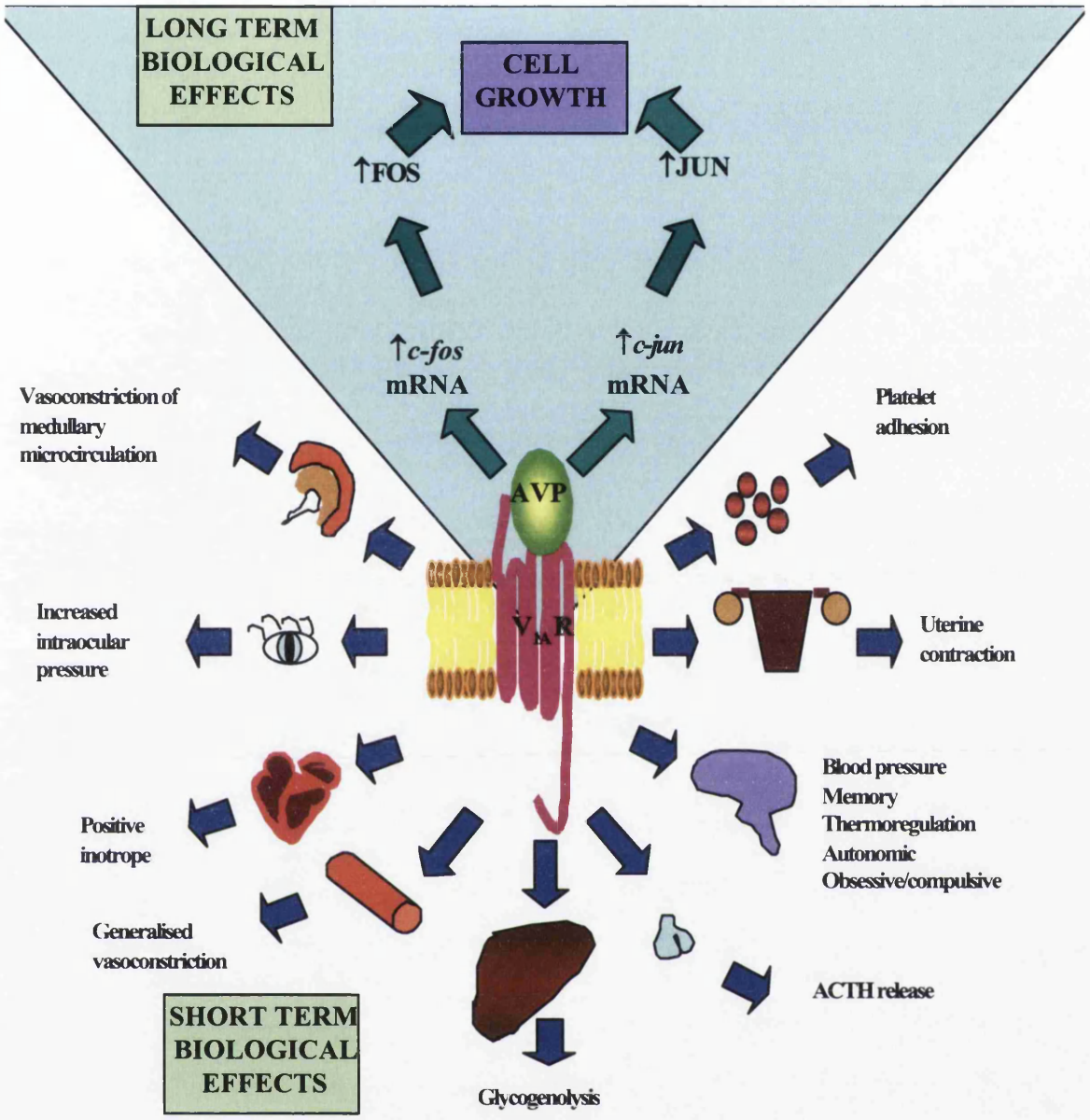

Figure 1.3. A representation of the biological actions of AVP at the $V_{1A}R$.

1.3.3 V_2 receptor (V_2R)

1.3.3.1 Molecular biology

Molecular cloning of the X chromosome and deduction of the amino acid sequence for the human and rat V_2R was accomplished in 1992 (184, 185) along with the genomic organization of the human V_2R gene (186). In addition the rat V_2R promoter region has also been sequenced by inverse PCR mediated cloning (187). The translated V_2R protein has the characteristics of a G-protein coupled, 7 transmembrane domain, receptor consisting of 7 hydrophobic regions connected by 3 extracellular and 3 intracellular loops. The molecular weights of the rat and human V_2Rs are similar (40,518 d compared with 40,285 d respectively) and the human V_2R displays approximately 86% sequence homology with the rat V_2R (187). The coding region of the rat and human V_2R genes is interrupted by 2 short introns. In rat kidney there are 2 mRNA variants, V_{2L} and V_{2S}, shown to arise from a single V_2R gene by alternative gene splicing (188). The long (V_{2L}) form encodes the AC-coupled receptor. The short (V_{2S}) form of the receptor lacks the nucleotide sequence encoding the putative seventh transmembrane domain and is inactive on the AC pathway within transfected cells (188). Quantitative RT-PCR on micro-dissected nephrons demonstrated that neither V_{2L} nor V_{2S} are expressed in glomeruli and proximal tubules, whereas they are present in the ascending limb of the Loop of Henle and the CD (188). In all nephron segments the ratio of V_{2S} to V_{2L} mRNA is constant at 15% and therefore high V_{2S} levels are only observed within the CD (188). The V_2R has a single site for N-glycosylation at asparagine 22 located at the extracellular N-terminus (189). Likewise, utilising such techniques as site-directed mutagenesis and sialidase treatment of the receptor protein, it has been shown that O-glycosylation is present on most of the serines and threonines at the amino terminal end (190). From transfection experiments, the functional significance of these post-translational modifications remains unclear as mutant

V_2Rs, that lack the target amino acids for N- and O-glycosylation, function in a manner indistinguishable from the wild type receptor (190).

NDI is a rare clinical condition resulting from mutations in the V_2R and aquaporin-2 genes (AQP-2). It is characterised by an inability of AVP to concentrate urine and therefore regulate water homeostasis and this in turn leads to polyuria, severe dehydration and in severe cases, even death (191). It has 2 types of inheritance, X-linked and autosomal recessive. More than 130 mutations in the V_2R gene (the X linked type) have been described for this disease and these account for 95% of cases. Genetic linkage studies have localised the NDI locus to the q28-qtr region of the X chromosome (192, 193). The other 5% (autosomal recessive type) of patients have normal V_2Rs but have mutations that disrupt the gene encoding for AQP-2 (194-198). The majority of these are single amino acid mutations that result in protein configurational changes and the trapping of the abnormal proteins within the sER (199). The number of receptors present on the cell surface are thus reduced or abolished. Other mutations still allow the receptor to be present on the cell surface but may alter the ligand-receptor binding affinity or reduce the coupling efficiency to G_s (199-202). One of the first characterised mutations of the V_2R, associated with NDI, is a substitution of arginine 137, found at the cytoplasmic end of transmembrane domain III, for histidine (R137H). The functional consequence of this mutation has been shown to be a constitutive arrestin-mediated desensitisation in that, unlike the wild-type V_2R, the non-signalling R137H receptor is phosphorylated and sequestered in arrestin-associated intracellular vesicles even in the absence of agonist (203). Current treatment strategies for NDI include the limitation of fluid and dietary sodium intake as well as thiazide and amiloride administration (199). In most cases these control the disease but are non-curative. Current research into curative treatments is

focusing primarily on gene therapy (204) but also on chemical chaperones that restore the routing of AQP-2 mutants to the apical membrane of the CD (195). Once *in situ* these receptors retain the ability to function as normal (205).

1.3.3.2 Second messengers

The activation of the V_2R by AVP serves to increase water reabsorption from the urine into the bloodstream and this is effected as follows. Activated V_2Rs stimulate AC via the stimulatory G-protein Gs. This results in an increase in the second messenger cAMP that activates protein kinase A (PKA) and starts a phosphorylation cascade leading to the insertion of the AVP sensitive water channel AQP-2 into the apical cell membrane (21, 22, 206-210). To date, the mechanism by which an increased cAMP stimulates the microtubular-dependent reversible translocation of AQP-2 from cytoplasmic vesicles to apical cell membrane has not been clarified. Figure 1.4.

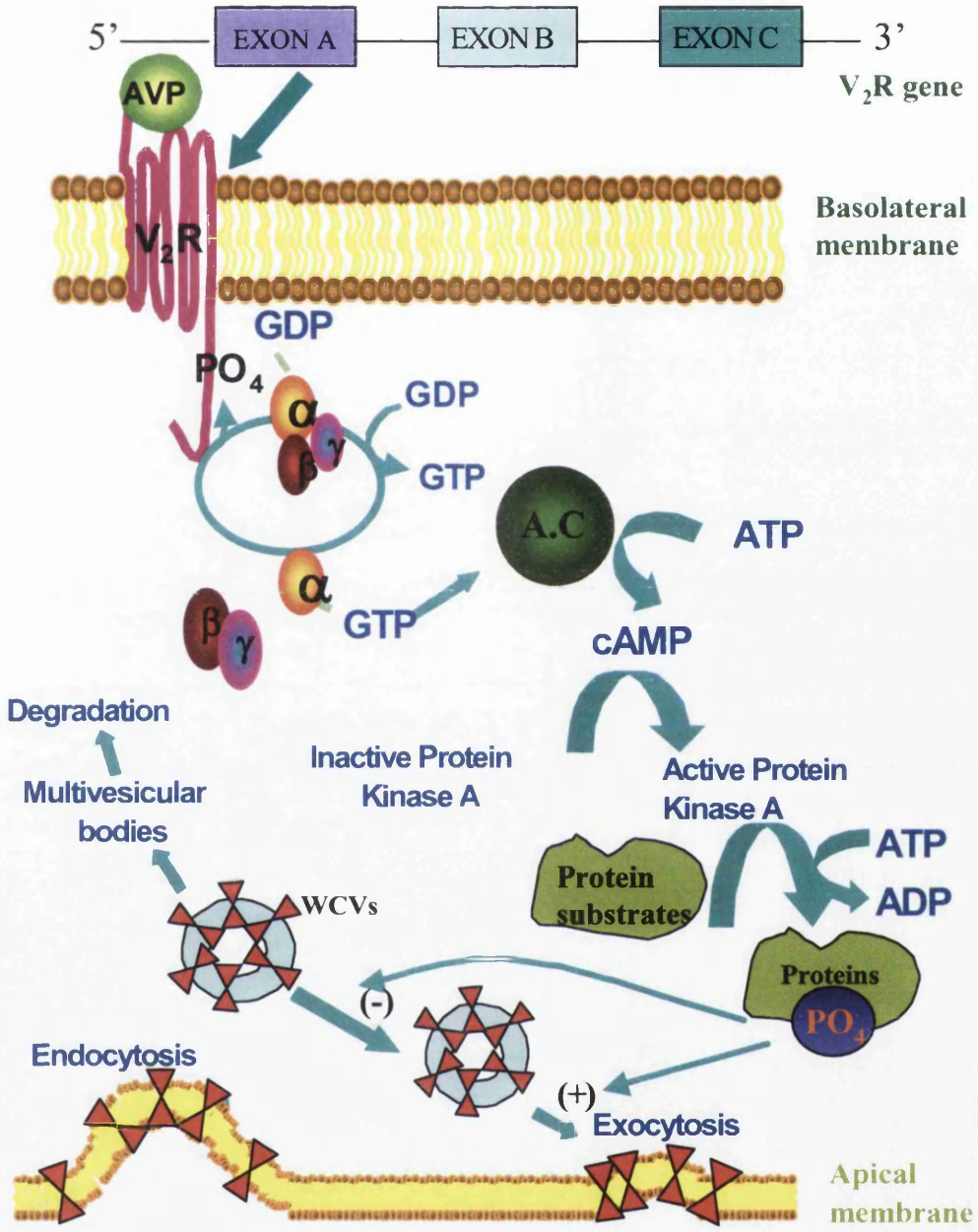

Figure 1.4: A representation of the V_2R and its interaction with second messengers to effect its principal action of enhancing water retention in the kidney. WCV=Water carrying vesicle, also known as AQP-2.

1.3.3.3 Localisation and distribution

In contrast to the wide distribution of the V_{1A} and V_{1B}Rs, functional V_2R protein has only been demonstrated in the principle cells of the CDs and inner medullary CD cells of the kidney (210, 211). More specifically the V_2R protein has been localised to the basilateral component of cells of the cortical and medullary CDs as well as cortical and medullary thin ascending limb and distal convoluted tubule.

In rat, using ISHH, V_2R transcripts have been detected in high amounts in the renal inner and outer medulla, primarily associated with CDs. Sparser labelling has been found in the renal cortex with no "grains" being seen in the glomeruli (109). Further studies by the same group, again using ISHH, demonstrated the presence of V_2R mRNA in rat at 16 and 19 days gestational age with labelling being localised to the cells of developing medullary and cortical CDs. After birth the V_2R mRNA is located in cells of differentiating thick limbs of the loops of Henle, papillary surface epithelium, overlying macula densa, and short distal nephron segments (109). In contrast to kidney, liver did not express V_2R mRNA at any time (116, 134). In the brain V_2R mRNA was found to be present in the newborn rat but this expression decreased with time such that none could be detected in rats older than 2 weeks of age (134). Others have found V_2R mRNA in the hippocampus of the adult rat brain, an area associated with memory and learning (212). Using RT-PCR, DNA sequencing and Northern blot analysis; V_2R mRNA has been identified in the human lung (213).

1.3.3.4 Biological actions

Renal: The principle action of the V_2R is to modulate salt and water homeostasis via the retention of water through the CDs of the kidney. Regulation of water permeability by AVP has been shown in the cortical CD (214), the outer medullary CD (215, 216), the initial intramedullary collecting duct (IMCD)(217), and the terminal IMCD (218, 219). In contrast, urea permeability is regulated via AVP, but only in the final segment of the CD and the terminal IMCD (217). The predominant action of AVP in the CD, via the V_2R, is to increase the permeability of the apical membrane and this is achieved by the insertion of the water channel AQP-2 into that membrane (Figure 1.4).

AVP acting at the renal V_2R, at concentrations seen following dehydration or low dose infusion, produces significant renal vasodilatation in conscious dogs without significant change in mean arterial pressure, cardiac output or heart rate (220). The mechanism for this particular AVP effect remains unclear at this stage. It may involve blockade of vascular V_2 or V_2-like receptors or be mediated through an AVP reduction in renal sympathetic tone effected from the area postrema (221), as central facilitation of the arterial baroreflex to AVP has been shown to be mediated by a V_2-like receptor (222, 223).

Other biological actions: The existence of non renal actions for AVP have been proposed indirectly based on observations from a number of physiological studies. For instance, in humans, AVP has been shown to stimulate forearm dilatation and that this is caused by V_2R mediated endothelial derived relaxing factor or nitric oxide release from vascular endothelial cells (224, 225). Additionally, the AVP V_2R agonist 1-deamino-8-D-AVP (dDAVP) induces von Willebrand factor release from endothelial cells, megakaryocytes and peripheral blood monocytes (226, 227). This response is reduced in patients with NDI

which further supports the role of AVP in these findings (198). AVP also stimulates cAMP accumulation in human mononuclear cells (228).

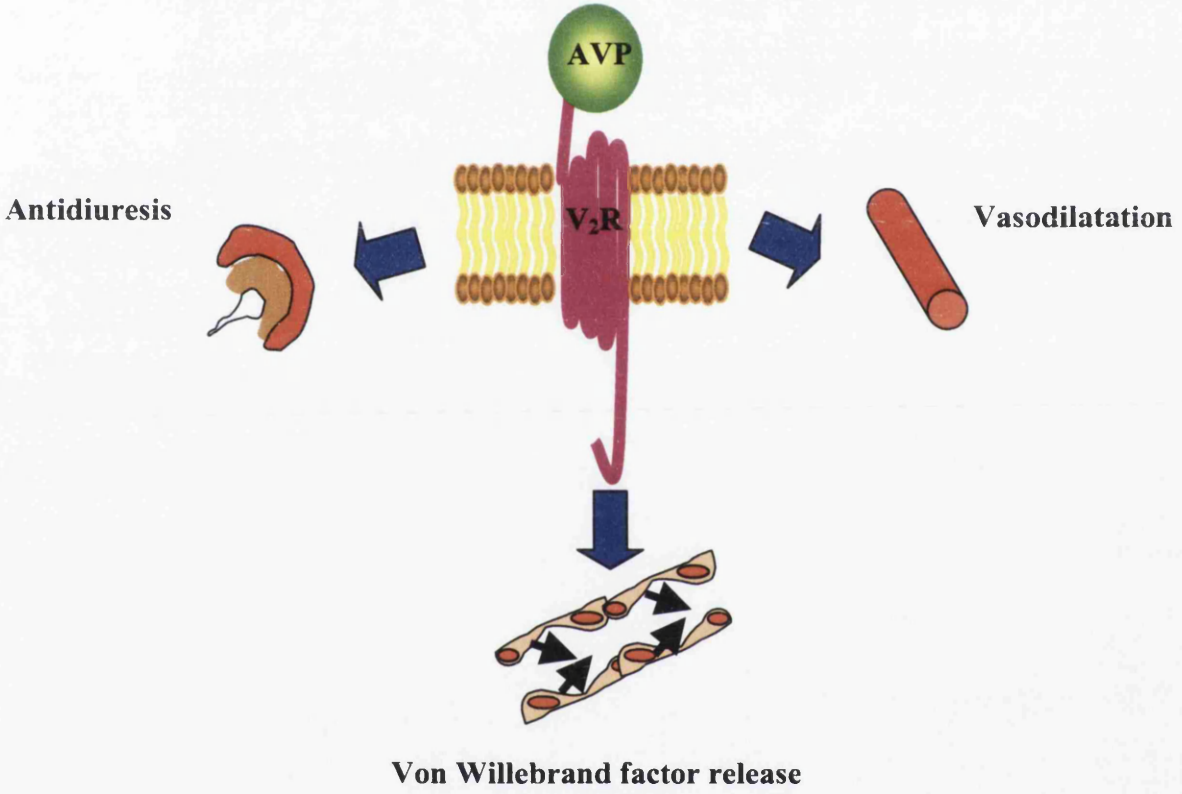

Figure 1.5. A representation of the biological actions of AVP at the V_2R.

1.3.4 V_{1B} receptor ($V_{1B}R$)

1.3.4.1 Molecular biology

Molecular cloning and deduction of the amino acid sequence for the human $V_{1B}R$ was accomplished in 1994 (229, 230) closely followed by the rat $V_{1B}R$ (231). The translated $V_{1B}R$ protein, as with the V_{1A} and V_2R, is a G-protein linked receptor with the classical 7 transmembrane domains consisting of 7 hydrophobic regions connected by 3 extracellular and 3 intracellular loops. The molecular weights of the rat and human $V_{1B}Rs$ are similar (47,031d and 46,977d respectively) with the rat and human $V_{1B}Rs$ having approximately 81% sequence homology (229, 230). The genomic organization of the $V_{1B}R$ closely resembles that of the V_2R with the intron at the same location between the first and second transmembrane domains in their coding regions (186, 188, 231). The N-terminal region preceding the first transmembrane domain contains a single glycosylation site at Asn^{21} (229). In humans the residues Lys-Val-Lys (231-233) and Lys-Ile-Arg (271-278), in the third intracellular loop, are conserved both in the V_{1A} and $V_{1B}Rs$ but not in the V_2R and may be important for the coupling of receptor and the PLC second messenger system (230).

1.3.4.2 Second messengers

AVP activation of the $V_{1B}R$, in a manner analogous to the $V_{1A}R$, results in activation of G-proteins and this in turn switches on PLC. PLC then catalyses the hydrolysis of phosphatidylinositol (4,5) bisphosphate and regulates target cell action via firstly, the generation of IP_3 and mobilisation of intracellular calcium and secondly, PKC activation via the generation of DAG (19, 20, 99, 232).

1.3.4.3 Localisation and distribution

The classical location of the $V_{1B}R$ is in anterior pituitary corticotrophs (233). Until very recently no specific antagonists have been available for this receptor and study of the $V_{1B}R$ has thus far proven very difficult. With the technique of immunohistochemistry, the $V_{1B}R$ protein has found to be widely spread throughout the brain, for instance, in the hypothalamus, amygdala, cerebellum, hippocampus and particularly in those areas with a "leaky" blood brain barrier or close to the circumventricular organs such as the medial habenula, SFO, organum vasculosum laminae terminalis and the median eminence (234). $V_{1B}R$ protein has also been described in rat and human adrenal medulla and in pheochromocytoma (130, 231, 235). $V_{1B}R$ mRNA transcripts have also been detected outside the brain in thymus, heart, lung, spleen, kidney, uterus, breast (231, 236, 237) and adrenal medulla (131). In March 2002, a novel non-peptide selective $V_{1B}R$ antagonist became available and has been characterised both *in vivo* and *in vitro* (47). This should further open the way to understanding the diverse functions of AVP as it acts through the $V_{1B}R$.

1.3.4.4 Biological actions

AVP classically acts through the pituitary $V_{1B}R$ to work in synergism with corticotropin-releasing factor in the regulation of the release of adrenocorticotropin hormone (ACTH) in response to stress (104, 238-240). In the adrenal medulla, $V_{1B}Rs$ within the chromaffin cells, regulate the release of catecholamine and are also involved in the secretion of ACTH. The widespread distribution of the $V_{1B}R$ within the brain would suggest that some of the central actions of AVP such as learning, memory and neuroendocrine activation, previously attributed to the $V_{1A}R$, may be resultant upon $V_{1B}R$ activation instead. In support of this $V_{1B}R$ knockout mice demonstrated behavioural changes (241).

1.4 NON-PEPTIDE AVP RECEPTOR ANTAGONISTS

Little more that 10 years ago it was extremely difficult to determine the role of AVP in CVDs because of a lack of suitable receptor antagonists. Whilst peptide AVP antagonists have been available for a long number of years now, their short half life, duration of action and parenteral administration has prevented their use in long-term study or their clinical development. Indeed much of the controversy that still surrounds the importance and function of AVP relates to the acute nature of previous studies using these peptide blockers as it is well recognised that initial responses to the blockade of homeostatic systems do not necessarily predict the more relevant long-term effects (50, 242). In addition, marked species heterogeneity exists for the peptide V_2R antagonists *in vivo* and this further complicated their clinical development as aquaretic agents (243). The development of non-peptidic, orally active, AVP receptor antagonists has made it possible to further investigate the role of AVP within disease since, for the first time, it is possible to block AVP receptors chronically and assess the functional consequences of this. Some of these non-peptide receptor antagonists have been researched extensively while others are currently under investigation (Table1.1). Potential therapeutic indications for AVP receptor antagonists comprise:

1. The blockade of the $V_{1A}R$ in arterial hypertension, CCF, Raynaud's syndrome, peripheral vascular disease and dysmenorrhoea.
2. The blockade of the V_2R in the SIADH, CCF, liver cirrhosis, nephrotic syndrome and any state of excessive retention of free water and subsequent dilutional hyponatraemia.
3. The blockade of $V_{1B}Rs$ in ACTH-secreting tumours.

Table 1.1: Summary of some of the non-peptide AVP receptor antagonists currently under clinical development.

Adapted from (42, 244, 245). Ki, or inhibition constant, is the concentration of antagonist required to inhibit 50% of the maximal V_{1A} or V_2R effects of AVP.

Drugname	OPC 21268	SR 49059	OPC 31260	SR 121463	OPC 41061	WAY-VPA985	VPA343	YM087
Route of administration	oral	oral	oral	oral	oral	oral	oral	oral
Ki, rat (nmol/l)	15 (kidney)	1.6(liver)	21.7(kidney)	1.42(kidney)	1.33(kidney)	0.48(kidney)	0.77(kidney)	0.9(V_{1A}) 0.4(V_2) (both kidneys)
Ki, human (nmol/l)	>1000 (multiple tissues)	6.3 (platelet)	25.4(kidney)	4.1(kidney)	1.12(kidney)	0.43(kidney)	Not determined	Not determined
Urine volume	Not determined	Not determined	Increased dose dependently	Increased dose dependently	Increased dose dependently	Increased dose dependently	Increased dose dependently	Increased dose dependently
Urine osmolality	Not determined	Not determined	Reduced dose dependently	Reduced dose dependently	Reduced dose dependently	Reduced dose dependently	Reduced dose dependently	Reduced dose dependently
Sodium excretion/24h	Not determined	Not determined	Unchanged	Unchanged	Unchanged at low dose	Unchanged at low dose	Unchanged at low dose	Unchanged
Agonist activity	No	No	No	No	No	No	No	No
AVP pressor response	Yes	Not determined	Not determined	Not determined	Not determined	Not determined	Not determined	Not determined

1.4.1 OPC 21268: $V_{1A}R$ antagonist

1-(1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2(1H)-quinolinone or OPC 21268 has selective effects at the $V_{1A}R$ and in 1991 was the first non-peptide AVP receptor antagonist to be described (244). It was developed by optimisation of a lead structure that was found by the screening of several thousand compounds (244). The drug was further characterised *in vitro* and *in vivo* in the rat (123) and since that time has been used widely and added a great deal to our understanding of the actions of the $V_{1A}R$ in disease. In rat, for instance, it has been shown to lower BP both acutely and chronically in mineralocorticoid hypertension (28) and to reduce BP in young SHR (50). The administration of OPC 21268 to rats with cold-induced brain injury and oedema exerted a significant protective effect, particularly in the areas where the maximum amount of blood-brain barrier breakdown was seen (246). OPC 21268, given to a rat model of diabetic nephropathy, significantly reduced albuminuria (247) and, again in rat, OPC 21268 also reduced the incidence of alcohol-induced gastritis (248).

After rapid ventricular pacing in the dog, a model of decompensated CCF, OPC 21268 significantly increased cardiac output and reduced total peripheral resistance and mean arterial BP (37). Unfortunately, there is great species variability in the response to OPC 21268 and its affinity for human $V_{1A}Rs$ is rather weak (249, 250). For instance OPC 21268 failed to prevent the AVP-induced contraction of human internal mammary arteries harvested from patients undergoing coronary artery bypass surgery (249). This has therefore greatly limited its clinical development. A recent study has demonstrated that the second transmembrane domain of the $V_{1A}R$ and in particular the amino acid residue Ala-342, which is absent in the human receptor, is responsible for the high affinity binding seen in the rat (251). This drug will be used alone and in combination with an ACE inhibitor in

a bid to look for additive benefits of dual therapy within a rat model of established hypertension in chapter 4 of this thesis.

1.4.2 SR 49059: $V_{1A}R$ antagonist

((2*S*)1-[(2*R*3*S*)-5-chloro-3-(2-chloro-phenyl)-1-(3,4-dimethyoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide) or SR 49059 was developed and characterised in 1993 (42). It has a marked affinity and selectivity for the $V_{1A}R$, no partial agonist activity and no significant effects at the V_2R (42, 252, 253). When studied in the Swiss 3T3 fibroblast model SR 49059 was shown to exhibit the most potent antiproliferative effect of any $V_{1A}R$ antagonist yet seen (254). Its main advantage over OPC 21268 is its effectiveness, not only at animal, but also human $V_{1A}Rs$ and thus is likely to prove a useful tool for studying the pathophysiological role of AVP in man and has greater potential for clinical development (255). In phase one trials it proved not only to be highly effective in reducing the $V_{1A}R$ dependent end-points of platelet aggregation and skin blanching but had an excellent side effect and safety profile also (256).

1.4.3 OPC 31260: V_2R antagonist

In 1992, through modification of the structure of OPC 21268, Yamamura *et al* developed and characterised a potent V_2R, non-peptide antagonist and named it (5-dimethylamino-1-[4-(2-methylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride) or OPC 31260 (43). It is a competitive AVP receptor antagonist with a renal V_2R: hepatic $V_{1A}R$ selectivity ratio of 25:1 (28, 123). Interestingly, OPC 31268 was the first non-peptide, AVP V_2R blocker effective in humans to increase free water clearance. Its mode of diuretic action, being different from that of previously known diuretic agents such as

frusemide and bendrofluazide, is characterized as an aquaresis. After the administration of a traditional diuretic agent water loss accompanies sodium excretion. With V_2R antagonism sodium is conserved while water alone is passed (43). With the discovery of OPC 31260, therefore, came the huge potential for the clinical development of drugs to combat the various medical conditions associated with water retention and hyponatraemia such as SIADH, CCF, liver cirrhosis and the nephrotic syndrome. Various animal and human studies have been undertaken using this drug and a number of these are outlined here. In dogs, the renal vasodilatory response resultant from exogenous AVP administration was blocked by OPC 31260 without any concomitant changes in cardiac output (220). OPC 31260 was found to be as effective as water restriction in the treatment of SIADH (257). Similar studies have shown good treatment efficacy in humans and rat although interestingly, lower doses were required to produce similar aquaretic responses in these species (257). In the male rat, pre-treatment with OPC 31260 significantly increased the vasopressor response to exogenously administered AVP, providing evidence for a V_2-like receptor related vasodilator effect in these animals (258). Short-term antagonism of the V_2R during the BP development phase in the SHR results in a paradoxical increase in BP that persists following treatment withdrawal, and suggests a role of the V_2R in preventing the development of hypertension in this animal model (49). In a 4 week study using a rat model of compensated CCF, chronic treatment with OPC 31260 resulted in a sustained and significant aquaresis but no survival benefits. Likewise no positive influences on cardiac remodelling or function were seen and plasma AVP levels were significantly elevated with this treatment (41). In human studies this drug has been shown to have beneficial aquaretic effects in SIADH, liver cirrhosis and in the prevention of cerebral oedema after subarachnoid haemorrhage (38, 259, 260).

1.4.4 YMO87: V_{1A}/V_2R antagonist

(methyl-1,4,5,6-tetrahydroimadazo[4,5-d][1]benzoazepine-6-carbonyl]-2-phenylbenzanilide monohydrochloride), more simply known as YMO87 or conivaptan, is a newly discovered non-peptide AVP receptor antagonist that has an affinity for both rat V_{1A} and V_2Rs that is equipotent to that seen with AVP itself (46, 169). It is a competitive antagonist at both the V_{1A} and V_2Rs with no agonist properties and is the first mixed AVP receptor blocker to be described (46). YM087 has been shown to be an effective dual blocker in both animal (rat and dog) and human models (169, 261-264). In cultured rat neonatal cardiomyocytes, YMO87 has been shown to prevent the AVP-induced increase in protein synthesis within this system and may therefore potentially be of clinical benefit in any diseases that involve cardiomyocyte hypertrophy (103). Likewise, in cultured rat vascular smooth muscle cells, YM087 potently and concentration-dependently inhibited AVP-induced hyperplasia and hypertrophy (169). In dogs with pacing-induced CCF, IV administration of YM087 not only promoted a marked water diuresis but also significantly increased cardiac output and significantly decreased left ventricular end-diastolic pressure and total peripheral resistance (265). In human studies, YM087 has been shown to be a highly effective treatment when given long-term to patients with SIADH who are refractory to water restriction. A more recent study has shown that a single IV dose of YM087, given to patients with advanced CCF (New York Heart Association class III and IV), resulted in a dose-dependent increase in urine output and also a significant reduction in pulmonary capillary wedge pressure and right atrial pressure when compared to placebo (266). These studies suggest that this drug may find clinical use as treatment for diseases associated with hyponatraemia and in particular CCF.

1.5 AVP IN PATHOPHYSIOLOGICAL STATES

AVP, its effects in CVD, as well as the regulation of its receptor system has received relatively little attention thus far and the precise role it has to play in conditions such as hypertension and DM remains controversial (41, 50, 242). Since the advent of the non-peptide receptor antagonists and selective radioligands, research has demonstrated the likelihood that AVP is important in these CVDs. Specifically relevant to this thesis is the role of AVP in hypertension and DM.

1.5.1 Hypertension

Given that AVP is a vasoactive peptide having vasoconstrictor effects through V_{1A}Rs in vascular smooth muscle and the brain and an antidiuretic action through activation of V_2Rs in the renal tubule, it seems likely that this hormone will have a role to play in the development and/or maintenance of hypertension. To date, however, surprisingly little is known regarding the precise role that this hormone has to play in the pathophysiology of this condition.

1.5.1.1 AVP and hypertension: evidence from animal models

Initially the discovery that SHR cross-bred with the BB rat still developed hypertension was a strong argument against the importance of AVP in genetic hypertension, but the subsequent finding of AVP-like immunoreactivity, indistinguishable from authentic AVP, in peripheral tissues of the BB rat casts doubt on the validity of this argument (35, 49, 50, 63). The development of non-peptide, orally active and selective AVP receptor antagonists have allowed reassessment of the role of AVP in both the development and maintenance of high BP but it remains controversial. Studies using the V_{1A}R antagonist,

OPC 21268, confirmed the importance of AVP in the maintenance of BP in volume expanded states such as mineralocorticoid hypertension where AVP levels are elevated (28) and demonstrated conclusively that AVP is not involved in the maintenance of BP in either renovasular (267), genetic hypertension (50) or more recently ACTH-induced hypertension (268). However the importance of AVP in the development of hypertension has been demonstrated in the SHR in a manner analogous to ang II (49, 50). This study also demonstrated a lack of additive BP lowering benefits of $V_{1A}R$ antagonism and ACE inhibition and interestingly that the use of V_2R selective antagonist OPC 31260, again during the developmental phases of hypertension, actually augmented hypertension in adult SHR. No study, as yet, has looked for any additive BP lowering effects of ACE inhibition and $V_{1A}R$ antagonism in the adult SHR with established hypertension, part of the remit of this thesis.

1.5.1.2 AVP and hypertension: evidence from human studies

The contribution of AVP to the maintenance and development of human hypertension is not well understood at this time. Results regarding plasma AVP levels in hypertensive patients are not consistent with high levels in some studies but normal or low levels in others (269-271). There does, however, appear to be a subset of hypertensive patients, those with associated low plasma renin levels, in whom AVP is consistently elevated. These patients include the elderly, African-Americans as well as patients with chronic renal failure and DM (27, 29, 272, 273). The importance of AVP as a pressor agent in low-renin hypertension has been shown in a number of studies. The first used an IV injection of a peptide V_1R antagonist and demonstrated that in elderly and in black participants the depressor responses to this antagonist infusion were significantly greater than in young and white volunteers (274). A second similar experiment showed that a

single IV injection of a $V_{1A}R$ antagonist lowered arterial BP in African-Americans but not in Caucasians (by 28±4 mmHg in African-Americans versus 5±3 mmHg in Caucasians, $P<0.05$ (29). A population-based study has shown that plasma AVP concentrations display a discernable relationship with arterial BP, particularly when renin levels are low (275). Furthermore, a single dose of a peptide $V_{1A}R$ antagonist in diabetic hypertensive patients with autonomic neuropathy led to a significant reduction in diastolic orthostatic BP, but not supine BP, suggesting that AVP plays a major role in modulating BP in the upright posture in these patients (276). Published data on the effect of non-peptide AVP receptor antagonists in human BP control has thus far been very limited. A study in essential hypertension demonstrated that plasma AVP levels were similar between normotensive and hypertensive subjects and that OPC 21268 did not produce any significant changes in BP or heart rate in these patients. This finding was independent of a low or high salt diet (277). Another group was able to demonstrate that a single oral dose of non-peptide $V_{1A}R$ antagonist, SR49059, given to a group of patients with mild essential hypertension resulted in a transient vasodilatory effect only, and no sustained BP reduction was seen (278). This group of patients included 12 black as well as 12 white subjects and a failure to see a fall in BP in the low-renin African-American group is in direct contrast to many of the studies outlined above. These discrepancies have been explained in terms of different antagonists used, different conditions of BP monitoring (supine verus orthostatic) and different demographic conditions such as age and gender.

Thus with regards to hypertension and AVP in man it seems likely at this stage that AVP plays a significant role in a subgroup of patients with low-renin hypertension such as the elderly, African-Americans and diabetic hypertensive patients. Currently, however, there is

little published data on long-term AVP receptor antagonism that would support this argument and this is something that clearly needs to be addressed.

1.5.2 Diabetes Mellitus (DM)

DM is a growing health concern of epidemic proportions with greater than 1.4 million people in the UK currently diagnosed (279) and an estimated further million who have the disease but remain unaware (280, 281). Moreover it is estimated that the prevalence of DM is likely to double between 1995 and 2010 (282). Diabetic nephropathy represents a major complication of DM and develops in about one-third of patients within 10-20 years after the onset of the disease and leads in most cases to end-stage renal failure with its associated high morbidity, mortality and associated national health expense (283-286). Indeed, diabetic nephropathy is the leading cause of end-stage renal disease in the Western world (286-288). The incidence of CVD is very high amongst diabetics with an annual mortality rate in the type 2 patient of as much as 4% per year as compared to an annual mortality rate of less than 1% in an age matched non-diabetic population (289). Although better blood glucose control and advances in drug therapy, such as the introduction of ACE inhibitors, reduces the likelihood of renal failure and cardiovascular complications it does not completely prevent them (15, 290). More effective strategies are therefore required. Several studies have shown that AVP is elevated in DM but the consequences of this have received little attention (23, 51, 125).

1.5.2.1 DM and an elevated plasma AVP

More than twenty years ago Zerbe *et al* were able to demonstrate that patients with type 1 DM and ketoacidosis had a marked elevation in plasma AVP concentration (23). Although

the degree of this elevation does vary from one study to another, all subsequent studies in humans with either type 1 or type 2 DM have confirmed this finding (24, 25, 291). In rats, streptozotocin (STZ)-induced DM results in a 2-to7-fold elevation in plasma AVP (51, 292-294) with there being a strong positive correlation between the dose of STZ given, the degree of resultant hyperglycaemia and the plasma AVP concentration seen. With a genetic form of DM (dbdb), an animal model for type 2 DM, rats exhibit an even greater elevation (293). In both rats and humans the correction of hyperglycaemia is accompanied by a significant reduction in plasma AVP and thirst. In DM the degree of elevation or reduction in plasma AVP, seen in response to various stimuli, is exaggerated. For instance, the rise in plasma AVP after hypertonic saline infusion (292) or after cigarette smoking and the fall in AVP observed after induction of hypoglycaemia is greater in diabetics as compared to control subjects (295). The usual circadian rhythmicity in plasma AVP observed in normal rats is lost with uncontrolled type 1 DM (296) and the AVP content of the neurohypophysis is reduced in patients with uncontrolled type 2 DM (297).

What factors might be responsible for this AVP elevation seen in DM? Thus far, several studies have investigated this (23, 292, 298, 299). During hyperglycaemia AVP remains inappropriately high, even when plasma sodium is in the hyponatraemic range, and might suggest a resetting of the hypothalamic "osmostat". It could be suggested that insulin deficiency transforms the osmoreceptor from glucose-insensitive to glucose-sensitive for its role in AVP release. Hyperglycaemia itself, however, does not seem responsible for the resetting of this "osmostat" as an IV infusion of hypertonic dextrose will not increase plasma AVP in diabetic subjects or healthy controls (300, 301). On the other hand, abrupt discontinuation of an insulin infusion in type 1 diabetics increases plasma AVP and this increase is delayed and significant only after 4 hours, despite the rapid rise in plasma

glucose seen (299). Besides glucose, amino acids represent other osmotically active solutes that are elevated in DM (302-304). This disturbance is secondary to the role that insulin normally plays in amino acid uptake into cells through their membranes (305, 306). It is possible that a chronic elevation in the plasma level of some amino acids could play a role in the high AVP secretion of DM (307). Plasma amino acid concentrations increase by 2-3mmol/l after a high protein meal while concentrations of sodium and urea remain little changed. Interestingly such a meal also triggers an increase in plasma osmolality and AVP and therefore it can be postulated here that amino acids represent the most likely osmotically active substances to trigger this (308). They may also play a role therefore in the elevated AVP levels seen in DM. Amino acid levels are also significantly disturbed in chronic renal failure (CRF) a disease that is also associated with an elevation in plasma AVP (267, 309).

1.5.2.2 Acute physiological benefits of an elevated plasma AVP in DM

In the short-term an elevation in plasma AVP plays a vital role in limiting the glucose-induced water loss and dehydration associated with uncontrolled DM. In uncontrolled DM the osmolar load is largely enhanced by glycosuria, glucose alone representing more than half of the excreted solutes (310). Under the influence of AVP, a considerable amount of the body's water, that is obligatorily filtered along with this glucose, is able to be reabsorbed in the CD and to such an extent that the final concentration of glucose in urine is far in excess of that in plasma (311). The AVP elevation in DM represents an appropriate adaptation by limiting, to some extent, the amount of water required for the excretion of a markedly elevated load of solutes (312).

1.5.2.3 Chronic deleterious effects of an elevated plasma AVP in DM

AVP is known to be elevated in chronic renal disease (272). Several studies have strongly suggested that when sustained for long periods of time this continuous, high urinary concentrating activity, may become deleterious to the kidney (313). Chronic infusion of AVP or its V_2R agonist dDAVP, in normal rats, induces glomerular hyperfiltration (314, 315) and kidney hypertrophy (316) and increases urinary albumin excretion (312). On the other hand, a chronic reduction in urinary concentrating ability slows the progression of CRF and reduces glomerulosclerosis, albuminuria and tubulointerstitial injury in BB rats with five-sixths nephrectomy (317). The mechanisms of these deleterious effects of AVP are not yet understood. AVP causes mesangial cell proliferation and extracellular matrix production via the $V_{1A}R$ (318, 319). AVP is involved in the renal regulation of BP control and contributes to increased renovascular resistance through $V_{1A}R$-induced vasoconstriction in the vasa recta of the renal medulla (106, 116) (320). AVP, through its actions on the renal V_2R, contributes to fluid retention and hyponatraemia. Since AVP is elevated in DM, and because of previously described deleterious effects of AVP in CRF, it seems likely that AVP is playing some role in the progression of renal disease in DM.

1.5.2.4 AVP receptors in DM

A recent study in type 1 diabetic patients demonstrated renal resistance to AVP in subjects with poorly controlled blood sugars and, that the severity of this, was inversely proportional to the degree of glycaemic control (321). This may go some way to explaining why those patients with poorly controlled DM fare less well in diabetic ketoacidosis (321). Whether this resistance effect is due to down-regulation of the V_2R number (B max), reduced V_2R affinity for AVP (Kd) or a change at the level of medullary AQP-2 remains unclear (321). A study in Sprague Dawley (SD) rats, made diabetic for 2

weeks, did not demonstrate any alteration in renal V_2R Bmax or Kd, while Bmax for both renal and hepatic $V_{1A}Rs$ were down-regulated with no alteration in Kd (51). In the db/db mouse model of genetic, or type 2 DM, after 10 weeks there was a down-regulation of hepatic $V_{1A}R$ mRNA while the brain message for the same receptor remained unchanged (322).

Early DM is characterised by impaired responses to pressor hormones and pressor receptor down-regulation. Whether these regulatory effects are maintained long-term and are still pertinent with associated hypertension remains to be determined and is part of the remit of this thesis.

1.5.2.5 AVP receptor antagonists- their potential role in the management of DM

The relatively recent availability of non-peptide, orally active analogues has allowed for study of the effects of chronic AVP receptor blockade in DM. Thus far these studies have been limited but have provided good evidence for the importance of AVP in a number of deleterious effects of this disease. For instance, a study in BB and control rats with STZ-DM showed urinary albumin excretion more than doubled in controls but rose by only 34% in the BB rats with DM. This was accompanied by a less intense kidney hypertrophy in the BB rats (312). In a short-term study in type 2 diabetics, $V_{1A}R$ antagonism using OPC 21268 significantly reduced microalbuminuria without affecting BP (323). In a model of focal glomerulosclerosis in hypercholesterolaemic rats with unilateral nephrectomy, OPC 21268 attenuated proteinuria and decreased glomerulosclerosis (324). On the other hand, a recent study using the $V_{1A}R$ antagonist SR 49059 demonstrated no change in diabetes-related vascular changes in the mesenteric arterial bed of the SD rat (294). The role of V_2R antagonism has been assessed in a 9 week study where STZ-DM

rats were given the selective, non-peptide, orally active V_2R antagonist SR 121463A. This treatment totally prevented the rise in albuminuria with time seen in untreated DM rats and blunted the DM-induced rise in kidney mass (325).

1.6 THE RENIN ANGIOTENSIN SYSTEM (RAS)

As compared to that of AVP, the role of the renin angiotensin system (RAS) in the regulation of BP and in CVDs has been well characterised. ACE inhibitors are widely used in the treatment of hypertension and are currently the 'gold standard' drug group used in the management of diabetic nephropathy (1, 9). In chapter 5 the ACE inhibitor ramipril will be given alone and in combination with $V_{1A}R$ antagonist OPC 21268. For these reasons a basic overview of the RAS will now be given.

The RAS is known to function on a number of levels, as a systemic circulating hormone, a local paracrine/autocrine system and as a neurotransmitter within the CNS (326-329). The primary active hormone, Ang II, is an octapeptide and is produced as the end result of the enzymatic cascade as shown in figure 1.5.

Angiotensinogen is a large glycoprotein, classically produced within the liver, and its secretion is stimulated by hormones such as glucocorticoids, steroids and by Ang II itself. Once released into the circulation this large precursor molecule undergoes enzymatic cleavage by renin to form Ang I. Renin is released from the juxtaglomerular cells of the kidneys in response to reductions in blood volume, BP or sodium concentration and renin's cleavage of angiotensinogen is the rate-limiting step in this cascade. Finally, Ang I is cleaved by the membrane-bound metalloproteinase ACE to form Ang II (330).

Ang II is one of the most potent vasoconstrictors in humans. It increases BP through vasoconstriction and also via direct and indirect actions at the kidney. Ang II acts directly in the proximal renal tubule to increase sodium and water reabsorption and indirectly stimulates the release of aldosterone from the adrenal cortex to result in the retention of

sodium and water in the distal tubules and CDs (331-334). Ang II also acts centrally to promote thirst, release AVP and stimulate the sympathetic nervous system (335). Within the heart Ang II acts as a mitogen, potently stimulating hypertrophy of myocytes (336) and hyperplasia of cardiac fibroblasts (337). Ang II acts through one of 2 receptor subtypes the angiotensin type 1 receptor (AT_1R) and the angiotensin type 2 receptor (AT_2R). The AT_1R is predominant in the adult and mediates the biological actions of Ang II, as outlined above, through a variety of signal transduction mechanisms including the activation of phospholipase C and A_2 and inhibition of AC (338). AT_2Rs are predominant in the fetus, but are also present in adult tissues such as the adrenals, ovaries, uterus and brain (338). They exert biological effects generally opposed to those mediated by the AT_1Rs and are currently thought to activate the tyrosine kinase second messenger system (339). Specific receptor blockers are currently available and the AT_1R blockers such as losartan, candesartan and irbesartan are increasingly being used clinically as alternatives to ACE inhibitors. AT_2R blockers are available for use in the laboratory and include PD 123177 and PD 123319 (340).

ACE, as stated earlier, is a membrane bound metallopeptidase and is produced most notably in the endothelium of somatic cells but also within parenchyma and inflammatory cells. The enzyme is normally (>90%) anchored to the cell membrane by a hydrophobic domain and has a large extracellular domain containing the 2 extracellular catalytic sites that are inhibited by ACE inhibitors (341). A lower molecular weight form of ACE, with a single catalytic site, is found in germinal cells (342). ACE can also be found floating free within plasma and other body fluids after post-translational cleavage by the enzyme secretase has removed the catalytic from the anchoring transmembrane and cytosolic domains (343). ACE has broad substrate specificity in that, as well as the cleavage of Ang

I, it can also inactivate other peptides such as bradykinin and substance P and it is this that produces the side effects of angioedema and cough associated with ACE inhibitor treatment (344, 345). Ang II can also be formed via alternative non-ACE routes and can be produced directly from angiotensinogen via the action of non-renin enzymes such as tissue plasminogen activator (tPA), cathepsin G and tonin (346).

All components of the RAS shown have been described in a wide variety of tissues including kidney, brain, heart and adrenal gland and this lends weight to the argument that Ang II can exert its biological actions not only as an endocrine hormone but also in a paracrine and autocrine fashion (330).

Activation of the RAS with increased local production of Ang II and increased bradykinin degradation has important implications in a number of CVDs. Conditions such as hypertension and DM result in endothelial dysfunction and are associated with the activation of ACE and in particular tissue ACE (330). Endothelial dysfunction describes a broad spectrum of abnormalities including disruption of the balance of vasodilation and vasoconstriction, vascular smooth muscle growth and increased oxidative stress and inflammatory markers (330). In essential hypertension increased plasma renin activity and hence increased Ang II has been implicated as a risk factor that contributes to the maintenance of the disease state and as a risk factor for vascular injury to the heart, kidney and brain (347). In DM levels of ACE and Ang II are also elevated, although often with a low to normal plasma renin (348). Proteinuria is a common sequelae to both of the above CVDs and, as well as being a principal predictor of renal damage, is also a strong marker for CVD (349). Treatment with ACE inhibitors has been shown consistently to reduce proteinuria in these patients as compared to other antihypertensive agents that lower BP to

a similar level (350). The lack of an antiproteinuric effect by other antihypertensive agents suggests that renal protection and hence CVD protection may occur through a BP-independent mechanism. Much evidence for this hypothesis exists in the literature from large multicentre clinical trials such as the HOPE (Heart Outcomes Prevention Evaluation Study) (351), CAPPP (Captopril Prevention Project) (352) and ABCD (Appropriate Blood Pressure Control in Diabetes) trials (353).

Irrespective of the huge impact that ACE inhibitors, and now also AT_1R blockers, have had on the treatment strategies for CVDs such as hypertension and DM, mortality and morbidity rates remain unacceptably high. It is evident therefore that novel therapeutic approaches are necessary to improve quality of life and survival amongst these patients.

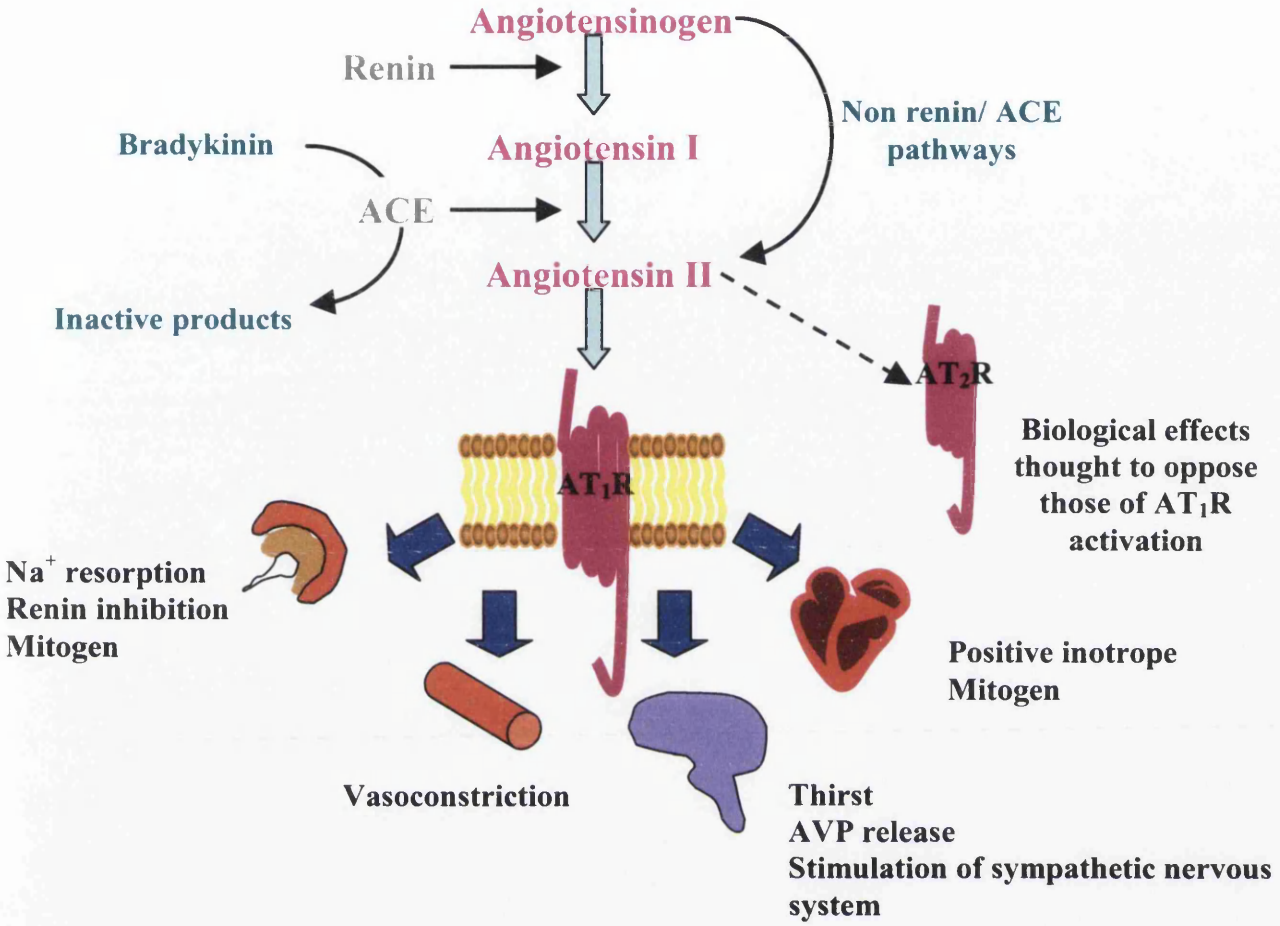

Figure 1.6: The renin angiotensin cascade leading to the formation of Ang II and its biological actions at the AT_1 and AT_2Rs.

1.7 THESIS HYPOTHESIS

As this review highlights there is strong evidence that AVP does have a part to play in the pathogenesis of CVDs but that its precise role remains unclear. To date, the regulation of AVP and its receptor system in long term DM, and in particular that associated with hypertension, has yet to be established. Once the regulation of the AVP system in this disease becomes clearer then any potential role for the blockade of AVP receptors by non-peptide receptor antagonists can be elucidated. The development of these specific, non-peptide AVP receptor blockers, as an additional treatment strategy for CVDs, is currently at a very early stage and new possible drugs are continually being discovered and characterised. ACE inhibitors, as described above, are well established in their role as antihypertensive agents, while $V_{1A}R$ antagonists alone are known not to lower BP in the adult hypertensive rat. Combination therapy with the 2 treatment groups has been shown not to be additive in the developmental phases of hypertension in adolescent SHR but has never been studied in the adult animal with established hypertension. Therefore the aims of this thesis are as follows:

1. To study, in rat, a novel group of 5 AVP non-peptide receptor antagonists made available from industry, and in particular to characterise *in vitro* and *in vivo* the drug whose agonist displacement profile most closely resembles that of AVP itself.

2. It is hypothesised that chronic dual ACE enzyme and $V_{1A}R$ inhibition will have additive effects in experimental hypertension that will not be predicted by selective ACE or $V_{1A}R$ inhibition. This thesis studied the effects of each treatment alone, and in combination, on

SBP (via telemetry), organ weights, metabolic, biochemical and hormonal parameters in a hypertensive rat model.

3. It is hypothesised that there is disregulation of the AVP receptor system in long standing experimental hypertensive DM. This thesis looked at the regulation of AVP and its $V_{1A}R$, at the mRNA and protein level, in a rat model combining hypertension and 8 weeks of STZ-DM.

Chapter 2

General materials and methods

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Contents

2.1 ETHICS

All experimental procedures involving animals were approved by the Austin and Repatriation Medical Centre Ethics Committee and were performed according to the National Health and Medical Research Council of Australia guidelines for animal experimentation.

2.2 ANIMAL MODELS

Rats were obtained from the Austin Biological Research Laboratories, Austin and Repatriation Medical Centre, Melbourne, Australia. Animals were housed at 23 to 25°C in a 12-hour light/dark cycle with access to a standard rat chow (0.6% sodium, 2% chloride, 2% calcium, Norco, Melbourne) and tap water *ad libitum*, unless otherwise indicated.

2.2.1 Spontaneously hypertensive rat

The spontaneously hypertensive rat (SHR), first developed in 1963, resulted following genetic inbreeding of rats with higher than normal blood pressure (354, 355). Analogous to essential hypertension the SHR develop cardiac hypertrophy in response to a slow and progressive rise in arterial pressure and peripheral resistance (figure 2.1) (356, 357). Increases in arterial pressure and peripheral resistance follow a characteristic pre-hypertensive phase (up to 6 weeks old), a developmental phase (6-12 weeks old) and an established phase where systolic blood pressure (SBP) is elevated to approximately 200-220mmHg by 16 weeks (figure 2.1). As there is no direct genetic control for the SHR it was decided that Sprague Dawley (SD) rats would be used as the normotensive control to the SHR. To confirm their inbred status, laboratory SHR colonies are regularly tested for polymorphic markers.

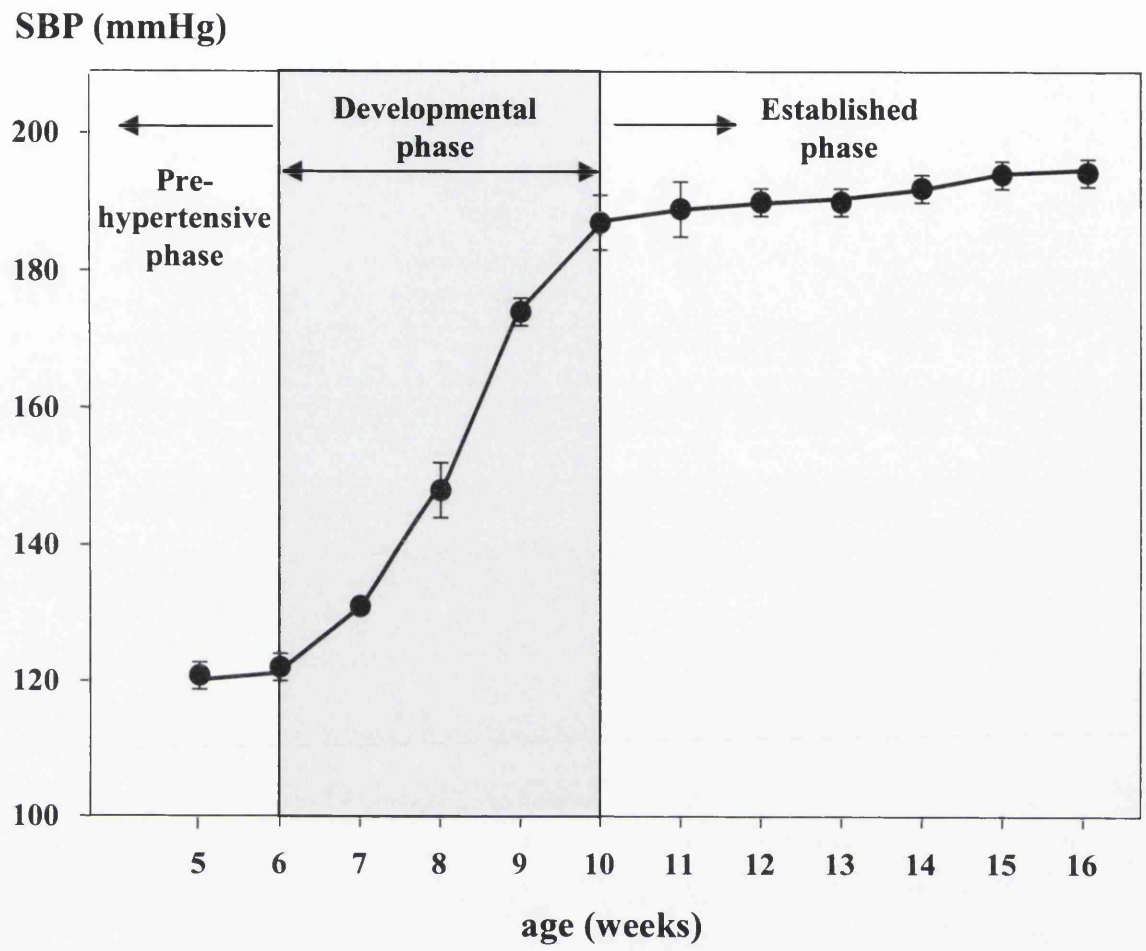


Figure 2.1 The development of blood pressure in the SHR. Modified from (50).

2.2.2 Streptozotocin-induced diabetes mellitus (STZ-DM)

The STZ-DM rat provides a useful and well-characterised model in which to study DM (358). These animals develop proteinuria, hypertension, glomerular basement membrane (GBM) thickening, mesangial expansion, glomerular hypercellularity and tubulointerstitial changes. Studies using this animal model have clearly demonstrated that various interventions including intensified glycaemic control and insulin, pancreatic transplantation and ACE inhibition will attenuate albuminuria and renal injury (359-361). However this model is not associated with hypertension, a common accompaniment of renal injury in man (12-14). Since most patients with diabetic nephropathy have associated systemic hypertension, it is important to also have a model of diabetic nephropathy with systemic hypertension. To address this, STZ-diabetes was induced in the SHR. This model developed an accelerated from of renal injury with rapid onset of albuminuria and more severe glomerular ultra structural injury (362).

SHR aged 8 weeks and weighing between 200 and 250g were fasted overnight and randomised to receive either STZ, at a dose of 45mg/kg, or citrate buffer (non-diabetic controls) by intravenous injection in the tail vein. Diabetic state was confirmed 48 hours later by tail vein glucose readings (Advantage, Boeringer Mannheim, Munich, Germany). Only animals with a plasma glucose level>17mmol/l were included in the study. Diabetic animals were treated with daily 4 Units, Ultralente Insulin (Novo, Copenhagen, Denmark) subcutaneously to allow weight gain and prevent ketoacidosis without rendering the animals euglycaemic. Tail vein glucose readings were taken every second week to confirm on-going diabetic state. Again, any animal with a plasma glucose<17mmol/l, at any stage, was excluded from the study.

2.3 DRUGS

Drugs A-E are vasopressin (AVP) receptor antagonists and were a generous gift from R. W Johnson Pharmaceutical Research Institute, (New Jersey. USA). OPC 21268, a vasopressin type 1A receptor ($V_{1A}R$) antagonist and OPC 31260, a vasopressin type 2 receptor (V_2R) antagonist, were generous gifts from Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). YM087, a mixed V_{1A} and V_2R antagonist, was a gift from Yamanouchi Pharmaceutical Co. Ltd (Japan). Ramipril, an ACE inhibitor, was purchased from Aventis Pharmaceuticals (New Jersey, USA). Perindopril, an ACE inhibitor, was purchased from Servier Pharmaceutical Co. Ltd (Cedex, France).

2.4 CHEMICALS

Aprotinin, STZ and Tris-HCL were obtained from Sigma Chemical Co (St Louis, USA) and bovine serum albumin (BSA) from CSL Ltd. (Victoria, Australia). AVP was obtained from Pensula laboratories Inc. (California, USA). All other reagents were purchased from BDH Chemicals (Victoria, Australia) or Ajax chemicals (New South Wales, Australia).

2.5 *IN VIVO* TECHNIQUES

2.5.1 Tail cuff SBP measurements

SBP was measure by tail-cuff plethysmography (38L flatbed recorder, model 229 Amplifier, IITC Life Science, Woodland Hills, USA) in warmed, lightly restrained rats.

2.5.2 Telemetry

SHR, aged eight weeks, weighing between 180-220g were synchronized to 12:12 hrs of light (0700-1900) and dark (1900-0700) for at least 5 days. Prior to implantation the devices (Data Sciences International, St. Paul, MN, USA, model TA11PA-C40) were calibrated to read within ±3mmHg. The rats were anaesthetized with a combination of Nembutal (Rhone Merieux, Pinkemba, Australia) 30mg/ml, Brietal Sodium (Eli Lilly, Indianapolis, USA) 40mg/ml and Atropine Sulphate (Delta West Pty. Ltd., Bentleigh, Australia) 6mg/ml and injected with a dose of 5ml/kg intraperitoneally. A 3-4cm abdominal incision was made and the abdominal aorta exposed and clamped inferior to the bifurcation of the renal artery. An additional clamp was also placed superior to the femoral artery bifurcation to prevent backflow from the legs. The sensor's catheter was inserted 1 cm into the clamped area, glued into place with Histoacryl® (Braun, Mengulsen, Switzerland) and the radio-frequency transmitter sutured to the abdominal wall. Each rat received post-operative pain relief in the form of morphine sulphate (David Bull, Mulgrave, Australia) (5mg/kg) intraperitoneally. After allowing a minimum of 5 days for full recovery the rats were assigned to treatment groups. Each rat was housed individually and exposed to standard food chow and water *ad libitum*, with the plastic cage (30cm x 40cm x13cm) placed on top of a receiver (RPC-1) connected to a BCM-100 consolidation matrix. This receiver then transmitted parameter and waveform information to the Dataquest Gold Acquisition system (Data Sciences International, St. Paul, MN).

2.5.3 Metabolic measurements

Rats were placed into metabolic cages (Iffa Credo, L'Arbresele, France) for 24 hours prior to study days to allow for acclimatisation. On day of study, rats were weighed and food and water measured at the beginning of the 24-hour study period. At the end of the 24-

hour period, food and water intake were measured and urine was collected for analysis of urinary volume, sodium and osmolality.

2.6 *IN VITRO* TECHNIQUES

2.6.1 Radio ligand preparation

2.6.1.1 Iodination of the AVP $V_{1A}R$ antagonist $d(CH_2)_5$,sarcosine^7-AVP

The radioligand used to localise the $V_{1A}R$ was ^{125}I[β-mercapto-β,β-cyclopentamethylene propionic acid), 7-sarcosine]-AVP (abbreviated, ^{125}I[$d(CH_2)_5$,sarcosine^7]AVP) (Auspep, Melbourne, Australia). $d(CH_2)_5$, sarcosine^7-AVP is a specific V_1R antagonist with potent antipressor activity (pA_2=7.93) and minimal antidiuretic activity (0.12U/mg) or antioxytocic activity (pA_2=6.13) (105, 363, 364). $d(CH_2)_5$, sarcosine^7-AVP was radio-iodinated by modification of the chloramine T method (105, 365). The reagents for the iodination were dissolved in 0.25mol/l borate buffer pH 7.5 and $d(CH_2)_5$, sarcosine^7-AVP was iodinated at room temperature using 1mCi of ^{125}I (Amersham Radiochemicals, Amersham, England) and chloramine T (5mg/ml). The reaction was stopped after 20 seconds with sodium metabisulphite. The reaction mixture was then transferred onto a Sep-pak, C18 cartridge, which had been pretreated concurrently with 2mls of 0.08% trifluoroacetic acid (TFA). After 5 successive washes with 2mls of 0.08% TFA, the sample was eluted with 80% acetonitrile containing 0.08% TFA. The eluate was then dried under a steady stream of high-purity nitrogen and stored overnight at 4°C. On the following day the sample was reconstituted in 350µl of mobile phase (32% acetonitrile with 0.08% TFA) for high performance liquid chromatography (HPLC) purification (Waters Associates, Milford MA). Fractions from the largest peak were pooled and dried

down under a steady stream of high purity nitrogen and then resuspended in 0.01mol/l acetic acid containing 0.1%BSA.

2.6.1.2 Radiolabelled AVP V_2R antagonist [^{3}H]des-Gly-$NH_2{}^9$-d$(CH_2)_5$[D-Ile^2,Ile^4]AVP

The selective V_2R antagonist radioligand, [Phe-3,4,5-^{3}H]^9-des-Gly-NH_2, I-(β-mercapto-β,β-cyclopentamethylene propionic acid) [2-D-Ile, 4-Ile]AVP (abbreviated, [^{3}H]des-Gly-$NH_2{}^9$-d$(CH_2)_5$[D-Ile^2,Ile^4]AVP) was obtained from Dupont Company (Boston, Mass. USA)(specific activity range: 45-80 Ci/mmol) (366). The radiochemical purity of this antagonist is checked using HPLC on a Vydac proteins and peptides C18 column with the mobile phase 0.2% TFA:acetonitrile (60:40). The rate of decomposition, stored at -20°C, is 1% every 3 months.

2.6.1.3 Iodination of the ACE inhibitor MK-351A

^{125}I MK-351A was the radioligand used to assess ACE inhibition by *in vitro* autoradiography. MK-351A is a derivative of the ACE inhibitor lisinopril. It was iodinated by the chloramine T method and purified by SP Sephadex C25 column chromatography as previously published (367).

2.6.2 *In vitro* autoradiography

At the conclusion of each experiment kidneys were snap frozen in isopentane at -40°C for *in vitro* autoradiography of AVP V_{1A} and V_2Rs and ACE. Frozen tissue sections (20μM in thickness) were cut on a cryostat (Microm, Germany) at -20°C, thaw mounted onto 1%

gelatin coated slides and dessicated at 4°C overnight to remove moisture before freezing at -80°C.

2.6.2.1 AVP $V_{1A}R$ *in vitro* autoradiography

Frozen slide mounted sections were equilibrated to room temperature and preincubated at 4°C for 15 minutes in a 100mmol/l Tris-HCL buffer (pH 7.4) containing 10mmol/l $MgCl_2$ and 0.1% BSA (buffer A). These slides were then transferred to separate slide mailers containing 10mls of incubation buffer (buffer A with 0.5mg/ml bacitracin and 100IU/ml aprotonin) and approximately 35pmol/l $^{125}I[d(CH_2)_5$, sarcosine$^7]$-AVP ($1.4x10^6$cpm) for 16 hours at 4°C. Non-specific binding was determined following parallel incubations containing 2μmol/l of unlabelled AVP. The binding reaction was terminated by four successive 30 second washes in ice-cold buffer A at 0°C followed by a 30 second wash in ice-cold distilled water. The slides were dried under cool air, loaded into X-ray cassettes and exposed to Agfa Scopix CR3 film (Agfa Gevaert, Ghent, Belgium) for 7-10 days. Quantitation of binding density was determined by computerised densitometry (MCID, Imaging Research, Ontario, Canada) using radioactive standards that were corrected for decay and fitted to calibration curves to convert density of the autoradiographs to d.p.m per mm^2.

2.6.2.2 AVP V_2R *in vitro* autoradiography

Frozen slide mounted sections were equilibrated to room temperature and preincubated at 4°C for 15 minutes in a 100mmol/l Tris-HCL buffer (pH 7.4) containing 10mmol/l $MgCl_2$ and 0.1% BSA (buffer A). These slides were then transferred to separate slide mailers containing 10mls of incubation buffer (buffer A with 0.5mg/ml bacitracin and 100IU/ml aprotonin) and 2nmol/l [^{3}H]des-Gly-$NH_2{}^9$-d$(CH_2)_5$[D-Ile^2,Ile^4]-AVP for 60 minutes at

20°C. Non-specific binding was determined following parallel incubations containing 2μmol/l of unlabelled AVP. The binding reaction was terminated by four successive 30 second washes in ice-cold buffer A at 0°C followed by a 30 second wash in ice-cold distilled water 0°C. The slides were dried under cool air, loaded into X-ray cassettes and exposed to fujibas ^{3}H film for 10-14 days. The films were read in the Fujibas 3000 phosphorimager (Fuji Photo Film Company Ltd, Tokyo, Japan). The generated computer images were then quantitated by computerised densitometry as described in section 2.6.2.1.

2.6.2.3 ACE *in vitro* autoradiography

Slide mounted sections were preincubated in 10mmol/l phosphate buffer, pH 7.4, for 15 minutes at room temperature and then incubated in 10mmol/l sodium phosphate buffer, pH 7.4, containing 150mmol/l sodium chloride and 0.2% BSA with ^{125}I MK-351A (0.3mCi/ml) for 1 hour at room temperature. Non-specific binding was measured in parallel incubations containing 1 mmol/l EDTA. Following incubation, sections were transferred through 4 successive 1 minute washes at 4°C in buffer. After the 1 minute washes sections were dried under cold air, placed in X-ray cassettes and exposed to Agfa Scopix CR3 X-ray film (Agfa Gevaert, Ghent, Belgium) for 1-4 days. Quantitation of binding density was determined as mentioned in section 2.6.2.1.

2.6.3 Renal membrane preparation

For both AVP V_{1A} and V_2R binding studies, rats were killed by decapitation and kidneys were removed and membranes prepared as previously published (366, 368). Briefly, kidneys were dissected to remove cortex and coarsely minced, suspended in a 0.25mol/l sucrose buffer containing 20mmol/l Hepes, 1mmol/l EGTA (pH 7.4), 5μg/ml antipain and

10μmol/l PMSF (buffer 1) at 4°C and homogenised using an Ultra-Thurrax (Jankel and Kunkel-IKA-WERK) dispenser at 18-20000rpm (3 times for 20 seconds). The homogenate was centrifuged at 1500rpm for 5 minutes at 4°C. The resultant supernatant was then centrifuged at 14000rpm for 20 minutes. The supernatant was then discarded and the pellet resuspended in 5mls of a 0.25mol/l sucrose buffer containing 20mmol/l Hepes, 0.5mg/ml bacitracin and 100IU Aprotinin (buffer 2) and centrifuged at 14000rpm for a further 20 minutes. The supernatant was again discarded and the pellet resuspended in 1ml of buffer 2 and stored in liquid nitrogen prior to use.

2.6.4 Liver membrane preparation

For AVP $V_{1A}R$ binding studies, rats were killed by decapitation and livers removed and membranes prepared as previously described (369). Briefly, the connective tissue was removed and livers minced before being suspended in buffer 1 from section 2.6.3 at 4°C. Livers were then homogenised using an Ultra-Thurrax (Jankel and Kunkel-IKA-WERK) dispenser at 20-24000rpm (3 times for 20 seconds) and the homogenate was centrifuged at 8500rpm for 15 minutes at 4°C. The pellet was discarded and the resultant supernatant was then centrifuged at 8500rpm for 15 minutes at 4°C. The pellet was discarded and the resultant supernatant was then centrifuged at 20000rpm for 60 minutes. The supernatant was then discarded and the pellet resuspended in 10mls if buffer 2 from section 2.6.3 and centrifuged at 20000rpm for a further 60 minutes. The supernatant was again discarded and the pellet resuspended in 5ml of buffer 2 and stored in liquid nitrogen prior to use.

2.6.5 Radioligand binding studies

2.6.5.1 AVP $V_{1A}R$ binding

The *in vitro* inhibition of selective $V_{1A}R$ antagonist radioligand binding was determined by incubating liver (60μg) or kidney (250μg) membranes from untreated rats in a buffer containing 100mmol/l Tris-HCL, 10mmol/l $MgCl_2$, 0.1% BSA, 0.5mg/ml bacitracin and 100IU/ml aprotinin (pH 7.4) with ^{125}I-labelled [$d(CH_2)_5$, sarcosine^7]-AVP (liver, 0.5nmol/l; kidney, 1nmol/l) for 1 hour at 20°C. Bound and free ligand was separated by filtration through Whatman GF/B glass fibre filters using a Brandel automatic filtration apparatus (Biochem Research and Development Laboratories Inc, Gaithersburg, MD). The tubes and filters were washed 3 times with 2.5litres of filtration buffer (100mmol/l Tris-HCL, 10mmol/l $MgCl_2$ and 0.1% BSA (pH 7.4). The filters were then placed into Wassermann tubes and counted in a gamma counter (LKB 1260 Multigamma II). Specific binding was calculated as total binding minus non-specific binding in the presence of 1μmol/l unlabelled AVP. The *in vitro* displacement of ^{125}I-labelled[$d(CH_2)_5$, sarcosine^7]-AVP binding to liver or kidney membranes was measured in the presence or absence of AVP or experimental drug in the concentration range 0.01pmol/l to 0.1mmol/l.

2.6.5.2 AVP V_2R binding

The *in vitro* inhibition of selective V_2R antagonist radioligand binding was determined by incubating kidney (500μg) membranes from untreated rats in a buffer containing 100mmol/l Tris-HCL, 10mmol/l $MgCl_2$, 0.1% BSA, 0.5mg/ml bacitracin and 100IU/ml aprotinin (pH 7.4) with 2nmol/l [^{3}H]des-Gly-$NH_2{}^9$-$d(CH_2)_5$[D-Ile^2,Ile^4]-AVP for 2 hours at 20°C. The incubation was stopped with the addition of 2.5mls of filtration buffer

(100mmol/l Tris-HCL, 10mmol/l $MgCl_2$ and 0.1% BSA (pH7.4) and bound and free ligand separated by filtration through Whatman GF/B glass fibre filters using a Brandel automatic filtration apparatus (Biochem Research and Development Laboratories Inc, Gaithersburg, MD). The tubes and filters were washed 3 times with 2.5litres of filtration buffer. The filters were dried and dissolved in 3.5mls of scintillant (Filter-count, Packard Instruments Co.Inc, Downers Grove, IL) and β radiation was measured using a Packard Tricarb 4530. Specific binding was calculated as total binding minus non-specific binding in the presence of 1μmol/l unlabelled AVP. Displacement of [^{3}H]des-Gly-NH_2^9-d$(CH_2)_5$[D-Ile^2,Ile^4]-AVP binding to rat kidney membranes by unlabelled AVP or experimental drug was measured using 2nmol/l [^{3}H]des-Gly-NH_2^9-d$(CH_2)_5$[D-Ile^2,Ile^4]-AVP in the absence and presence of AVP or drugs A-E in the concentration range of 0.01pmol/l to 0.1mmol/l.

2.6.5.3 AVP V_{1A}R radioligand binding kinetics

The effect of DM on binding site density (Bmax) and the apparent affinity (Kd) of the liver V_{1A} and kidney V_{1A}R was determined by analysis of the saturation binding of the selective V_{1A}R radioligand ^{125}I[d$(CH_2)_5$, sarcosine^7]-AVP. Liver and kidney membranes prepared from diabetic and control animals were incubated with ^{125}I-labelled[d$(CH_2)_5$, sarcosine^7]-AVP as described in section 2.6.5.1. Liver specific binding was approximately 70% and that for kidney approximately 50%. The Bmax and K_d values were determined by Scatchard analysis.

2.6.6 Blood collection

Rats were killed by decapitation and trunk blood collected into pre-chilled lithium heparin tubes (containing 100μl heparin, 25,000U/L) for the measurement of plasma AVP, sodium

and osmolality and into pre-chilled tubes containing EDTA/aprotinin (kallikrein inhibitor 500U/ml) for the measurement of plasma renin activity (PRA).

2.6.7 Hormonal and biochemical analysis

Plasma AVP was extracted using acetone and petroleum ether and measured by radioimmunoassay as previously described (370). The inter-assay and intra-assay coefficients of variation were less than 8% and the limit of detection was approximately 1pmol/l. PRA was measured by radioimmunoassay (371). The intra-assay coefficient of variation for the PRA assay was 9% and the specific binding 70%. Plasma ACE was measured using a fluorimetric assay (372). Plasma sodium was measured using an Instrument Laboratory Ilyte ion specific electrode analyse (Milan, Italy) and plasma osmolality was determined using an Advanced Instruments Micro-Osmometer model 330 (Ma. USA). Rat glucose was assayed with a glucose oxidase method (373) and glycohaemoglobin was determined by an automated HPLC method (Primus CLC330) (374). Urinary albumin concentration was measured by a double-antibody radioimmunoassay involving a rabbit anti-rat antibody (Organon Teknika, Durham, NC) as previously described (375).

2.7 MOLECULAR TECHNIQUES

2.7.1 Real-time Polymerase Chain Reaction (PCR)

2.7.1.1 Extraction of total Ribonucleic acid (RNA)

Total RNA was isolated using the Rneasy Kit (Qiagen, Dorking UK). The RNA was then stored at -80°C until used. Briefly, 30mg of tissue was thawed and homogenised in 850µl

of buffer RLT with 1% βmercaptoethanol, homogenised thoroughly and any precipitate discarded. The tissue RNA and deoxyribonucleic acid (DNA) were precipitated out by the addition of 70% ethanol. This was followed by DNase treatment and then a series of spins and ethanol elution steps. The RNA yield was then measured spectrophotometrically as follows. RNA purity and concentration were determined spectrophotometrically by adding 2µl of the aforementioned RNA solution to 198µl of diethylpyrocarbonate(DEPC)-treated water and measuring its absorbance at 260 and 280nm. The amount of RNA in the original (undiluted) sample was calculated using the following formula:

$$\text{RNA } (\mu g/\mu l) = \frac{\text{Optical density (260nm) x dilution factor}}{1000}$$

The RNA purity was calculated using the following equation:

$$\text{Absorbance ratio} = \frac{\text{optical density (260nm)}}{\text{optical density (280nm)}}$$

An absorbance ratio of 1.8-2.0 indicated a high degree of purity of RNA.

2.7.1.2 Reverse transcription of total RNA (cDNA synthesis)

0.3µg of RNA was reverse transcribed in a reaction mixture which contained a final concentration of 1X 1st strand buffer (0.1mol/l KCL, 6mmol/l $MgCL_2$, 0.05mol/l Tris-HCL

pH 8.4 and 10mmol DTT) (Boehringer-Mannheim, Germany), 500μmol/l of the deoxyribonucleotides dCTP, dGTP, dATP and dTTP (Progen Industries Ltd, NSW, Australia), 25 units of avian myoblastoma virus-reverse transcriptase (AMV-RT) (Boehringer-Mannheim, Germany) and sterile DEPC water in a final volume of 30μl. For control purposes the above reaction mixture was repeated in the absence of AMV-RT to validate synthesis from mRNA. Tubes were incubated at 25°C for 10 minutes, 60 minutes at 42°C and 5 mins at 95°C. Termination of the reaction was completed with the addition of 0.6ml EDTA (pH 8.0) and samples stored at -80°C until used.

2.7.1.3 PCR primers and probes

Specific AVP $V_{1A}R$ and V_2R cDNA oligonucleotide forward and reverse primer sequences and internal probes (FAM-chromophore) were designed using Primer Express applications-based primer design software (PE Applied Biosystems, CA, USA). TaqMan® ribosomal RNA (rRNA) control reagents containing rRNA probe (VIC-chromophore) and forward and reverse primers were used for the detection of the housekeeping gene 18S rRNA. The primer and probe concentrations had previously been optimised for PCR use following the guidelines within the TaqMan® Universal PCR master mix protocol (www.perkin-elmer.com.ab).

2.7.1.4 Plate set up and PCR reaction

25ng of cDNA for both liver and kidney was added, in duplicate, to each well of a 96-well reaction plate to which was added 22.5μl of a reaction mix containing 12.5μl of TaqMan® Universal PCR master mix (mix contains Amplitaq gold™, AmpErase UNG®, nucleotides dCTP, dATP, dGTP, dUTP, $MgCl_2$, glycerol, passive reference 1 and optimized buffer

components), TaqMan® 18S probe (final concentration 175nmol/l), 18S forward primer (final concentration 20nmol/l), 18S reverse primer (final concentration 40nmol/l), Taqman® $V_{1A}R$ probe (final concentration 150nmol/l), $V_{1A}R$ forward primer (final concentration 900nmol/l), $V_{1A}R$ reverse primer (final concentration 900nmol/l) and filter sterilized DEPC-treated water. For each gene of interest a "no cDNA control" was added and 2 wells per 96-well plate were allocated to be "no template controls". The reaction plate was capped, briefly spun and placed into the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems CA, USA) and amplification conducted as follows: Firstly, 2min at 50°C for UNG® digestion followed by 10min at 95°C to activate AmpliTaqGold® and then 40 cycles of 15 seconds at 95°C (denature) and 1 min at 60°C (anneal and extend).

2.7.1.5 Quantitation of PCR products

The method used to obtain relative gene expression data is the comparative CT method previously described by K. Livak (Applied Biosystems; Sequence Detector User Bulletin 2). The C_T value represents the "threshold cycle" for each gene. This method uses a "calibrator" sample for comparison of every unknown sample's gene expression. It can be any sample chosen to represent 1X expression of the gene of interest. In the diabetic study the calibrator sample is derived from the control SHR animals and gene expression of the diabetic SHR samples are compared to it. This method of relative quantitation is based on the assumption that the rate of C_T change and the rate of target copy change is identical for the gene of interest and the housekeeping gene and that a doubling of target results in one cycle decrease in the C_T.

Firstly, 18S C_T values were subtracted from $V_{1A}R$ C_T values, in each well, to give a ΔC_T value. $\Delta\Delta C_T$ values were achieved by subtracting the average ΔC_T value for control rats from the ΔC_T values of corresponding tissues from the DM rats. The expression of the $V_{1A}R$ genes in the diabetic animals relative to controls was evaluated using the formula $2^{-\Delta\Delta CT}$.

2.8 STATISTICAL ANALYSIS

Results are presented as mean±SEM. Data was analysed using one- and two-way analysis of variance (ANOVA) and the Fisher test where appropriate using the Statview® programme (Brain power Inc, Calabass, CA). Albuminuria data was logarithmically transformed before statistical analysis. Significant differences were obtained when $p<0.05$.

Chapter 3

In vitro and *in vivo* characterisation of novel non-peptide vasopressin receptor antagonists

CHAPTER 3

In vitro and *in vivo* characterisation of novel non-peptide vasopressin receptor antagonists

Contents

3.1 SUMMARY

1. This chapter reports on study 1; the *in vitro* characterisation of non-peptide, orally active, vasopressin (AVP) V_{1A} and V_2 receptor ($V_{1A}R$ and V_2R) antagonists, drugs A-E, in the rat and study 2; the *in vivo* characterisation of drug A.

2. Study 1. Drugs A-E caused a concentration-dependent displacement of the selective $V_{1A}R$ antagonist radioligand, ^{125}I-labelled [d$(CH_2)_5$,sarcosine^7]AVP from $V_{1A}Rs$ in both rat liver and kidney medulla membranes. The concentration of drug that displaced 50% of specific radioligand binding, or $IC_{50}s$ for each drug, at the above receptors, was determined. Drug A was most effective followed by drugs E, C, D and B at the liver $V_{1A}R$ and drug A was again most potent at the renal $V_{1A}R$ followed by drugs E, C, B and D. Drugs A-E also caused a concentration-dependent displacement of the selective V_2R antagonist radioligand [^{3}H]desGly-NH_2^9[d$(CH_2)_5$, D-Ile^2, Ile^4]AVP from V_2Rs in rat kidney medulla membranes, drug A being most potent followed by drugs E, B, C, and D.

3. Study 2: From study 1, drug A had a pharmacological profile most closely resembling AVP at both the V_{1A} and V_2Rs and therefore underwent further *in vitro* and *in vivo* characterisation in this second study.

Oral administration of Drug A (1, 3, 10 mg/kg) dose-dependently inhibited AVP binding to liver $V_{1A}Rs$ and kidney V_{1A} and V_2Rs at 2h. Significant inhibition was seen at the kidney V_{1A} and V_2R at 10mg/kg ($P<0.01$) but at 3mg/kg at the liver $V_{1A}R$ ($P<0.05$). Oral Drug A

(10 mg/kg/day) inhibited AVP binding to liver V_{1A} ($P<0.05$) and kidney V_{1A}Rs ($P<0.01$) for 4h and to kidney V_2Rs for 8h ($P<0.01$; 2 and 4 hours. $P<0.05$; 8 hours).

Oral administration of Drug A (10, 30mg/kg) for 5 days in normotensive male and female rats caused a dose-dependent aquaresis on day 1 (acute response) ($P<0.01$ at 10mg/kg and 30mg/kg for both male and female rats). By day 5 (chronic response) this aquaresis had diminished ($P<0.05$ at 10mg/kg in female rats and $P<0.05$ at 30mg/kg dose in male rats).

Oral administration of drug A (10mg/kg) to normotensive female rats for 3 days did not have any hypotensive effects.

Plasma sodium and osmolality results were not elevated over control at either dose while plasma AVP was raised significantly at a dose of 30mg/kg ($P<0.05$ in female: $P<0.01$ in male). V_{1A}R *in vitro* autoradiography demonstrated significant inhibition of binding of the selective V_{1A}R antagonist radioligand, ^{125}I-labelled [d$(CH_2)_5$,sarcosine^7]AVP, by drug A at the renal V_{1A}R ($P<0.05$ at 10mg/kg and $P<0.05$ at 30mg/kg). V_2R *in vitro* autoradiography was not done in view of there being a clinically detectable V_2R diuretic response to both 10 and 30mg/kg doses.

4. These results show that Drug A is an orally effective, V_{1A} and V_2R antagonist that may be useful in the treatment of conditions characterised by vasoconstriction and fluid retention such as congestive cardiac failure (CCF).

3.2 INTRODUCTION

CCF is a progressive disease characterised by neurohormonal activation, salt and water retention causing dyspnoea and oedema and ventricular dilation and hypertrophy, a process known as remodelling (376). Despite the impact of ACE inhibitors to attenuate this remodelling and improve survival (377, 378), the 5 year mortality rate remains unacceptably high. This has prompted the search for novel therapeutic approaches (379-381). AVP receptor antagonists represent one such new approach and several non-peptide AVP receptor antagonists have been developed. OPC 21268 and SR 49059 are selective $V_{1A}R$ antagonists. OPC 21268 blocks the pressor effects of AVP and lowers blood pressure (BP) in mineralocorticoid hypertension in the rat (86, 123), whilst SR 49059 is a potent and selective $V_{1A}R$ antagonist in the human internal mammary artery (382). OPC 31260, OPC 41061 and SR 121463A antagonise the effect of AVP at the V_2R to cause an aquaresis (41-44). YM087 is a mixed AVP receptor blocker antagonising the action of AVP at both the V_{1A} and V_2Rs (46). *In vivo*, the $V_{1A}R$ antagonistic properties of YM087 are demonstrated by its ability to block the pressor response to AVP in rats and dogs (169, 383). In humans, a more recent study has shown that a single intravenous dose of YM087 given to patients with advanced CCF (New York Heart Association class III and IV) resulted not only in a dose-dependent increase in urine output but also a significant reduction in pulmonary capillary wedge pressure and right atrial pressure when compared to placebo (266). This highlights the fact that it is the mixed V_{1A} and V_2R antagonists in particular that are likely to play an important part in heart failure management in the near future. To date YM087 is currently the only available such drug and the search is on for others.

The high oral bioavailability of non-peptide AVP receptor antagonists allows long-term studies to be performed to further elucidate the role of AVP in cardiovascular diseases such as hypertension, CCF and renal failure and also in CNS disorders such as anxiety. The acute effects of an intervention do not necessarily predict long-term efficacy and to date there have been no studies assessing the long-term efficacy of any of the drugs under investigation here, specifically of drug A.

This study is split into two parts. Study 1 investigates the AVP receptor antagonist properties of Drugs A-E *in vitro*, in the rat, using selective AVP V_{1A} and V_2R radioligands. Study 2 further characterises drug A *in vitro* and *in vivo*, in the rat, using selective AVP V_{1A} and V_2R radioligands and assesses the effect of 5 days oral administration of drug A on BP, renal and plasma parameters in the normotensive rat.

3.3 EXPERIMENTAL PROTOCOL

3.3.1 Ethics

This experiment was approved by the Austin and Repatriation Medical Centre Ethics Committee and performed according to the National Health and Medical Research Council of Australia guidelines for animal experimentation.

3.3.2 Animals

Both female and male Sprague-Dawley (SD) rats (250-300g) were used. Male SD rats (250-300g) were included in the 5 day metabolic parameter study. Systolic blood pressure (SBP) was measured in female rats using the indirect tail-cuff method as described in section 2.5.1.

3.3.3 Drugs

Drugs A-E were a generous gift from R. W. Johnson Pharmaceutical Research Institute (New Jersey. USA). They were dissolved in organic solvent, dimethylsulfoxide (DMSO), and distilled water (ratio 4:1) and stored at a concentration of 1mmol/l at -20°C prior to all *in vitro* experiments. For animal gavage, drug was suspended in 0.5% methylcellulose (BDH, Poole, UK). A sonication step prior to gavage was included in later experiments as will be described. OPC 21268 was a gift from Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan) and suspended in 0.5% methylcellulose. YMO87, also known as conivaptan, was a gift from Yamanouchi Pharmaceutical Co. Ltd (Japan) and was suspended in 0.5% methylcellulose for gavage.

3.3.4 Radioligand

The selective $V_{1A}R$ antagonist ^{125}I[1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 7-sarcosine]AVP (abbreviated: ^{125}I[d$(CH_2)_5$, sarcosine^7] AVP) and the selective V_2R antagonist radioligand, [Phe-3,4,5-^3H]9-des-Gly-NH_2, [1-(β-mercapto-β,β-cyclopentamethylene propionic acid) 2-D-Ile,4-Ile]AVP (abbreviated: [^3H]desGly-$NH_2{}^9$[d$(CH_2)_5$, D-Ile^2, Ile^4]AVP were used as described in section 2.6.1.

3.3.5 Study 1: *In vitro* characterisation of AVP receptor antagonists: Drugs A-E

These experiments were designed to investigate the inhibition by drugs A-E of selective V_{1A} and V_2R antagonist radioligand binding to liver and kidney medullary V_{1A} and kidney medullary V_2Rs using the protocols outlined in sections 2.6.5.1 and 2.6.5.2.

3.3.6 Study 2: Dose response and time course of AVP receptor binding with oral drug A

To assess the *in vivo* inhibition of drug A on AVP binding to liver and kidney medullary $V_{1A}Rs$ and kidney medullary V_2Rs, rats were gavaged with vehicle (0.5% methyl cellulose) or drug A (1, 3, 10 mg/kg) and killed 2h later for the dose response study (n=3-4 rats/group).

For the time course study, rats were gavaged with 10mg/kg of drug A and killed at varying time points (0, 1, 2, 4, 8, 16, and 24 h) after gavage (n=3-4 rats/time point).

In each study, blood, liver and kidneys were collected. One kidney was snap frozen and the other used for immediate membrane preparation (section 2.6.3). Liver membranes were

prepared (section 2.6.4). The effect of drug A on the binding characteristics of V_{1A} and V_2Rs in the kidney and of V_{1A}R binding sites in the liver was determined by membrane binding and/or *in vitro* autoradiography (sections 2.6.2.1, 2.6.2.2, 2.6.5.1 and 2.6.5.2)

3.3.7 Effect of oral drug A on renal parameters and BP

3.3.7.1 Metabolic studies

Female rats: Initial problem solving experiments: Female SD rats (300g, n=24) were randomised to receive vehicle or drug A (10mg/kg; 30mg/kg) by oral gavage. The initial batch of drug A had been finished in preceeding experiments and so a second was made available from the pharmaceutical company for this group of studies. On day 1 of treatment rats were placed into metabolic cages and water intake, urinary volume, urine sodium and osmolality measured for 24 h. A surprisingly low acute diuresis resulted and a 30mg/kg bd dose of drug A was introduced, as well as the known acute aquaretic agents YMO87 and OPC 31260. The resultant aquaresis with these known agents was good. The new batch of drug A was compared *in vitro* to the old by comparing the displacement of specific V_{1A}R antagonist [$d(CH_2)_5$, sarcosine^7] AVP in liver membranes and finally the new drug was sonicated prior to oral gavage for metabolic and BP studies.

On days 1 and 5 of treatment all rats were placed into metabolic cages and water intake, urinary volume, urine sodium and osmolality measured for 24 hours. On day 6 rats were then killed by decapitation and trunk blood collected as before. Kidneys were removed (n=4/group), rapidly frozen in dry-ice chilled isopentane and stored at -80°C until used for AVP V_{1A}R *in vitro* autoradiography (section 2.6.2.1).

Male rats: Male SD rats (300g, n=24) were randomised to receive vehicle or drug A (10mg/kg; 30mg/kg) by oral gavage. On days 1 and 5 of treatment all rats were placed into metabolic cages and water intake, urinary volume, urine sodium and osmolality measured for 24 hours. On day 6 rats were then killed by decapitation and trunk blood collected as before. Kidneys were removed (n=6/group), rapidly frozen in dry-ice chilled isopentane and stored at -80°C.

3.3.7.2 BP studies

Female SD rats (n=16) were gavaged with vehicle and 10mg/kg of drug A for 3 days and had SBP determined by tail cuff plethysmography on days 1 and 3, 120 minutes post gavage.

3.4 STATISTICAL ANALYSIS

Data were analysed by analysis of variance (ANOVA) using the Statview SE=Graphics programme (Brainpower, Calabasas, CA). Comparisons of group means were performed by Fisher's least significant difference method. Data are shown as means±SEM unless otherwise specified. $P<0.05$ was viewed as statistically significant.

3.5 RESULTS

3.5.1 Study 1. *In vitro* inhibition by drugs A-E of AVP binding

Each drug dose-dependently displaced selective $V_{1A}R$ antagonist radioligand ^{125}I-labelled [d$(CH_2)_5$,sarcosine^7]AVP at V_{1A}Rs in both rat liver and kidney medulla membranes (Figure 1). The concentration of drug required to displace 50% of specific AVP binding, or IC_{50}, for each drug is given in table 3.1. Each drug also caused a concentration-dependent displacement of the V_2R antagonist [^{3}H]desGly-NH_2^9[d$(CH_2)_5$, D-Ile^2, Ile^4]AVP to V_2Rs in kidney medulla membranes (Figure 3.1). The IC_{50} for each drug, as compared to AVP, at the kidney V_2R, is also given in table 3.1.

From the above, drug A is seen to have the "best" pharmacological profile in that it most closely resembles AVP in its ability to displace the V_{1A} and V_2R selective radioligands. Specifically, the concentration of drug A that displaced 50% of specific AVP binding (IC_{50}) was 5.8±0.1 nmol/l for liver $V_{1A}R$ and 30±0.1 nmol/l for kidney V_{1A}Rs (n=3-5). The IC_{50} for AVP was 0.87±0.03 nmol/l for liver $V_{1A}R$ and 3.9±0.14nmol/l for kidney $V_{1A}R$ (n=3-5). The IC_{50} for drug A, at the renal V_2R, was 3.22±0.1 nmol/l (n=3-5) and that of AVP 0.48±0.1 nmol/l, for kidney V_2Rs (n=3-5) (Table 3.1).

3.5.2 Study 2. *In vivo* treatment with drug A on *in vitro* AVP binding

Oral administration of drug A (1, 3, 10 mg/kg) dose-dependently inhibited AVP binding to liver and kidney $V_{1A}R$ and kidney V_2Rs at 2h, with significant inhibition reached at the 10mg/kg dose ($P<0.01$)(Figure 3.2).

After oral administration, drug A (10 mg/kg/day) inhibited AVP binding to liver ($P<0.05$) and kidney ($P<0.01$) V_{1A}Rs for 4h and to kidney V_2Rs for 8h ($P<0.05$). There was a rebound increase in V_2R binding at 24 hours ($P<0.01$)(Figure 3.3).

Drug A had no effect on plasma sodium or osmolality at any dose (1, 3 or 10 mg/kg), 2h following oral administration (Table 3.2) and drug A (10 mg/kg) had no effect on plasma sodium or osmolality at any of the time points studied (Table 3.2). Plasma AVP levels were significantly reduced by drug A at a dose of 10 mg/kg at the 8 and 24 hour time points following gavage ($P<0.01$) (Table 3.2).

3.5.3 Effect of oral drug A on renal parameters and BP

3.5.3.1 Metabolic studies

Female rats: Problem solving experiments. In initial metabolic studies an aquaresis was not seen with an oral drug dose of less than 30mg/kg/day (data not shown) and this was surprising in view of the preceding *in vitro* and *in vivo* data. Within the laboratory the mixed V_{1A}/V_2R antagonist, YMO87, and the selective V_2R antagonist, OPC 31260, had previously been extensively studied and these drugs known to produce significant diuresis within the laboratory set up (46, 384) . They were included in a repeat experiment (figure 3.4). A predicted diuresis occurred with OPC 31260 (10mg/kg)($P<0.01$) and YMO87 (1mg/kg) showed a significant reduction in urinary osmolality ($P<0.05$) whereas, once more, drug A demonstrated no aquaresis until the higher dose of 30mg/kg (figure 3.4). An explanation beyond the set up of the metabolic cages was therefore sought.

The metabolic studies were undertaken using a second batch of drug A, the first having been completely used during the initial *in vivo* and *in vitro* experiments. A possible explanation might be that the second drug had been manufactured under slightly different conditions and was somehow less potent then the first. To investigate this a liver $V_{1A}R$ displacement curve with AVP, old and new "batch" drug A, was undertaken. The curve produced by the second batch of drug A almost overlay that from the original (IC_{50} old=4.02x10nmol/l; IC_{50} new=6.61x10nmol/l) and therefore this second theory was discounted (Figure 3.5).

In appearance the second batch of drug A was more granular than the first and to take this into account the metabolic studies were repeated once more with drug sonication prior to gavage. This brought the drug into a very fine suspension and was effective in promoting an aquaresis at lower doses of drug A (initial data not shown).

Acutely, female rats exhibited a dose-dependent increase in urine volume from a dose of 10mg/kg once daily ($P<0.01$). Urinary osmolality was significantly reduced from a dose of 10mg/kg ($P<0.01$). A significant increase in urinary sodium was seen at 10mg/kg ($P<0.05$) but not so at the higher dose of 30mg/kg (figure 3.6 & table 3.3). Chronically, after 5 days of daily oral treatment, there was a significant increase in urinary volume at 10mg/kg and 30mg/kg ($P<0.05$). Urinary osmolality was reduced at 10mg/kg ($P<0.05$) and 30mg/kg ($P<0.01$) (Figures 3.6 & table 3.2). Urinary sodium remained unchanged (figures 3.6 & table 3.3).

Male rats: On day 1, male rats exhibited a dose-dependent increase in urine volume at 10mg/kg/day ($P<0.01$). Urinary osmolality was significantly reduced at 10mg/kg and

30mg/kg ($P<0.01$). No significant natriuresis was seen at any dose (figure 3.7 & table 3.3). Chronically, oral drug A (10mg/kg and 30mg/kg), for 5 days caused no significant increase in urine output at 10mg/kg but did increase urine output at a dose of 30mg/kg ($P<0.05$). Urinary osmolality was similarly reduced by a dose of 30mg/kg ($P<0.05$). No natriuresis was seen after 5 days (figure 3.7 & table 3.3).

3.5.3.2 BP studies

Oral drug A had no effect on SBP in female normotensive rats on days 1 and 3 of treatment at a dose of 10mg/kg (Figure 3.8).

3.5.3.3 Plasma data

After 5 days of oral dosing plasma sodium and osmolality were unchanged in both the male and female rats. Plasma AVP was significantly elevated in female rats but only at a dose of 30mg/kg (5.4±0.4pmol/l versus 7.1±0.4pmol/l; vehicle versus drug A: $P<0.05$). In male rats, plasma AVP was significantly elevated at a dose of 30mg/kg only (8.5±0.8pmol/l versus 12.8±2.1pmol/l; vehicle versus drug A: $P<0.01$)(Table 3.4).

3.5.3.4 *In vitro* autoradiography

In female rats, after 5 days of oral treatment with drug A, significant inhibition of binding of the selective $V_{1A}R$ antagonist radioligand ^{125}I-labelled [d$(CH_2)_5$,sarcosine^7]AVP by drug A at the renal $V_{1A}R$ was seen at 10mg/kg ($P<0.05$) and 30mg/kg ($P<0.01$)(Figure 3.9).

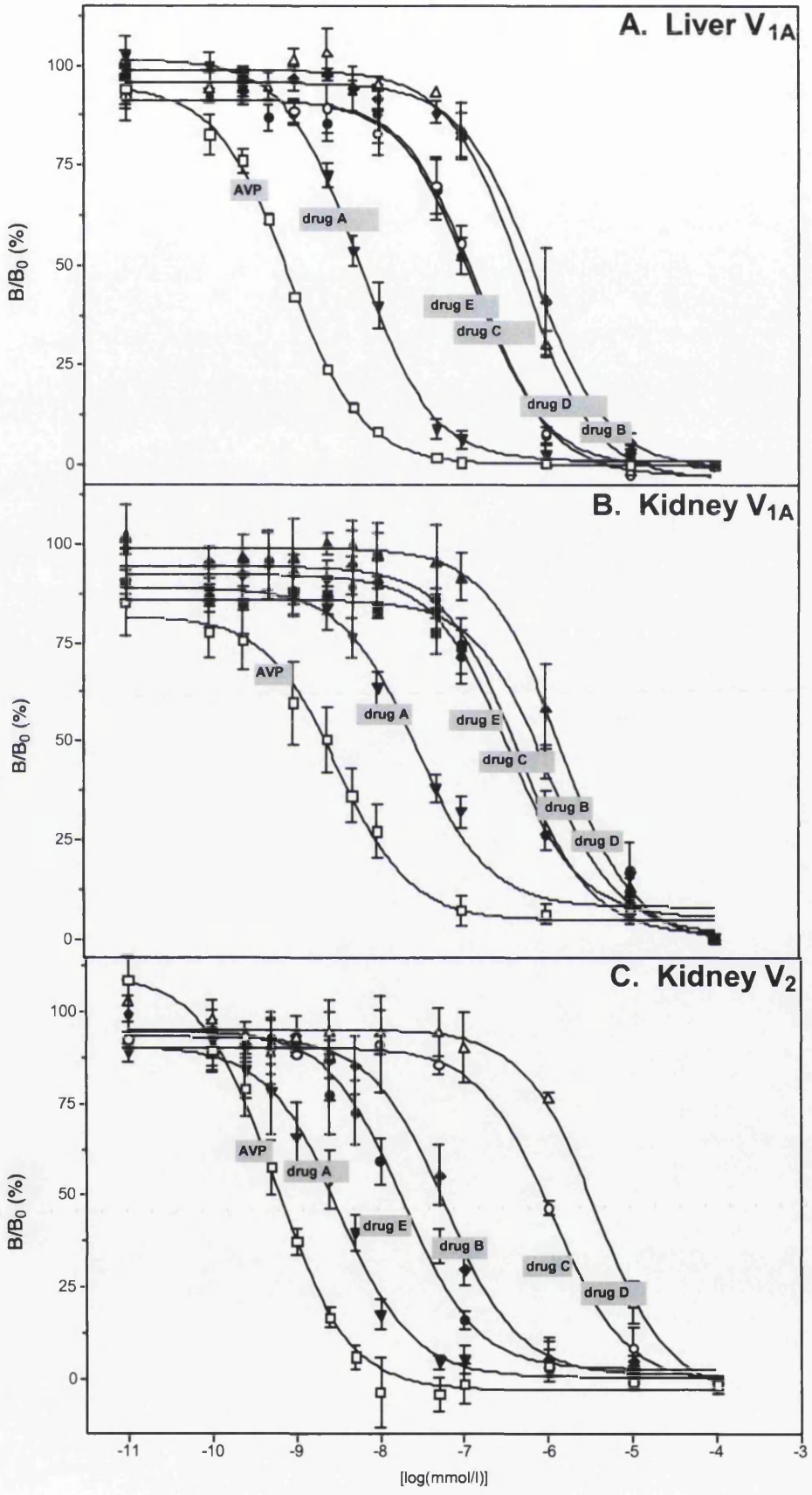


Figure 3.1. Displacement of specific V_{1A} and V_2R antagonist binding with drugs A-E.

Figure 3.1. Displacement of specific V_{1A} and V_2R antagonist binding with drugs A-E.

Figure 3.1A. Displacement of the specific binding of the $V_{1A}R$ antagonist ^{125}-I-labelled[d$(CH_2)_5$, sarcosine^7]AVP from rat liver membranes by increasing concentrations of unlabelled AVP and drugs A-E.

Figure 3.1B. Displacement of the specific binding of the $V_{1A}R$ antagonist ^{125}-I-labelled[d$(CH_2)_5$, sarcosine^7]AVP from rat kidney medulla membranes by increasing concentrations of unlabelled AVP and drugs A-E.

Figure 3.1C. Displacement of the specific binding of the V_2R antagonist [^{3}H]desGly-$NH_2{}^9$[d$(CH_2)_5$, D-Ile^2, Ile^4]AVP from kidney medulla membranes by increasing concentrations of unlabelled AVP and drugs A-E. B and B_0 represent the amount of specific binding in the presence and absence of unlabelled compound respectively.

Each point represents the mean±SEM of 3-5 separate determinations performed in triplicate.

Table 3.1. IC_{50}'s of drugs A-E in rat.

Liver and kidney membranes were prepared from untreated rats and incubated with ^{125}I-labelled[d$(CH_2)^5$, sarcosine^7]AVP in the presence of increasing concentrations of AVP and drugs A-E to determine IC_{50} at liver V_{1A} and renal V_{1A}Rs. Kidney medullary membranes from untreated rats were also incubated with [^{3}H]desGly-$NH_2{}^9$[d$(CH_2)^5$, D-Ile^2, Ile^4]AVP in the presence of increasing concentrations of AVP and drugs A-E and IC_{50} values were obtained.

Each value represents the mean±SEM of 3-5 separate determinations performed in triplicate.

AVP/DRUG	RECEPTOR TYPE		
	LIVER V_{1A} (nmol/l)	RENAL V_{1A} (nmol/l)	RENAL V_2 (nmol/l)
AVP	0.87±0.03	3.4±0.1	0.58±0.07
DRUG A	5.8±0.4	29±0.9	3.31±1.4
DRUG B	740±9	1020±10	57.3±0.8
DRUG C	157±7	463±9	106±1.3
DRUG D	520±7	1495±12	3650±14
DRUG E	138±6	354±13	18±0.9

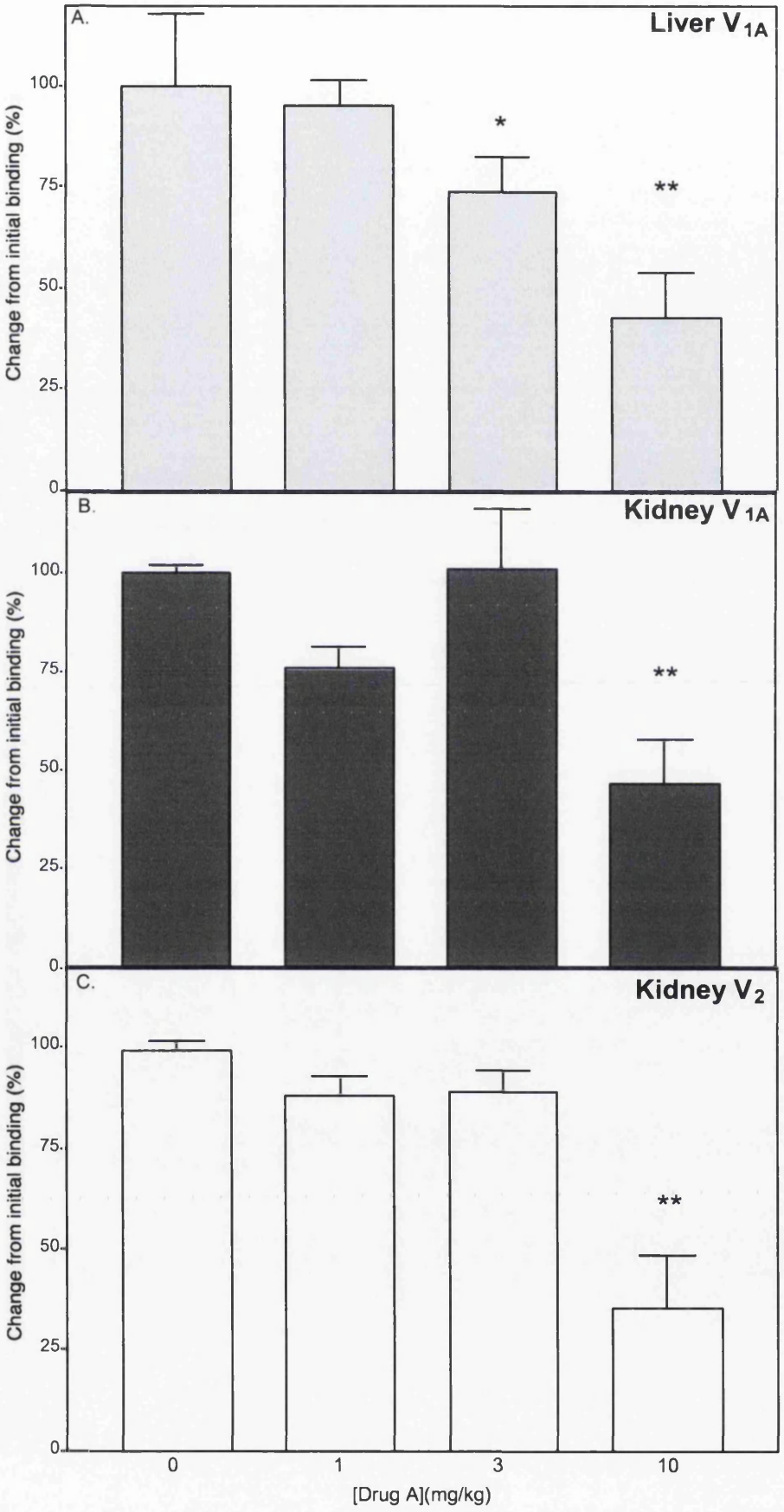


Figure 3.2. Dose response with oral drug A in rat

Figure 3.2. Dose response with oral drug A in rat

Figure 3.2A. Effect of orally administered drug A at 0, 1, 3 and 10 mg/kg doses on the *in vitro* specific binding of ^{125}I-labelled [d$(CH_2)_5$, sarcosine^7]AVP to liver V_{1A}Rs.

Figure 3.2B. Effect of orally administered drug A at 0, 1, 3 and 10 mg/kg doses on the *in vitro* specific binding of ^{125}I-labelled [d$(CH_2)_5$, sarcosine^7]AVP to kidney medullary V_{1A}Rs.

Figure 3.2C. Effect of orally administered drug A at 0, 1, 3 and 10 mg/kg doses on the *in vitro* specific binding of [^{3}H]desGly-NH_2^9[d$(CH_2)_5$, D-Ile^2, Ile^4]AVP to renal medullary V_2Rs.

Results are expressed as percentage change from initial binding at time zero. Each point represents the mean±SEM of three separate determinations performed in triplicate.

*$P<0.05$, **$P<0.01$ versus time zero.

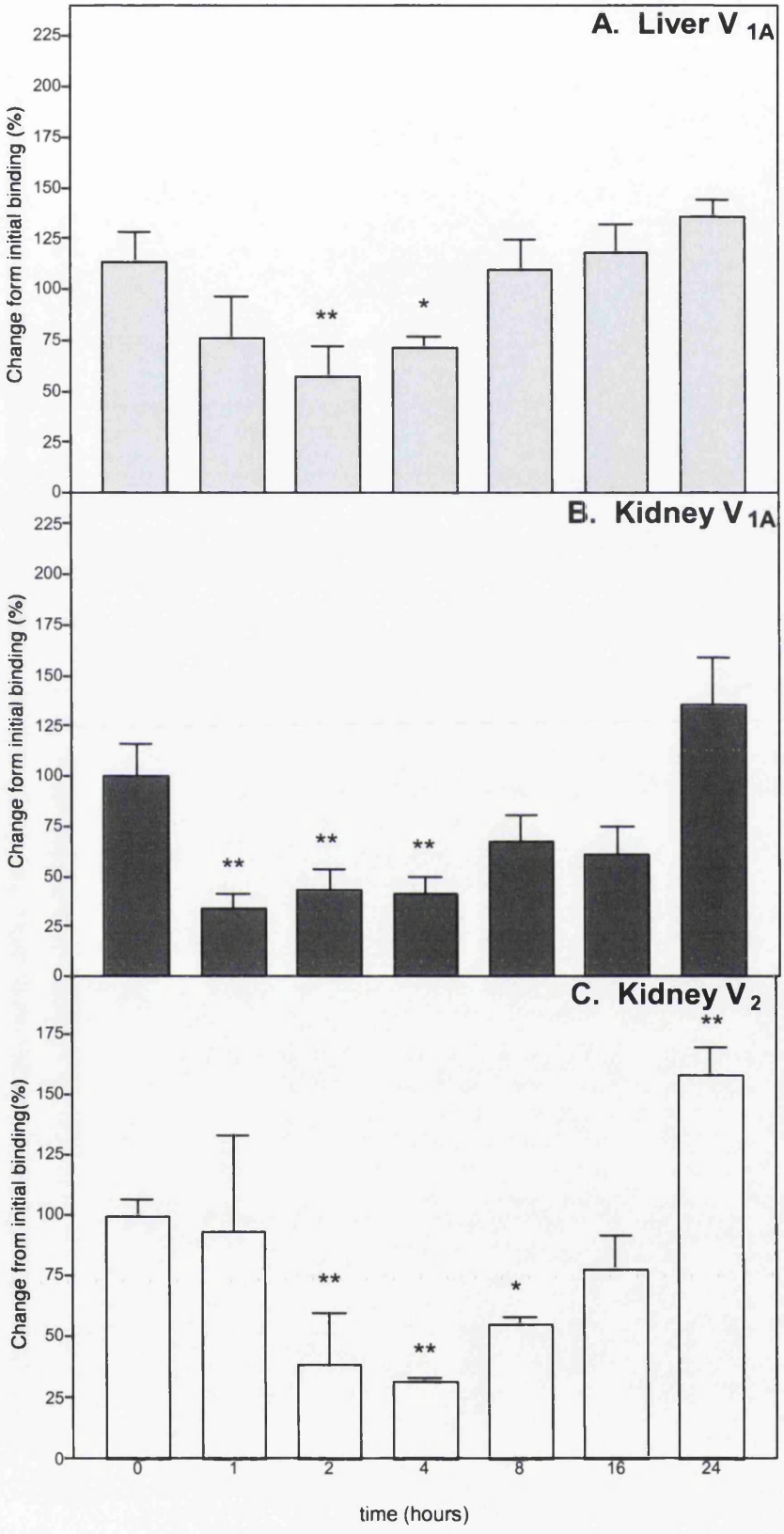


Figure 3.3. Time course of oral drug A in rat

Figure 3.3. Time course of oral drug A in rat

Figure 3.3A. Effect of oral Drug A (10mg/kg) on the *in vitro* specific binding of $V_{1A}R$ antagonist ^{125}I-labelled [d$(CH_2)_5$, sarcosine^7]AVP to liver with increasing time from gavage.

Figure 3.3B. Effect of oral Drug A (10mg/kg) on the *in vitro* specific binding of $V_{1A}R$ antagonist ^{125}I-labelled [d$(CH_2)_5$, sarcosine^7]AVP to kidney with increasing time from gavage.

Figure 3.3C. Effect of oral Drug A (10mg/kg) on the *in vitro* specific binding of V_2R antagonist [^{3}H]desGly-NH_2^9[d$(CH_2)_5$, D-Ile^2, Ile^4]AVP to kidney with increasing time from gavage.

Each point represents the mean±SEM of 3-4 separated determinations per time point performed in triplicate. *$P<0.05$, **$P<0.01$ versus time zero.

Table 3.2. Effect of orally administered drug A (1,3,and 10 mg/kg) at 2 hours, and 10mg/kg at 1-24 hours at various time points on plasma sodium, AVP and osmolality.

Each value represents the mean±SEM of four separate determinations per time point performed in triplicate. **P<0.01 versus time zero.

Plasma sodium (mmol/l)

time	vehicle	1mg/kg	3mg/kg	10mg/kg
0				138±0.5
1				139±0.8
2	137±0.6	138±0.8	137±0.6	139±0.6
4				137±0.2
8				137±0.2
16				136±0.8
24				137±0.4

Plasma osmolality (mosm/kg)

time	vehicle	1mg/kg	3mg/kg	10mg/kg
0				295±1.3
1				297±0.7
2	298.35±2.2	297.6±1.07	297.5±2.64	297±1.7
4				293±0.8
8				291±0.4
16				293±1.9
24				293±2.3

Plasma AVP (pmol/l)

time	vehicle	1mg/kg	3mg/kg	10mg/kg
0				4.7±0.1
1				4.1±0.3
2	3.5±0.6	2.9±0.6	2.0±0.1	4.1±0.4
4				4.3±0.5
8				3.3±0.3 **
16				3.9±0.3
24				2.8±0.1 **

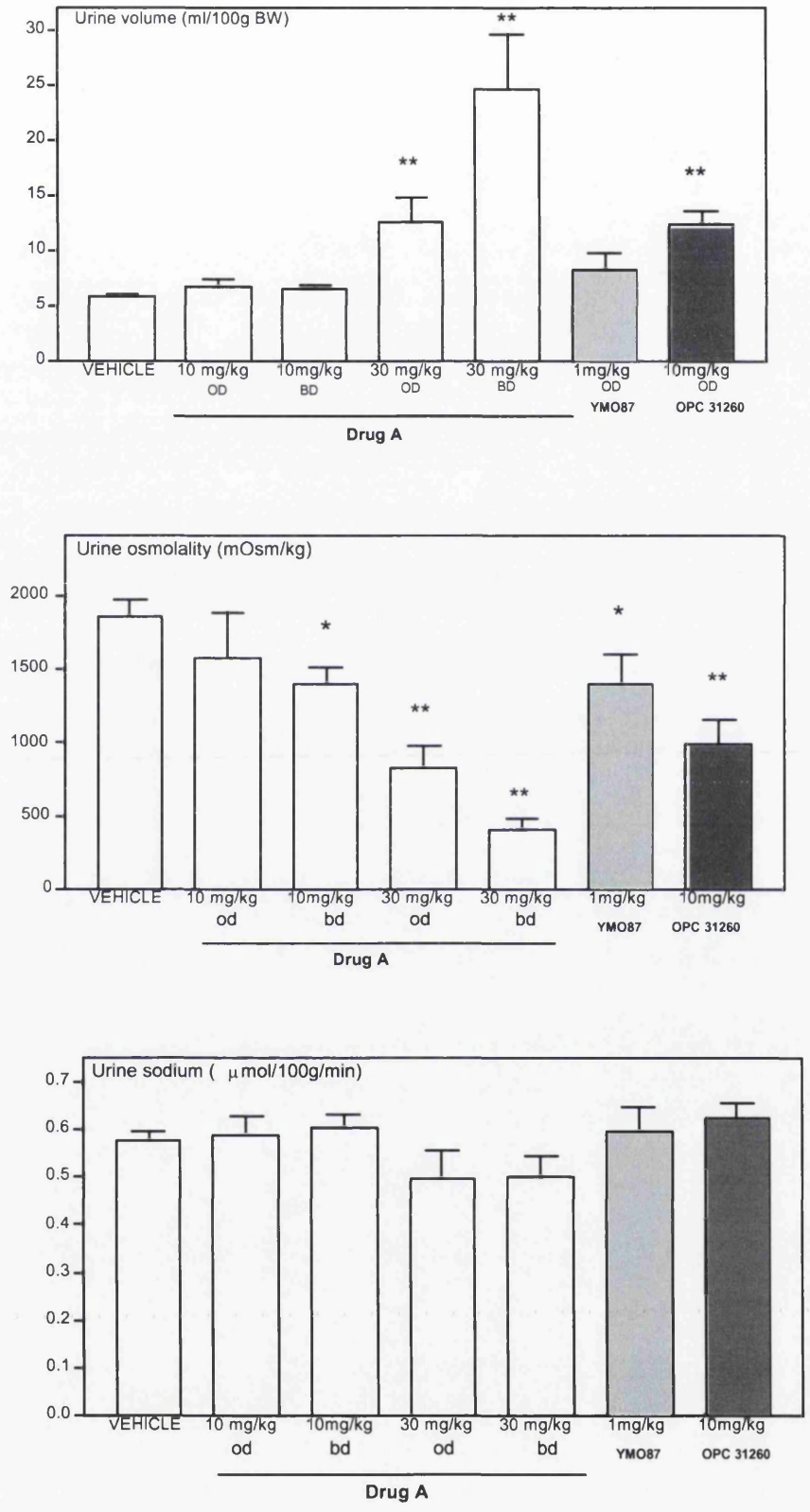


Figure 3.4. A comparison of the diuretic effects of increasing doses of drug A as compared to previously characterised AVP receptor antagonists

Figure 3.4. A comparison of the diuretic effects of increasing doses of drug A as compared to previously characterised AVP receptor antagonists YMO87 (mixed $V_{1A}R$ and V_2R) and OPC 31260 (V_2R selective antagonist).

Each point represents the mean±SEM of 4 separate determinations. *P<0.05, **P<0.01 versus vehicle.

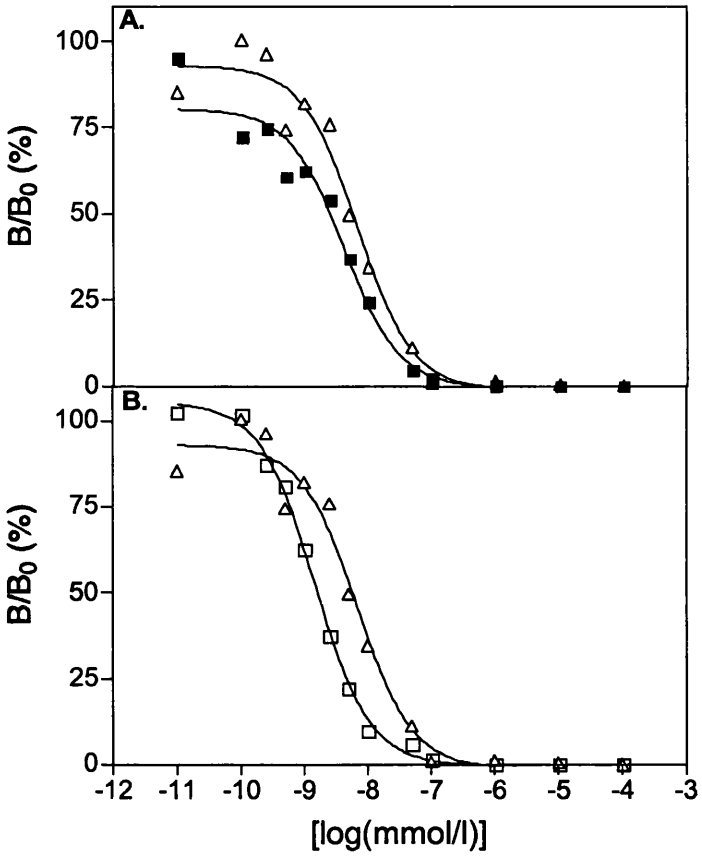


Figure 3.5. A comparison of the displacement of specific $V_{1A}R$ antagonist binding by "old" and "new" drug A.

Displacement of the specific binding of the $V_{1A}R$ antagonist ^{125}I-labelled [d$(CH_2)_5$, sarcosine^7]AVP from rat liver membranes by increasing concentrations of

A. Unlabelled AVP (■) and drug A-new( )

B. Drug A -old( ) and drug A-new( )

Each point represents one determination performed in triplicate.

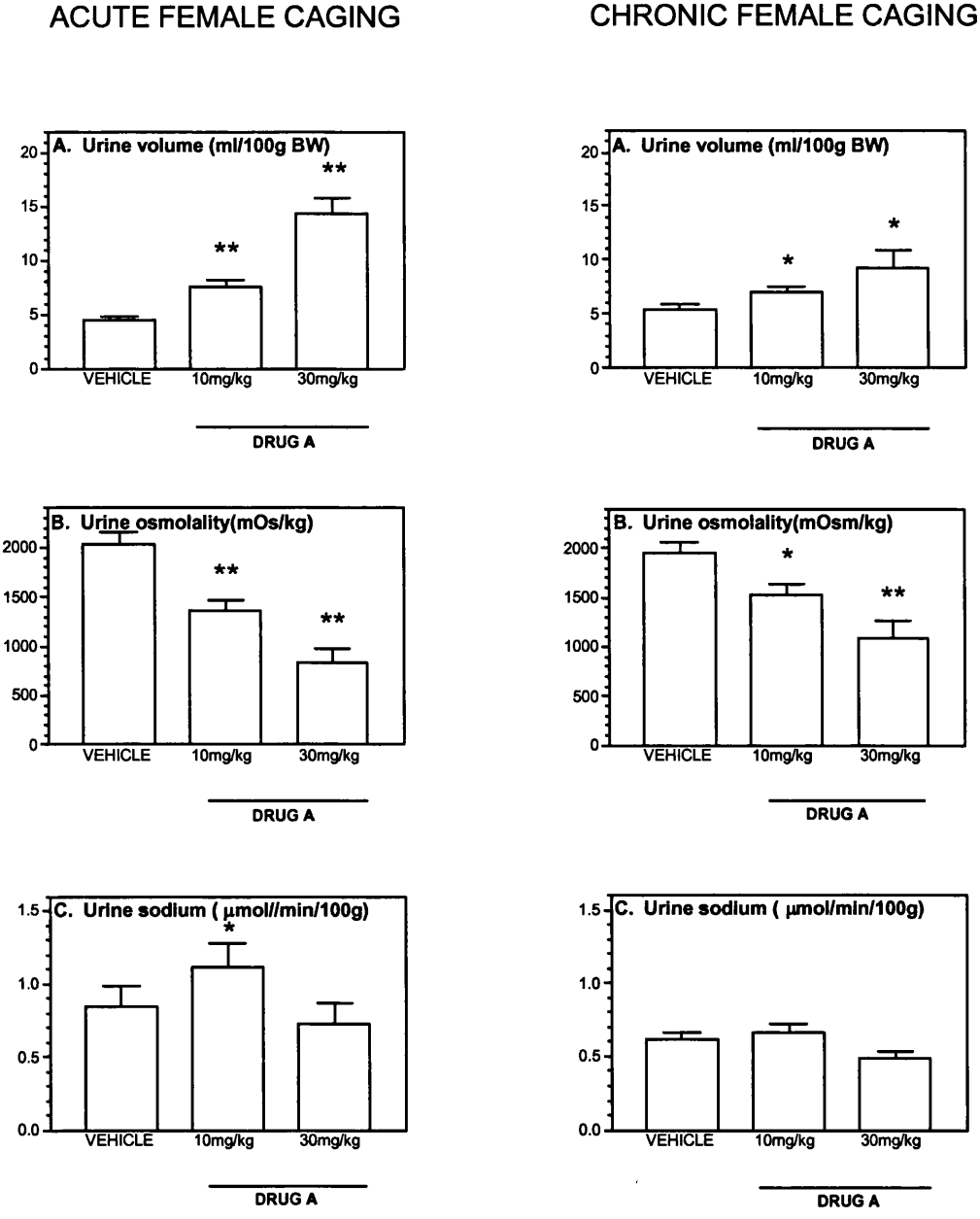


Figure 3.6. Results of metabolic studies to assess the acute effects of AVP V_2R blockade by drug A.

Female SD rats were placed into metabolic cages on days 1(acute) and 5(chronic) of treatment and A. Urine volume, B. Urine osmolality and C. Urine sodium were assessed for 24h.

Each point represents the mean±SEM of 6-8 separate determinations. *P<0.05, **P<0.01 versus vehicle. BW=Body weight.

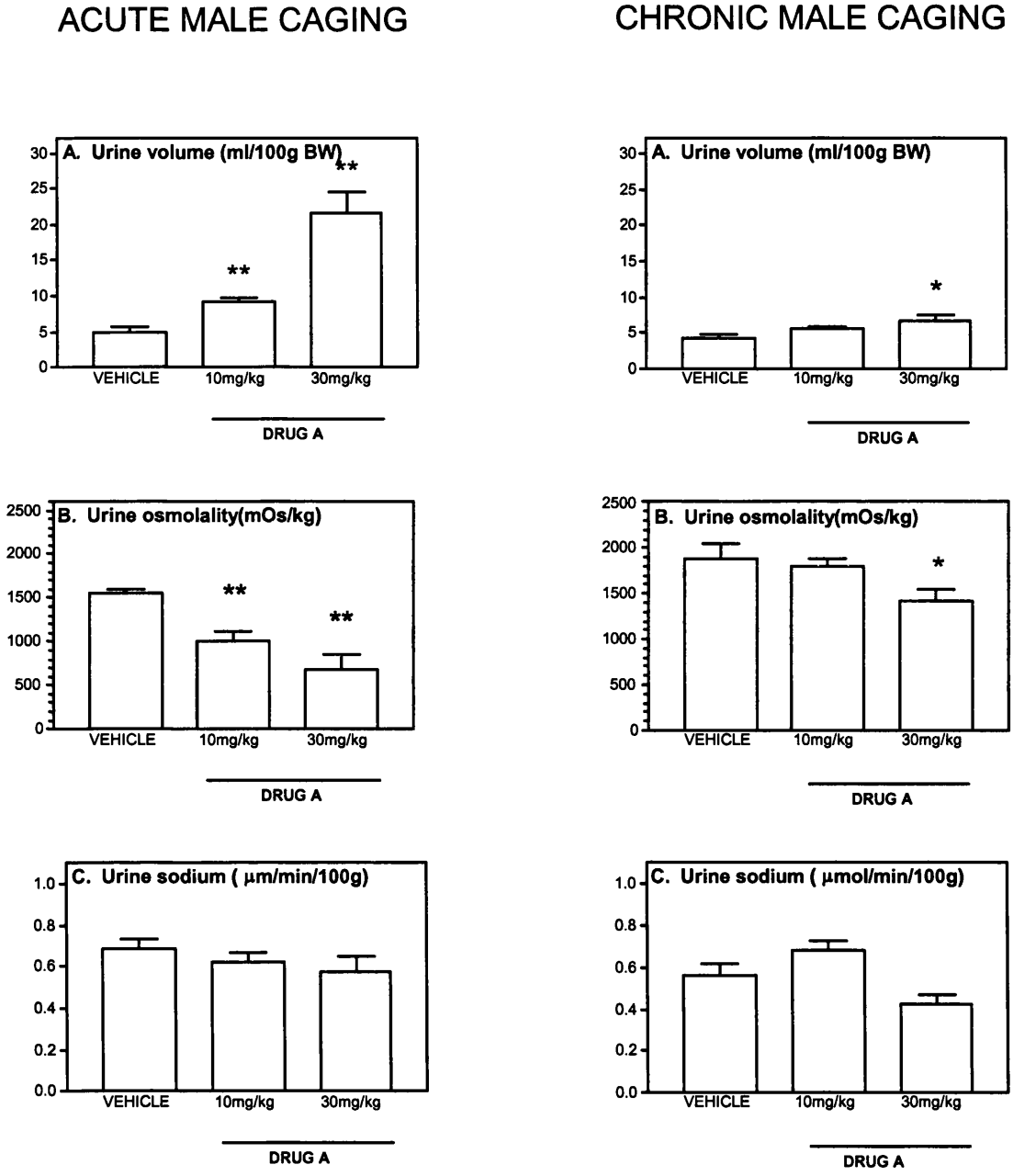


Figure 3.7. Results of metabolic studies to assess the acute effects of AVP V_2R blockade by drug A

Male SD rats were placed into metabolic cages on days 1(acute) and 5(chronic) of treatment and A. Urine volume, B. Urine osmolality and C. Urine sodium were assessed for 24h.

Each point represents the mean±SEM of 6-8 separate determinations. *P<0.05, **P<0.01 versus vehicle. BW= Body weight.

Table 3.3. Acute and chronic metabolic data represented as actual values for both male and female, acute and chronic studies.

Each point represents the mean±SEM of 6-8 separate determinations. *P<0.05, **P<0.01 versus vehicle.

	ACUTE FEMALE			CHRONIC FEMALE		
	Vehicle	10mg/kg	30mg/kg	Vehicle	10mg/kg	30mg/kg
Urine volume (ml/100g)	4.53±0.29	7.90±0.55**	14.36±1.51**	5.37±0.40	6.95±0.52*	9.21±1.63*
Osmolality (mOsm/kg)	2184±47	1364±103**	639±97**	1924±105	1524±112*	1094±164**
Sodium (µmol/min/100g)	0.49±0.05	0.65±0.03*	0.46±0.03	0.62±0.05	0.67±0.06	0.49±0.05

	ACUTE MALE			CHRONIC MALE		
	Vehicle	10mg/kg	30mg/kg	Vehicle	10mg/kg	30mg/kg
Urine volume (ml/100g)	4.9±0.7	9.1±0.7**	21.4±3.1**	4.4±0.5	5.6±0.4	6.8±0.8*
Osmolality (mOsm/kg)	1541±66	1012±101**	388±71**	1880±165	1790±94	1413±128*
Sodium (µmol/min/100g)	0.69±0.05	0.62±0.05	0.58±0.07	0.56±0.06	0.68±0.04	0.43±0.04

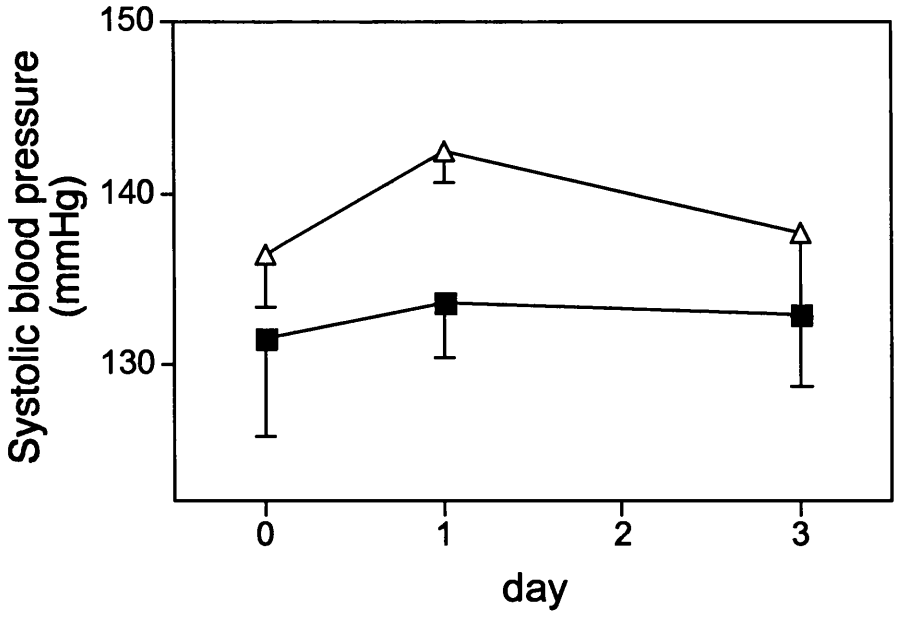


Figure 3.8. BP in female normotensive rats after acute and chronic treatment with drug A.

SD rats were blood pressured by tail cuff plethysmography pre-treatment, and on days 1 and 3 of treatment with drug A (■=vehicle, △=drug A 10mg/kg).

Each point represents the mean±SEM of 6 separate determinations performed in triplicate.

Table 3.4. Effect of orally administered drug A (10 mg/kg/day and 30mg/kg/day) on plasma parameters after 5 days of oral dosing in female and male SD.

Each point represents the mean±SEM of 6-8 separate determinations. *P<0.05, **P<0.01 versus vehicle.

	CHRONIC FEMALE			CHRONIC MALE		
	Vehicle	10mg/kg	30mg/kg	Vehicle	10mg/kg	30mg/kg
Plasma sodium (mmol/l)	136.8±0.2	136.8±0.2	144.7±7.1	134.3±0.5	133.1±0.8	131.3±3.3
Plasma osmolality (mOsm/kg)	297.2±0.9	295.4±1.2	297.7±1.0	294.8±0.7	289.6±2.5	286.5±3.9
Plasma AVP (pmol/l)	5.4±0.4	5.6±0.5	7.1±0.4*	8.5±0.8	7.1±0.7	12.8±2.1**

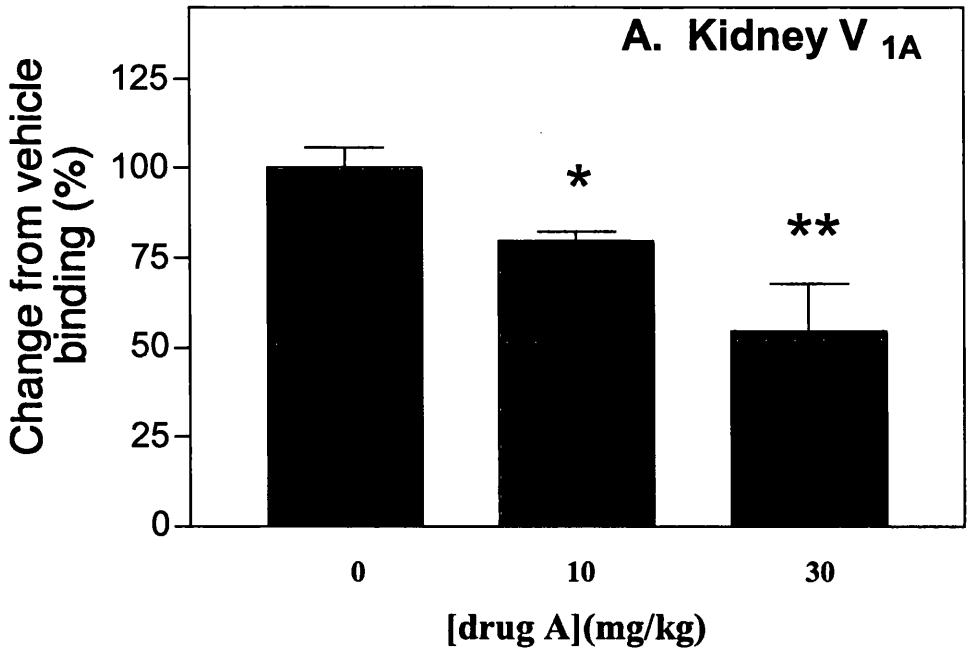


Figure 3.9. *In vitro* $V_{1A}R$ autoradiography after 5 days of oral drug A in female rat.

Figure 3.9. Effect of orally administered drug A at 0, 10 and 30mg/kg doses on the *in vitro* specific binding of ^{125}I-labelled [d$(CH_2)_5$, sarcosine^7]AVP to kidney medullary V_{1A}Rs.

Results are expressed as percentage change from the binding seen with vehicle treated rats.

Each point represents the mean±SEM of 4 separate determinations performed in triplicate.

*P<0.05, **P<0.01 versus vehicle.

3.6 DISCUSSION

The results of this study demonstrate that, *in vitro,* drugs A-E act as competitive inhibitors of AVP at liver (order of potency A, E, C, D, B) and kidney V_{1A}Rs (order of potency A, E, C, B, D) and the kidney V_2Rs (order of potency A, E, B, C, D). Drug A is the best competitive inhibitor of AVP at liver and kidney V_{1A}Rs and the kidney V_2Rs. The IC_{50} values calculated from the radioligand displacement curves indicate that drug A binds to the liver V_{1A}R at approximately 17% of the affinity of AVP and has approximately 12.5% the affinity for the renal V_{1A}R. At the kidney V_2R drug A has approximately 14% the affinity of AVP.

The *in vivo* time course and dose response studies show that oral drug A is an effective V_{1A}R and V_2R antagonist. The inhibitory effect of drug A was dose- and time-dependent with maximal responses at 10mg/kg. In liver membranes the effect of drug A was not evident until 2 hours post gavage and its inhibitory effect lasted up to 4 hours. For the renal V_{1A}R drug effect was seen after 1 hour and again lasted up until 4 hours post dosing, while for the renal V_2R, inhibitory effect was first seen after 2 hours and lasted up until 8 hours post gavage. These results suggest that drug A is a good inhibitor of both V_{1A} and V_2Rs *in vitro* and that the most suitable dosage regime would be two to three times daily.

From assessment of SBP in this study, 3 days of oral drug A did not cause a significant hypotensive effect in normal, hydrated, female rats while *in vitro* autoradiography confirmed blockade of the V_{1A}Rs by drug A. It is known, however, that blockade of the V_{1A}R does not reduce BP in normal animals due to the buffering effects of an intact sympathetic nervous system and activation of the renin angiotensin system (385). Drug A was an effective V_2R antagonist to cause significant aquaretic responses with 5 days of

oral treatment and these responses were different in both male and female rats. Acutely, male rats had a more pronounced aquaretic response than females at both 10 and 30mg/kg doses. After 5 days, however, the aquaretic response in both sexes was "blunted". In females this reduction in urine output was less than that seen in male rats and aquaresis remained significant at both 10 and 30mg/kg doses. In the male rats, on the other hand, a significant aquaresis after 5 days was only seen with a dose of 30mg/kg.

A number of possible explanations exist for this turning down of the aquaretic response to AVP with chronic dosing of drug A. The most likely can be explained by the phenomenon known as tachyphylaxis. With some drugs the intensity of response to a given dose may change during the course of treatment. In these cases, responsiveness usually decreases as a consequence of continued drug administration to produce a state of relative tolerance to the drug's effects. When responsiveness diminishes rapidly after administration of a drug the response is said to be subject to tachyphylaxis. While chronic blockade of the V_2R using peptide receptor antagonists was not associated with persistent aquaresis (386), studies with non-peptide AVP receptor antagonists have, thus far, demonstrated no tachyphylactic effects. For instance, OPC 31260 had a sustained aquaretic effect in a coronary artery ligated rat model of heart failure (41) and YM087 remained a highly effective aquaretic agent after 7 days of treatment in female SD rats (46). Many different mechanisms may give rise to tachyphylaxis. They include: -

- Alteration in concentration of competing receptor ligands.
- Change in receptors.
- Change in signal pathways distal to the receptor.
- Increased metabolic degradation.
- Physiological adaptation.

In our study, the observed tachyphylaxis may conceivably result from an alteration in the AVP receptors and in particular receptor number. As can be seen in figure 3.3 there appears to be an up-regulation of all receptors by 24 hours post dosing and this increase in receptor number is significant for the renal V_2R, approximately 1.6 fold ($P<0.01$). It is conceivable that this "rebound" increase in the V_2R may further increase with each day of dosing and with increased dosing frequency such that a higher and higher dose of oral antagonist is required to produce its desired effect, in this case an aquaresis. Another possible explanation is that after repeated dosing with a mixed AVP receptor antagonist AVP is increased as part of a feedback type response. This being the case then, once more, a higher concentration of drug A than that which is effective acutely would be required to produce any desired aquaretic response chronically. This explanation would also fit nicely with the fact that acutely, male rats demonstrate a greater diuresis than females but that over the 5 days of our study that response was reduced more significantly than the female chronic response. With greater initial aquaresis, and possibly dehydration, this would result in higher plasma AVP levels in the male animals. Our plasma AVP results do not directly compare acute with chronic dosing nor do they look at male plasma AVP acutely, however, at a dose of 10mg/kg/day, in both male and female rats, no significant increase in plasma AVP after 5 days, when compared to vehicle treated rats, was seen. On the other hand at a dose of 30mg/kg there is significance and more so in male rats ($P<0.05$; female and $P<0.01$; male). This would reflect a greater degree of dehydration in those animals given the highest drug dose once more as a result of a greater aquaresis in the males. Interestingly, in the initial time course study, plasma AVP was significantly decreased after 8 and 24 hours with a single 10 mg/kg dose of antagonist which is surprising in view of the above. This is unlikely to have been seen at a dose of 30mg/kg had this been

included. In previous studies using the V_2R antagonists OPC 31260 (41) and YM087 (46), plasma AVP levels have been elevated after a single dose.

There was no significant rebound increase in $V_{1A}R$ number in both liver and kidney (figure 3.3) and thus there is likely to be sufficient $V_{1A}R$ blockade throughout the study to avoid any systemic hypertensive effects.

Obviously other mechanisms for tachyphylaxis may be at work here, but an investigation into signal pathways, metabolic degradation and physiological adaptation were beyond the remit of this particular study.

In conclusion, these studies confirm that drug A is a potent, non-peptide, orally effective V_{1A} and V_2R antagonist that has inhibitory effects on both the V_{1A} and V_2Rs following oral dosing. After 5 days of dosing this effect is not well sustained and it may be that longer-term studies are required before this drug's clinical usefulness can be properly assessed. If shown to be effective over the longer period it may prove useful in the management of CCF where increased AVP contributes to increased vascular resistance through its $V_{1A}R$ and to hyponatraemia via its effects at the V_2R to cause fluid retention.

Chapter 4

Combination treatment of V_{1A} receptor antagonism and Angiotensin Converting Enzyme inhibition in Spontaneously Hypertensive Rats

CHAPTER 4

Combination treatment of V_{1A} receptor antagonism with Angiotensin Converting Enzyme inhibition in the Spontaneously Hypertensive Rat

Contents

4.1 SUMMARY

1. This study assessed the effects of combination therapy with the V_{1A} receptor ($V_{1A}R$) blocker OPC 21268 and angiotensin converting enzyme (ACE) inhibitor ramipril on systolic blood pressure (SBP), organ weights, metabolic and hormonal parameters in the adult spontaneously hypertensive rat (SHR).

2. The study was divided into 2 separate experiments: Study 1 and study 2. In the first, male SHR had telemetry probes inserted into their abdominal aortas at 16 weeks of age and, after recovery and an initial baseline SBP period of 5 days, were then randomised to receive ramipril (1mg/kg/day), OPC 21268 (60mg/kg/day) or combination ramipril (1mg/kg/day) and OPC 21268 (60mg/kg/day) for 2 weeks with continuous SBP recording. In study 2, 16 week SHRs were randomised to the above 3 groups or vehicle and gavaged for 14 days. On days 10 and 11 these rats were placed in metabolic cages for 24 hour assessment of water and food intake as well as urinary parameters. After this 14 day period the rats were killed to assess cardiac mass, tissue enzyme inhibition, plasma biochemistry and plasma hormones.

3. Ramipril alone, or in combination, reduced SBP and relative left ventricular (LV) mass compared with OPC 21268 or vehicle treated animals ($P<0.01$). Ramipril significantly inhibited renal and plasma ACE ($P<0.01$) and increased plasma renin activity (PRA). There was no significant effect on plasma arginine vasopressin (AVP) in ramipril, OPC 21268 or combination treatment groups. OPC 21268 did not reduce SBP or LV mass despite significant $V_{1A}R$ blockade.

4. This study demonstrates that in an adult rat hypertensive model, 2 weeks of $V_{1A}R$ antagonism is not additive or synergistic to ACE inhibition in terms of BP lowering or reduction of cardiac mass.

4.2 INTRODUCTION

Hypertension is a common health problem within Western societies and is a major contributor to mortality and morbidity due to many cardiovascular diseases such as cerebrovascular accident, myocardial infarction and renal failure (1, 9). Increasingly the treatment thresholds for this condition are being lowered as it becomes clear that BPs previously considered "normal" are actually still high enough to contribute to cardiovascular risk. For instance a BP of 140/90, even without other cardiovascular risk factors, is now considered to be abnormal (9). In an attempt to reach these new "target" BPs increasing numbers of patients now require polypharmacy (387). A significant number of people remain hypertensive despite the current availability of a vast array of different numbers and groups of antihypertensive agents thus prompting the search for novel antihypertensive drugs (388).

AVP is a vasoactive peptide that mediates vasoconstrictor effects through AVP V_{1A}Rs in vascular smooth muscle and the brain, and its antidiuretic effects through the activation of V_2 receptors (V_2Rs) in the renal tubule. While it is clear that this nonapeptide has a role to play in the developmental stages of hypertension (50), its role in established hypertension as yet remains unclear. In rat models such as deoxycorticosterone acetate-salt (DOCA) hypertension, where AVP levels are elevated, this hormone has been shown to be important (120, 124). OPC 21268 or 1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2(1H)-quinolinone (Figure 4.1) is a non-peptide, orally active, selective AVP V_{1A}R antagonist which has little effect at the AVP V_2R and is described in more detail in chapter 1 (section 1.4.1) (123). OPC 21268 has been shown to lower BP chronically in DOCA hypertension (124), in Dahl salt sensitive (DSS) hypertension (389, 390) and also in the malignant phase of hypertension in the stroke-prone SHR (391).

Through chronic administration of this drug it is now clear that AVP does not reverse adrenocorticotrophin-induced hypertension in the Sprague-Dawley rat (268) and is not involved in renovascular hypertension (267). In male DSS rats it has been shown that the combination of OPC 21268 and ACE inhibitor captopril was not additive in terms of BP reduction, although each individual treatment attenuated hypertension (389). However, a 1 week period of $V_{1A}R$ antagonist OPC 21268, in adult SHR, failed to result in any BP lowering effects (50).

As compared to that of AVP, the role of the renin angiotensin system (RAS) in the regulation of BP and in cardiovascular diseases has been well characterised (392-394). ACE inhibitors are highly efficacious BP lowering agents in animals and humans and are widely used in the treatment of hypertension, both alone and in combination with other agents (1). They are currently the 'gold standard' drug group used in the management of diabetic nephropathy (286, 395) and cardiac failure (396). No studies thus far have looked at combination therapy of ACE inhibition with $V_{1A}R$ antagonism in the adult hypertensive rat to assess whether, at this mature stage, any additive BP lowering benefits exist.

The aims of this study were to examine the BP effects of 2 weeks of treatment with ACE inhibition and $V_{1A}R$ antagonism in adult SHR and to look for any additive benefits in terms of SBP, plasma and metabolic parameters and organ weights with this combination therapy.

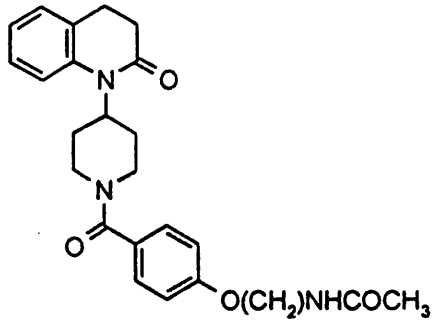


Figure 4.1. Representation of the chemical structure of OPC 21268. Modified from (397).

4.3 EXPERIMENTAL PROTOCOL

4.3.1 Ethics

This experiment was approved by the Austin and Repatriation Medical Centre Ethics Committee and performed according to the National Health and Medical Research Council of Australia guidelines for animal experimentation.

4.3.2 Drugs

OPC 21268 was a generous gift from the Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). Ramipril was purchased from Aventis Pharmaceuticals (New Jersey, USA). OPC 21268 was suspended in 0.5% methylcellulose (BDH Pharmaceuticals, Poole, UK) while ramipril was dissolved in water. Both of these drugs were administered orally by gavage.

4.3.3 Study 1

16 week male SHR had telemetry probes inserted (n=13), as described in chapter 2 (section 5.5.2). After a 7 day surgical recovery period, baseline 24 hour SBPs were recorded for a

further 5 days after which time the animals were randomised to the following treatment groups - OPC 21268 (n=5), ramipril (n=4) and ramipril plus OPC 21268 (n=4). Drugs were administered by gavage and dosing continued for 14 days. OPC 21268 was given twice daily at a dose of 30mg/kg with ramipril being given once daily at a dose of 1mg/kg. SBPs were sampled every 30 minutes for 30 seconds for the duration of this study. All stored BP data was gathered retrospectively from the telemetry computer and SBPs from each 8am-8am 24 hour period averaged. Excluded from subsequent analysis was any SBP of greater that 220 mmHg or less than 80 mmHg as they were presumed artefactual. For each treatment group the blood pressures for treatment days 1-14 were averaged and analysed as compared to the average overall baseline blood pressure. In addition, for each individual SHR, the daily drop in SBP relative to that animals average baseline pressure was calculated.

4.3.4 Study 2

16 week SHR's without telemetric probes *in situ* were randomised to receive vehicle, OPC 21268 (30mg/kg bd), ramipril (1mg/kg/day) or ramipril plus OPC 21268 (n=6/group). On days 10 and 11 of treatment, animals were placed into metabolic cages to assess 24 hour food and water intake, urine output and urinary sodium and osmolality (n=6/group). Rats were killed by decapitation on day 14, 1 hour post gavage, and trunk blood collected for the measurement of plasma sodium and osmolality, plasma AVP concentration and plasma ACE and PRA (n=6/group) as described in chapter 2 (section 2.6.7). Kidneys were collected, weighed and rapidly frozen in dry ice chilled isopentane for *in vitro* autoradiography (n=6/group) as described in chapter 2 (sections 2.6.2.1 and 2.6.2.3). Liver was removed and membranes prepared immediately and stored in liquid nitrogen prior to

$V_{1A}R$ binding studies (n=6/group) as described in chapter 2 (section 2.6.4). Heart was removed and dissected into left and right ventricles and weighed (n=6/group).

4.4 STATISTICAL ANALYSIS

Data were analysed by analysis of variance (ANOVA) with or without repeated measures using the Statview SE=Graphics programme (Brainpower, Calabasas, CA). Comparisons of group means were performed by Fisher's least significant difference method. Data are shown as means±SEM unless otherwise specified. $P<0.05$ was viewed as statistically significant.

4.5 RESULTS

4.5.1 Study 1

4.5.1.1 SBP

The average baseline SBP over 5 days was 162±4 mmHg (n=13). The mean baselines for each group being: 160.8±4.8 (OPC 21268), 165.7±10.0 (ramipril) and 160.2±5.5 (ramipril+OPC 21268) (n=4-5/group). There was no statistical difference between groups.

Day 1-14 average SBPs per group were: 162.9±5.1mmHg (OPC 21268), 145.7±9.7mmHg (ramipril) and 147.5±6.2mmHg (ramipril + OPC 21268). When compared to the average baseline of 162±4 mmHg, OPC 21268 was not statistically different while with ramipril alone, $P<0.01$, and ramipril plus OPC 21268, $P<0.05$. The difference in treatment BPs between ramipril alone and ramipril+OPC 21268 did not reach significance (Figure 4.2.).

The average changes in SBP per treatment group, as compared to that group's own average baseline BP were (day 14 data only given): +4.7±4.2mmHg (OPC 21268), –22.3±6.7mmHg (ramipril) and –12.9±1.3mmHg (ramipril+OPC 21268). Again at day 14 the change in SBP with OPC 21268 was not statistically different as compared to baseline data while ramipril and ramipril+OPC 21268 did reach significance ($P<0.01$ and $P<0.05$ respectively). The difference in treatment BPs between ramipril alone and ramipril+OPC 21268 did not reach significance (Figure 4.2).

4.5.2 Study 2

4.5.2.1 Metabolic studies

Food and water intake, urinary volume, sodium and osmolality were not significantly altered by any of the treatments. Body weight, likewise, was similar across all groups (Table 4.1).

4.5.2.2 Relative organ weights

As expected ramipril alone, or in combination with OPC 21268, significantly reduced heart and LV mass ($P<0.01$). OPC 21268 alone had no effect on total heart or LV mass. Combination treatment with ramipril and OPC 21268 showed no additional organ weight reduction over and above that seen with ramipril alone. Relative right ventricular (RV) and kidney weights were unaltered by any treatment (Figure 4.3).

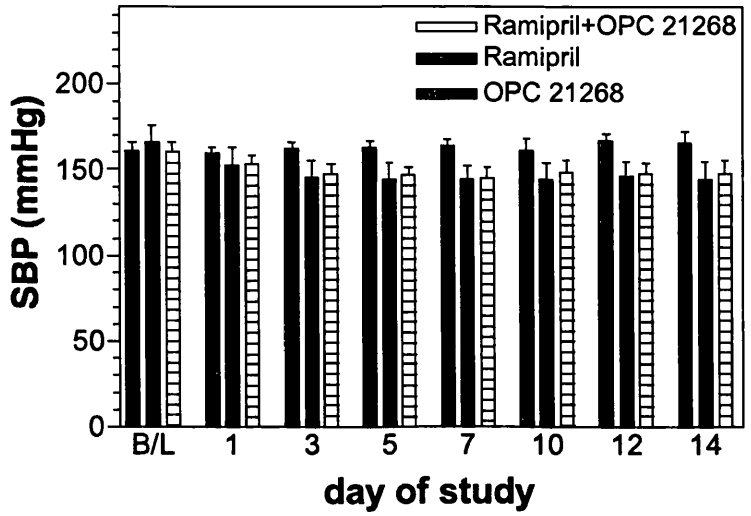
4.5.2.3 Plasma data

Plasma osmolality and AVP were unaltered by any treatment. As expected ramipril therapy resulted in a significant inhibition of plasma ACE activity ($P<0.01$) and a significant increase in PRA ($P<0.01$) (Figure 4.4).

4.5.2.4 *In vitro* ACE autoradiography and liver $V_{1A}R$ binding studies

ACE autoradiography confirmed the inhibition of renal tissue ACE by ramipril (Figure 4.5). Liver $V_{1A}R$ binding studies confirmed the inhibition of the $V_{1A}R$ by OPC 21268 (Figure 4.5).

A.



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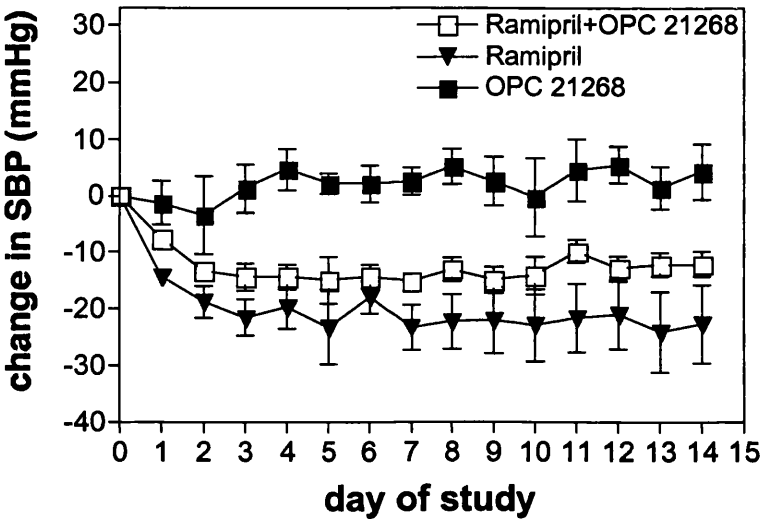


Figure 4.2. SBP telemetry data

A. The average baseline SBP's per group as well as those for days 1, 3, 5, 7, 10, 12 and 14 of drug treatment. Each bar represents the mean±SEM of 4-5 separate determinations. $P<0.01$ for drug treatment ramipril alone (compared to baseline). $P<0.05$ for drug treatment ramipril+OPC 21268 (compared to baseline).

B. Telemetry data expressed as a fall in SBP over baseline for each individual animal where each point represents the mean±SEM of 4-5 separate determinations. $P<0.01$ for drug treatment ramipril alone. $P<0.05$ for drug treatment ramipril+OPC 21268.

Table 4.1. Body weight and results from metabolic studies to assess differences in food, water consumption and renal parameters on days 10 and 11 between the 4 treatment groups shown, n=6/group. No statistical differences were seen.

	Treatment groups			
Parameter	Vehicle	OPC 21268	Ramipril	Ramipril+ OPC 21268
Body weight (g)	353±4	351±10	350±4	340±10
Food consumption (g/100g)	5.8±0.4	5.8±0.2	6.0±0.4	6.2±0.4
Water consumption (ml/100g)	34.0±12.7	35.2±13.9	40.0±14.6	35.9±13.1
Urine volume (ml/100g)	3.1±0.4	3.1±0.2	3.5±0.3	3.8±0.3
Urine sodium (μmol/min/100g)	3.1±1.6	3.5±1.1	2.6±0.9	2.2±0.8
Urine osmolality (mOsm/kg)	2022±316	2175±90	1964±140	2046±137

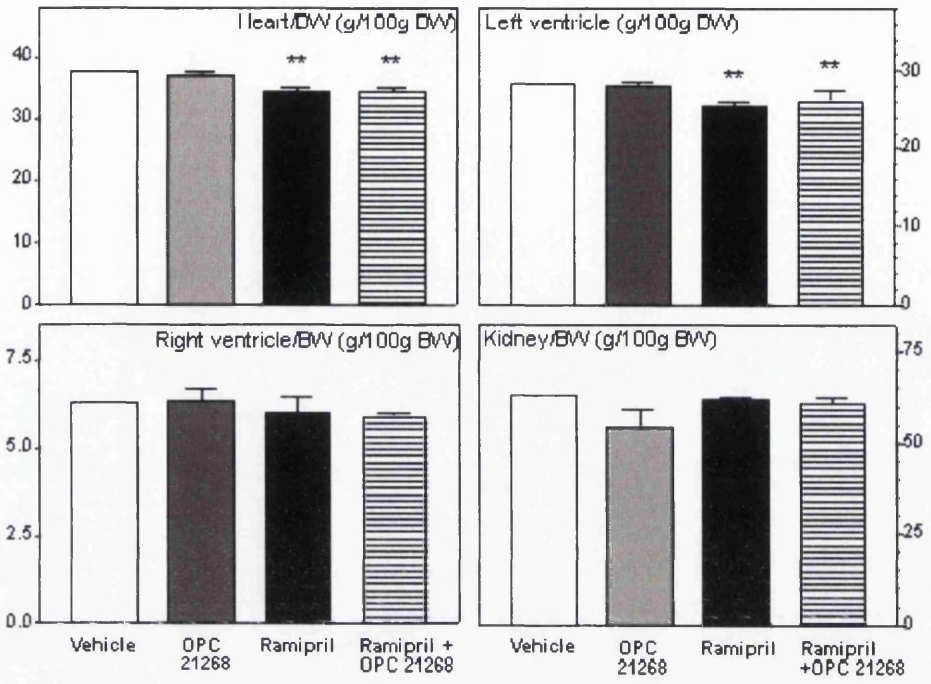


Figure 4.3. Body weight and relative organ weights per treatment group

Body weight and relative organ weight for each treatment group as given above.

Each point represents the mean±SEM of 6 separate determinations. **P<0.01 versus vehicle treated hypertensive controls. BW=body weight.

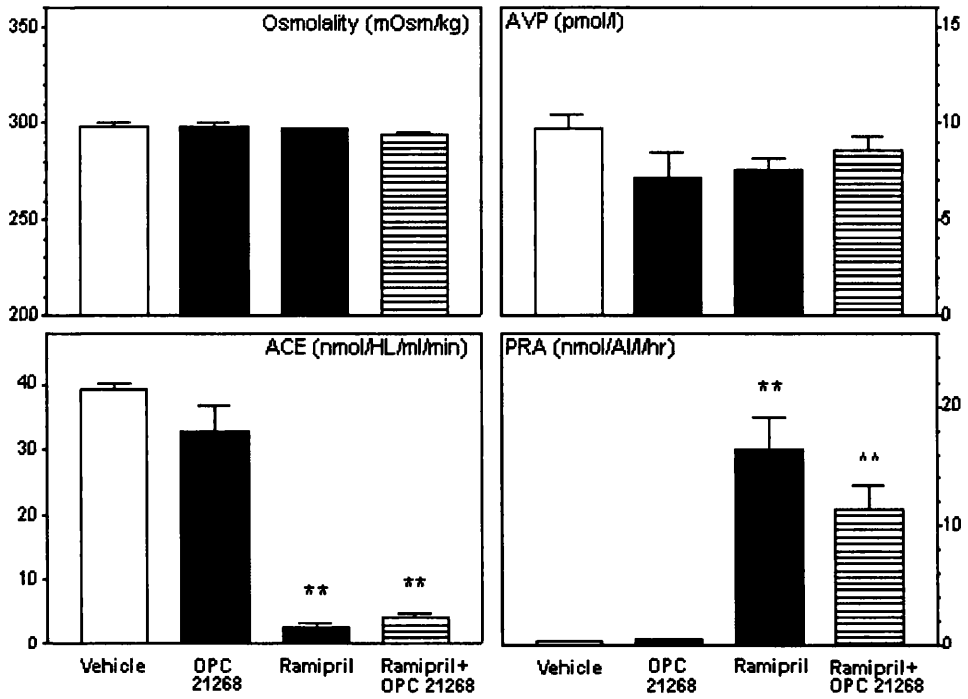


Figure 4.4. Plasma data per treatment group

Plasma data for each treatment group shown above.

Each point represents the mean±SEM of 4 separate determinations performed in duplicate.

**P<0.01 versus vehicle-treated hypertensive controls.

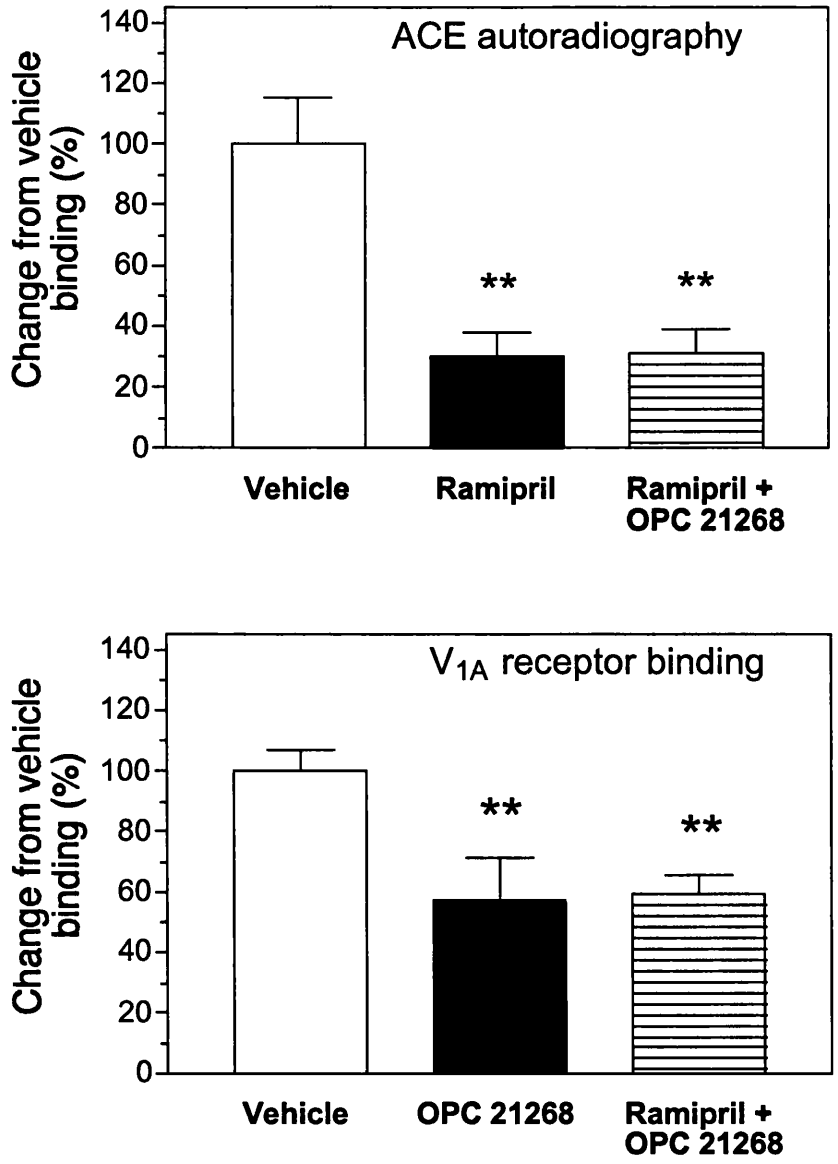


Figure 4.5. Renal ACE *in vitro* autoradiography and liver $V_{1A}R$ binding studies to validate treatment efficacy

Renal ACE *in vitro* autoradiography and liver $V_{1A}R$ binding studies demonstrating tissue ACE inhibition by ramipril and $V_{1A}R$ inhibition by OPC 21268 respectively.

Each point represents the mean±SEM of 5-6 separate determinations performed. *P<0.05 and **P<0.01 versus vehicle treated hypertensive controls.

4.6 DISCUSSION

The role of ang II in hypertension as a potent vasoconstrictor, antinatriuretic and trophic hormone is well characterised (392-394). Much less is certain as regards AVP. This study looked at BP effects of combination therapy with an ACE inhibitor and $V_{1A}R$ antagonist in adult SHR and demonstrated that $V_{1A}R$ antagonism alone did not lower BP. ACE inhibition and combination ACE inhibition with $V_{1A}R$ antagonism did result in BP lowering but there were no additive or synergistic effects with the addition of $V_{1A}R$ blockade. Likewise, heart and LV weights were reduced with ACE inhibition but not $V_{1A}R$ blockade alone and no additional benefits were seen with combination treatment. The lack of effect of OPC 21268 was not due to any failure to block the $V_{1A}R$ since binding studies clearly demonstrate $V_{1A}R$ inhibition. It appears therefore that AVP does not have a major role to play in the maintenance of BP in the adult SHR and has no role to play as add on therapy to ACE inhibition in this particular model of essential hypertension.

As stated in the introduction, our group has previously reported that the $V_{1A}R$ antagonist, OPC 21268, delayed the progression of hypertension in the young SHR in a similar manner to ACE inhibitors (50). No additive effects of $V_{1A}R$ antagonism and ACE inhibition given during this developmental stage were observed. These results suggested that AVP, via $V_{1A}Rs$ and angiotensin II, are involved in the pathogenesis of hypertension and may act via a common mechanism in male SHRs in the developmental stage. While it is clear that AVP antagonism has no role to play over and above ACE inhibition in the developmental phases of hypertension, its role in established hypertension as yet remains unclear. In rat models such as DOCA (124) and DSS hypertension (389, 390), where plasma AVP levels are elevated, then this hormone has been shown to play a role and $V_{1A}R$ blockade is therefore beneficial here. In models of essential hypertension, where

plasma AVP is not classically elevated, there have been reports that AVP receptor blockade lowers BP as well as reports to the contrary (385, 398) while, as stated in the introduction, a 1 week study conducted by our group could demonstrate no BP lowering effect of OPC 21268 alone on male adult SHR (50). To date, nobody has looked at the combined effects of $V_{1A}R$ with ACE antagonism in this animal model.

The mechanisms and sites of interaction of the AVP and RASs are not clearly understood at this time and few data are available concerning the possible interaction between angiotensin II and AVP. Klingbeil *et al i*nfused AVP and angiotensin II alone and in combination to determine whether a synergism could be found between these two peptides in the release of adrenocorticotropic hormone (399). Cowley *et al* infused AVP alone, or in combination with angiotensin II, to determine whether combined hormone administration did enhance each hormone's singular hypertensive action (400). These authors found that the effects of the combination of hormones were not enhanced compared with the response to the individual hormone infusions, thus providing parallels with the results of this present study. Zhang *et al* demonstrated a reduction in AVP levels with captopril treatment (401) while Okuno *et al* have shown that intraventricular and intravenous captopril infusions in SHR did not alter either central or peripheral AVP levels (402). This latter study concurs with our findings where AVP levels were unaltered by any of the treatments. This would indicate that these 2 hormonal systems work independently of each other in terms of BP control. At the receptor level, desensitisation of the $V_{1A}R$ is thought to be fast and accompanied by the sequestration of receptors inside the cell within both tissues and transfected cells (403). Thus the $V_{1A}R$ undergoes both homologous and heterologous desensitisation, the latter being triggered by angiotensin II. This phenomenum of hormone-stimulated sequestration has been demonstrated in vascular

smooth muscle cells that express both receptor types (404, 405). In the context of ACE inhibition where angiotensin II levels are lower, up-regulation of AVP $V_{1A}R$ cell surface numbers and thus a greater response to AVP receptor antagonism might be expected. No additive BP lowering effects of ACE inhibition and $V_{1A}R$ antagonism was demonstrated here and therefore, in the adult SHR, any such up-regulation doesn't seem to play a role. As well as their peripheral actions, angiotensin II and AVP also act centrally, through the circumventricular organs, to influence cardiovascular regulation. The area postrema, a circumventricular organ in the brainstem, mediates at least some of the central actions of these peptides. AVP appears to act in the area postrema to cause sympathoinhibition (406) and a shift in baroreflex control of the sympathetic nervous system to lower pressures. In contrast to AVP ang II has effects in the area postrema to blunt baroreflex control of heart rate and cause sympathoexcitation (406) and so, centrally, both hormones have opposing actions and it may be that in this hypertensive model central sympathoinhibitory influences and peripheral vasoconstriction are in balance with each other.

After 14 days of treatment only the animals receiving ramipril showed any reduction in relative cardiac organ weights. As described in chapter 1, both ACE inhibitors and $V_{1A}R$ antagonists have antimitogenic actions and it may be that a more prolonged period of $V_{1A}R$ antagonism is required before these structural benefits play a more important role and ultimately lower BP.

The above contrasts to the findings of a pilot study in diabetic SHR. This was undertaken in adult diabetic SHR on chronic perindopril treatment made available to us with telemetry probes *in situ.* Here, by introducing another disease into the equation, and one in particular that is associated with reduced PRA and elevated plasma AVP levels, a reduction in BP

with OPC 21268 was shown and this appeared to be additive to that seen with perindopril. In diabetes mellitus (DM) AVP is known to be elevated, along with its $V_{1A}R$, at least in the earlier stages of this disease. The AVP system in DM is "out of balance" therefore and may be contributing towards hypertension, a situation not seen in the non-diabetic SHR. Blockade of this up-regulated $V_{1A}R$ system in DM will therefore result in a lowering of BP. This pilot study and its results will be described in more detail in chapter 5.

The contribution of AVP to the maintenance and development of human hypertension is not well understood at this time and published data on the effect of non-peptide AVP receptor antagonists in human BP control has thus far been limited. Results regarding plasma AVP levels in hypertensive patients are not consistent, with high levels in some studies but normal or low levels in others (269-271). There does, however, appear to be a subset of hypertensive patients, those with associated low plasma renin levels, in whom AVP is consistently elevated. These patients include the elderly, African-Americans, as well as patients with chronic renal failure and DM (27, 29, 272, 273). These studies are discussed in more detail in chapter 1 (section 1.5.1.2).

In conclusion, this study demonstrates that in an adult rat model of hypertension, the combination of ACE inhibitor and $V_{1A}R$ antagonist, for 2 weeks, is not additive or synergistic in terms of BP lowering or heart weight reduction and $V_{1A}R$ antagonism alone had no hypotensive or organ weight effects in this model. It seems unlikely therefore that combination therapy has a role to play in blood pressure lowering and certainly in those members of the population with normal plasma renin levels.

Chapter 5

Regulation of the vasopressin V_{1A} receptor in experimental diabetes mellitus and hypertension

CHAPTER 5

Regulation of the vasopressin V_{1A} receptor in experimental diabetes mellitus and hypertension

Contents

5.1 SUMMARY

1. The aim of the following studies was to look at the effects on vasopressin (AVP) and the AVP V_{1A} receptor ($V_{1A}R$) system of 8 weeks of streptozotocin (STZ-) induced diabetes mellitus (DM) in male spontaneously hypertensive rat (SHR).

2. Male SHR (n=16) were made diabetic at 8 weeks of age, given daily subcutaneous injections of 4 units of long acting protophane insulin, weighed weekly and underwent fortnightly blood glucose measurements (DM-SHR). Male SHR (n=8) were given citrate buffer at 8 weeks of age and served as control animals (SHR). In weeks 4 and 8 systolic blood pressure (SBP) was assessed by tail cuff plethysmography and in week 8 rats were placed in metabolic cages for the measurement of food and water consumption as well as urine output (n=11 DM-SHR; n=8 SHR). The urine was subsequently analysed for urinary albumin excretion by radioimmunoassay. The rats were killed at the end of week 8 and plasma collected for the measurement of Haemoglobin A1c (HBA1c), glucose and AVP. Pieces of liver and kidney were snap frozen for real time PCR and fresh liver and kidney membranes prepared for subsequent kinetic binding studies using the $V_{1A}R$ selective radioligand $^{125}I[d(CH_2)_5$, sarcosine$^7]$-AVP.

3. After 4 and 8 weeks of DM, SBP was reduced in DM-SHR compared to SHR. (4 weeks: 180±4 mmHg versus 193±4 mmHg; $P<0.01$; 8 weeks: 186±4 mmHg versus 213±4 mmHg; $P<0.01$). Urinary albumin excretion rate in week 8 was significantly increased in DM-SHR (1.77x/÷1.1815 mg/24 hours) as compared to SHR (0.67x/÷1.21 mg/24 hours; $P<0.01$) while plasma AVP was significantly elevated in DM-SHR (11.2±0.7pmol/l) versus SHR (9.2±0.4pmol/l); $P<0.05$). mRNA for both liver and kidney

$V_{1A}R$ were unchanged from DM-SHR to SHR while, at the protein level, Scatchard analysis demonstrated up-regulation of both liver and kidney $V_{1A}R$ density (Bmax) in DM-SHR (Liver: 96.1±7.6 fmol/mg versus 66.7±8.2 fmol/mg; $P<0.05$; Kidney: 11.0±1.1 fmol/mg versus 8.04±0.61 fmol/mg; $P<0.05$)). No change in receptor affinity (Kd) was seen.

4. In this study up-regulation of liver and kidney $V_{1A}Rs$ occurs in the setting of stable AVP gene transcription. These results suggest that changes in receptor processing may be responsible for the differential regulation of AVP receptors that occurs in the 8 week diabetic, hypertensive rat. This study is the first to show that in a rat model combining DM and hypertension there is both an increase in plasma AVP and up-regulation of the $V_{1A}R$ in liver and kidney. As AVP is thought to contribute to the renal damage seen in diabetic nephropathy then this study provides a rationale for the use of $V_{1A}R$ antagonists in this disease.

5.2 INTRODUCTION

DM causes hypertonicity, polyuria and polydipsia and, long term, is almost always associated with complications of the cardiovascular system such as vascular disease, renal disease and hypertension (289, 407-409). AVP is central to control of fluid balance and blood pressure (BP) (385). Plasma AVP levels are elevated in a number of disease states that include DM (23, 51, 125) and hypertension (120). Increased AVP may have adverse actions by increasing peripheral resistance via constrictor actions at the $V_{1A}R$ and contributing to fluid retention and hyponatraemia through effects on the renal V_2R. In addition, AVP can act as a mitogen and in a manner analogous to angiotensin II may have deleterious effects when present long-term and in excess (30, 31). Hyperglycaemia itself initially promotes mesangial cell proliferation and extracellular matrix production but through time will inhibit them (410). Diabetic nephropathy has a high associated morbidity and mortality. It is characterised by a number of pathological changes not least of which are that of excessive extracellular matrix production, cell proliferation and contraction (286). Previous studies have looked at the regulation of AVP and its receptors in DM but these have tended to be short-term or inconclusive. For instance previous experiments revealed that in normotensive, Sprague Dawley (SD) rats with 2 weeks of STZ-DM, elevated plasma AVP levels were associated with a reduction of renal and hepatic $V_{1A}Rs$. In addition to producing a reduction in second messenger activation, down-regulation of the $V_{1A}R$ was shown to contribute to diminished $V_{1A}R$-mediated biological responses to AVP (51) and was paralleled by a reduction in receptor mRNA levels (125). The pathophysiological significance of reduced renal and hepatic $V_{1A}Rs$ in normotensive DM rats with little or no renal pathology is at this stage unclear. A recent study in the 10 week genetically diabetic (db/db) mouse, a model for type II DM, demonstrated significant down-regulation of hepatic $V_{1A}R$ mRNA and protein in the liver

of the db/db mouse (322). Renal $V_{1A}R$ regulation was however not assessed in this study. Thus with such limited information currently available on the regulation of the $V_{1A}R$ system in STZ-DM it is important to clarify the role of AVP and its receptors in DM, and in particular the $V_{1A}R$, prior to performing intervention studies with the now available non-peptide AVP receptor antagonists.

The general aim of this study was to assess the regulation of AVP and its $V_{1A}R$ both at a mRNA and protein level in a rat model combining hypertensive with long-term STZ-DM.

5.3 EXPERIMENTAL PROTOCOL

5.3.1 Ethics

This experiment was approved by the Austin and Repatriation Medical Centre Ethics Committee and performed according to the National Health and Medical Research Council of Australia guidelines for animal experimentation.

5.3.2 Drugs

OPC 21268 was a generous gift from Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). Perindopril was purchased from Aventis Pharmaceuticals (New Jersey, USA). OPC 21268 was suspended in 0.5% methylcellulose (BDH Pharmaceuticals, Poole, UK) while perindopril was dissolved in water. Both of these drugs were administered orally by gavage.

5.3.3 Study 1

5.3.3.1 AVP $V_{1A}R$ system regulation in the SHR after 8 weeks STZ-DM

To induce DM, male SHR (age 8 weeks) received tail vein injections of STZ (45mg/kg). Control rats received an equivalent volume of citrate buffer as described in chapter 2 (section 2.2.2). Rats were weighed weekly and diabetes monitored by fortnightly blood glucose determination. Any animal with a blood sugar of less than 17mmol/l at any stage was excluded from the study. The diabetic rats received 4 Units of long acting protophane insulin subcutaneously daily via a Novo Pen 3 (Novo Nordisk Pharmaceuticals Pty. Ltd). During the fourth and eighth week of DM SBP was determined using tail cuff plethysmography and during the eighth week of DM rats were housed in metabolic cages

for the measurement of food and water intake and renal parameters. After 8 weeks rats were killed (n=8 SHR and n=11 DM-SHR) and trunk blood collected for the measurement of plasma AVP, angiotensin converting enzyme (ACE), plasma renin activity (PRA), sodium, osmolality and HBA1c. Livers and kidneys were removed and fresh membranes prepared for receptor binding studies as described in sections 2.6.3, 2.6.4 and 2.6.5. Small cross sections of kidney and pieces of liver were removed and snap frozen in dry-ice chilled isopentane for real time PCR as described in section 2.7.1.

5.3.4 Study 2

5.3.4.1 AVP $V_{1A}R$ regulation in the SHR after 8 months STZ-DM

A separate study was undertaken by a collaborating group in which SHR were made diabetic for 8 months. Fresh livers and snap frozen kidneys were made available to allow us a preliminary look at AVP and $V_{1A}R$ regulation at this 8 month time point. From liver, fresh membranes were prepared for Scatchard analyses and the frozen kidneys used for *in vitro* $V_{1A}R$ autoradiography as described in chapter 2 (sections 2.6.2 and 2.6.5.3).

5.3.5 Study 3

5.3.5.1 SBP in DM-SHR with ACE inhibition and $V_{1A}R$ antagonism

A short pilot study was also undertaken in male DM-SHR made available from another study. These animals had telemetric probes *in situ* and were approximately 24 weeks of age. All had been diabetic since age 8 weeks (n=6). Half of the animals had been receiving perindopril therapy for the preceding 10 week period and that was on going (n=3), while the others had received no prior drug treatment (n=3). Baseline BPs were

monitored for 4 days, OPC 21268 (30mg/kg bd) given for 3 consecutive days followed by a 10 day "drug wash out period" during which time perindopril therapy continued. 24 hour SBP was monitored continuously throughout.

5.4 STATISTICAL ANALYSIS

Data were analysed by analysis of variance (ANOVA) with or without repeated measures using the Statview SE=Graphics programme (Brainpower, Calabasas,CA). Comparisons of group means were performed by Fisher's least significant difference method. Albuminuria data was logarithmically transformed before statistical analysis. Data are shown as means±SEM unless otherwise specified. $P<0.05$ was viewed as statistically significant.

5.5 RESULTS

5.5.1 Study 1

5.5.1.1 Characterisation of the diabetic model

Body weight

Of the 16 SHR initially injected with STZ, 11 were available by week 8; 2 rats having been excluded when their random blood sugars were persistently below 17mmol/l and the other 3 having died at various time points throughout.

All animals gained weight over the 8 week study period ($P<0.01$) but compared to control SHR, the DM-SHR gained significantly less weight ($P<0.01$)(Figure 5.1).

Blood pressure

Mean SBP was significantly higher in SHR compared to DM-SHR at both 4 and 8 weeks (4 weeks: 193±4 mmHg versus 180±4 mmHg; P<0.01; 8 weeks: 213±4 mmHg versus 186±4 mmHg; P<0.01) (Figure 5.2).

Metabolic parameters

Diabetic rats ate more, drank significantly more water (P<0.01) and passed significantly more urine (P<0.01) compared to control SHR. Urinary albumin excretion was likewise elevated when compared to control rats (P<0.01)(Figure 5.3). Urinary osmolality was significantly lower (P<0.01) whilst urinary sodium significantly higher in the diabetic animals (P<0.01)) (Figure 5.3).

Biochemical parameters

HBA1c and plasma glucose were significantly elevated in the diabetic animals compared to their controls (P<0.01) (Figure 5.4). Plasma osmolality was significantly elevated (P<0.01) while plasma sodium significantly reduced (P<0.01) in DM-SHR compared to SHR (Figure 5.4).

Hormonal parameters

Plasma AVP (P<0.05) and ACE (P<0.01) were significantly elevated in DM-SHR as compared to SHR. No statistical differences were found in PRA (Figure 5.5).

Organ weights

There was no statistical difference in relative heart weight between diabetic and control SHR after 8 weeks (Figure 5.6).

Liver and kidney weights were both significantly increased in the diabetic group. ($P<0.01$) (Figure 5.6). Mesenteric weights were no different (Figure 5.6).

5.5.1.2 Hepatic and renal $V_{1A}R$ mRNA

No alteration in the quantity of hepatic or renal $V_{1A}R$ mRNA was found between DM-SHR and SHR (Figure 5.7).

5.5.1.3 Hepatic and renal $V_{1A}R$ regulation

Using Scatchard analysis, renal $V_{1A}R$ number (Bmax) was significantly increased by approximately 20% ($P<0.05$) in DM while receptor affinity (Kd) was unchanged. Likewise hepatic $V_{1A}R$ number was found to be higher by approximately 50% in the DM-SHR ($P<0.05$) but the affinity for AVP did not alter (Figure 5.8).

5.5.2 Study 2

After 8 months of DM plasma AVP was not significantly elevated in DM-SHR as compared to control SHR at this time point (DM-SHR; 8.29±0.4pmol/l, n=18 versus SHR; 7.20±0.4pmol/l, n=11). Scatchard analysis performed on liver membranes could demonstrate no differences in Bmax or Kd between the 2 groups (Figure 5.9). *In vitro* autoradiography on kidney, although able to give less information than Scatchard analysis, did reveal a down-regulation in receptor density after 8 months of combined DM and hypertension although this did not reach significance (Figure 5.9).

5.5.3 Study 3

SBP is expressed as mean 24 hour BP in figure 5.10. In DM-SHR treated with OPC 21268 alone, a 2-4% fall in SBP over the treatment period was seen. This lowering of BP appeared to persist and if anything was slightly increased for a number of days after cessation of therapy then the mean 24 hour SBP gradually climbed back to baseline levels by day 14 (n=3). In the DM-SHR, treated with chronic ACE inhibition, the addition of $V_{1A}R$ blockade for 3 days gave an approximate 6% fall in mean BP and this also slowly returned to baseline levels over the next 10 days (n=3).

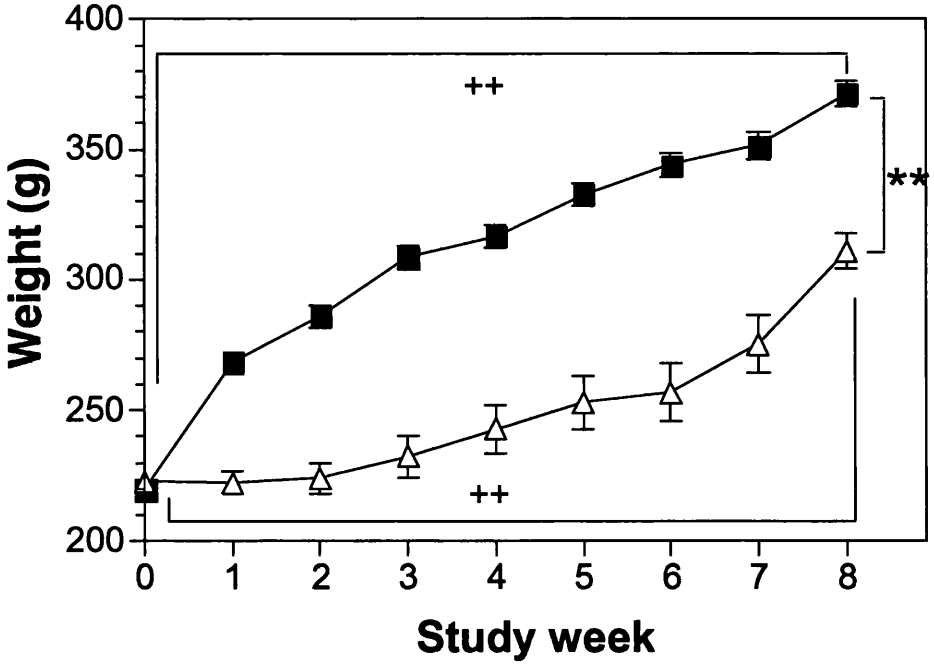


Figure 5.1. Weekly animal weight versus time in SHR(■) versus DM-SHR(△).

Each bar represents the mean±SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. **P<0.01 (between group comparison in week 8). ++P<0.01 (intragroup comparison; week 8 as compared to baseline).

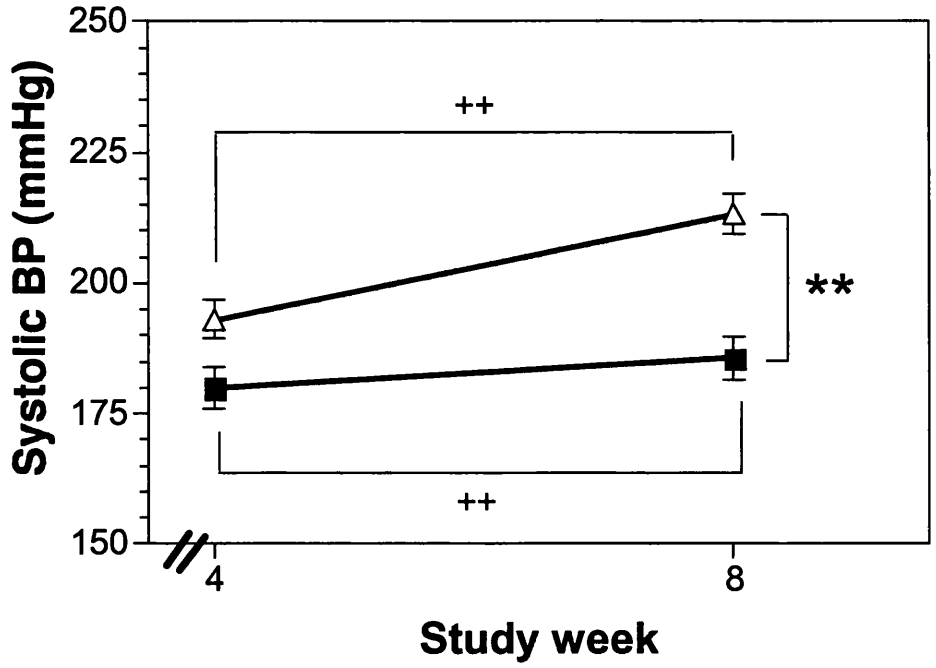


Figure 5.2. SBPs as measured by tail cuff plethysmography in SHR(△) and DM-SHR( ) in study weeks 4 and 8.

Each point represents the mean±SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. **P<0.01 (between group comparison in week 8). ++P<0.01 (intragroup comparison; week 8 as compared to baseline).

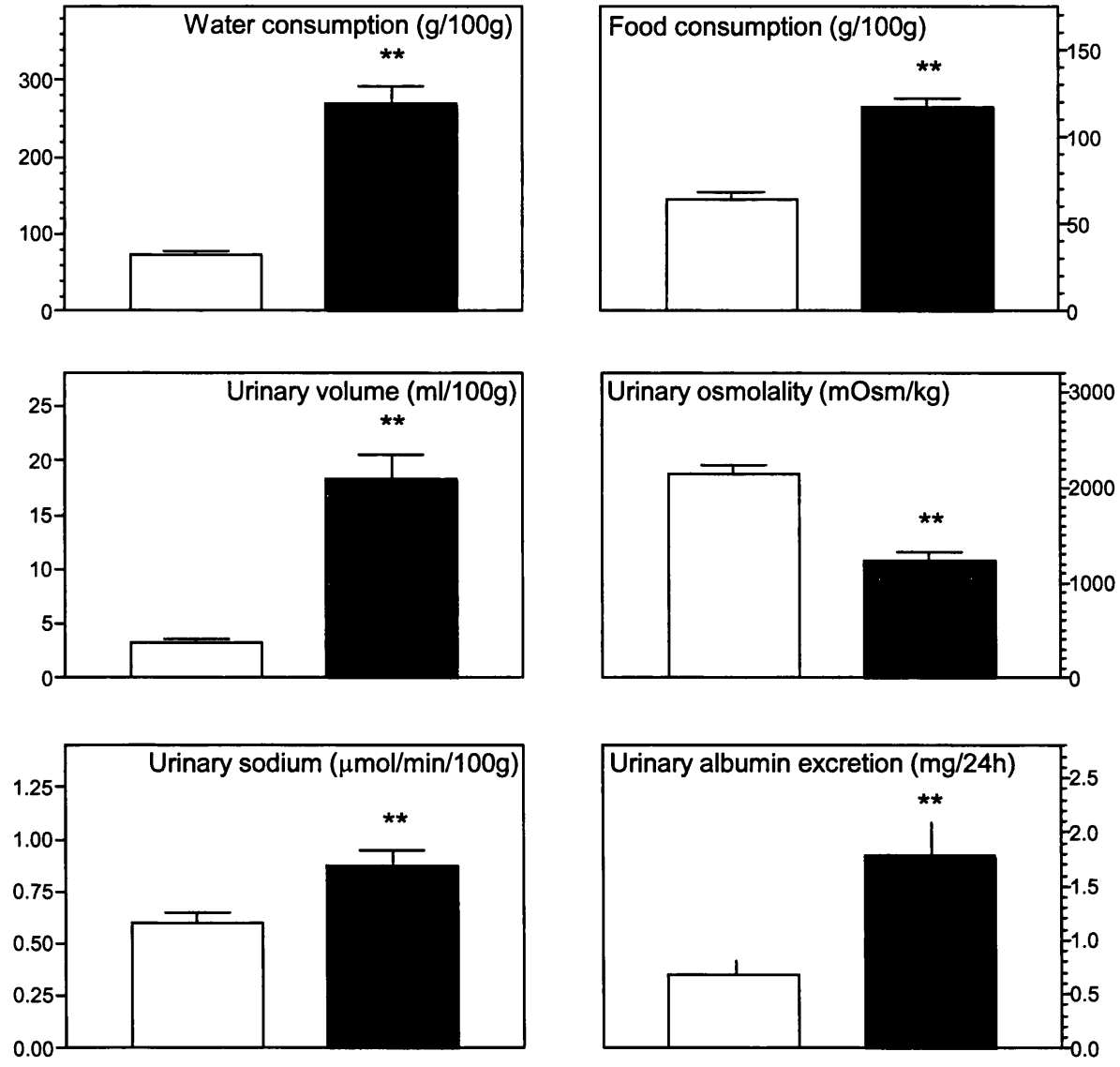


Figure 5.3. Parameters derived from metabolic caging of SHR (open bars) and DM-SHR (closed bars) in week 8 of DM.

For urinary albumin excretion each bar represents the mean x/÷SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. All other bars represent the mean±SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. **P<0.01.

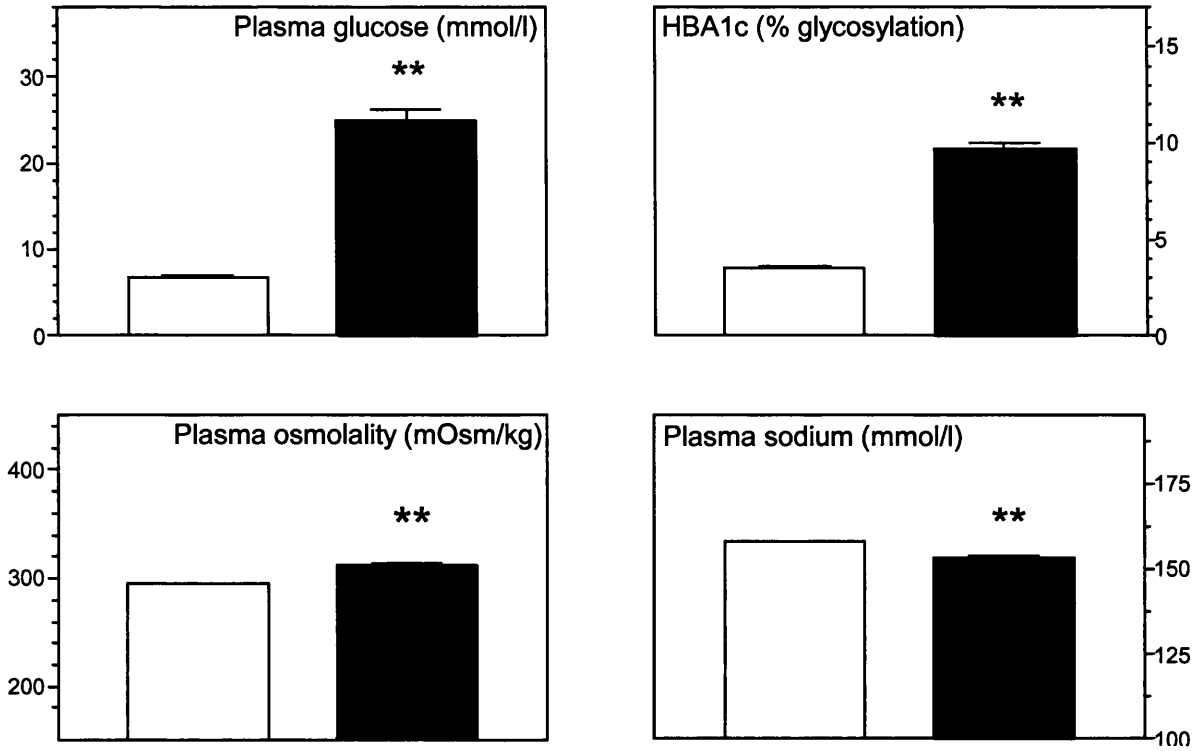


Figure 5.4. HBA1c, plasma glucose, osmolality and sodium in SHR (open bars) versus DM-SHR (closed bars) after 8 weeks of STZ-DM.

Each bar represents the mean±SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. **P<0.01.

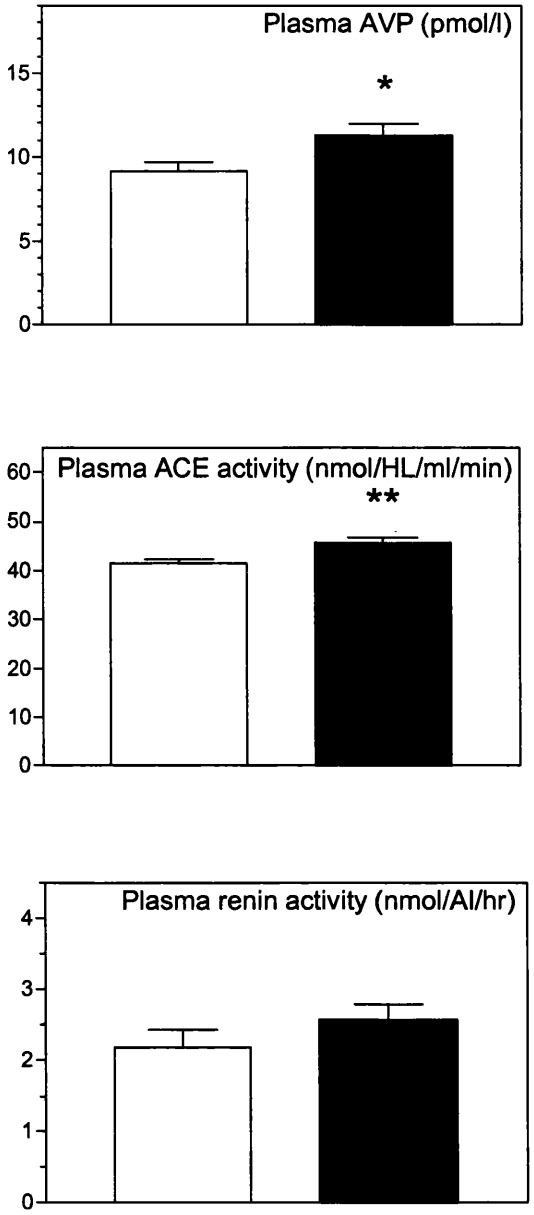


Figure 5.5. Hormonal parameters in SHR (open bars) versus DM-SHR (closed bars) after 8 weeks of STZ-DM.

Each bar represents the mean±SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. **P<0.01.

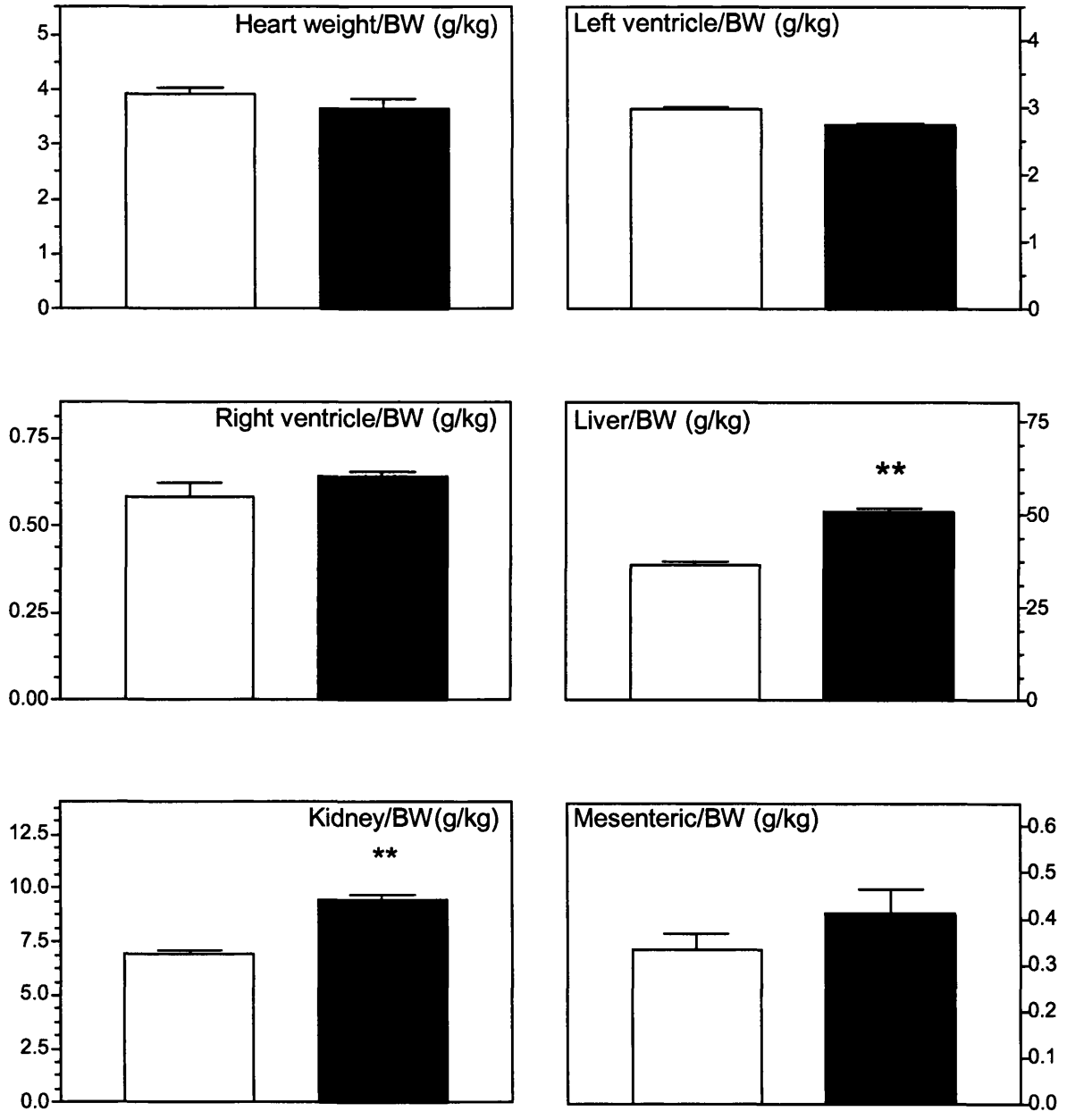


Figure 5.6. Relative organ weights in SHR versus DM-SHR after 8 weeks of STZ-DM.

Each bar represents the mean±SEM of 8 separate determinations for SHR and 11 separate determinations for DM-SHR. **P<0.01.

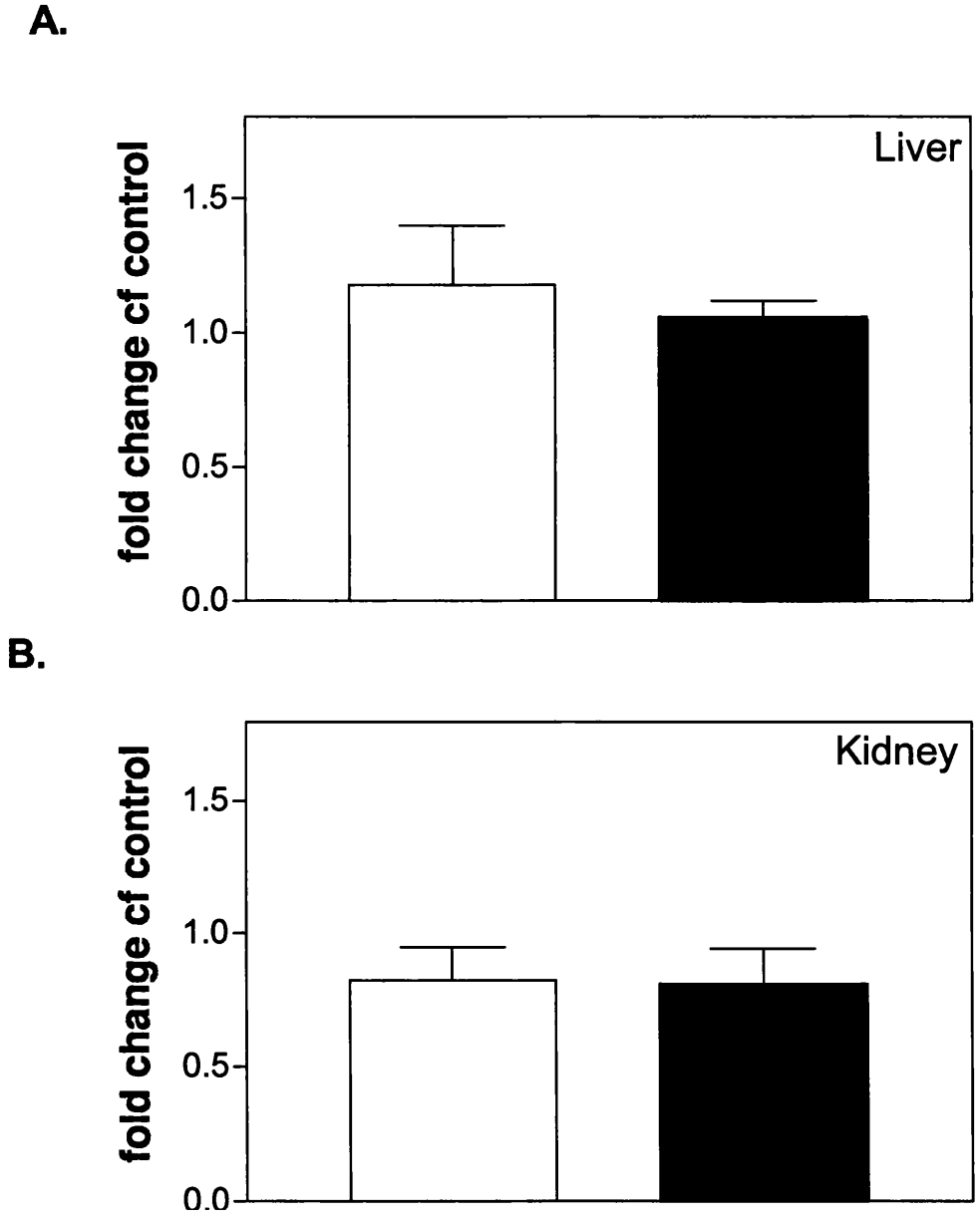
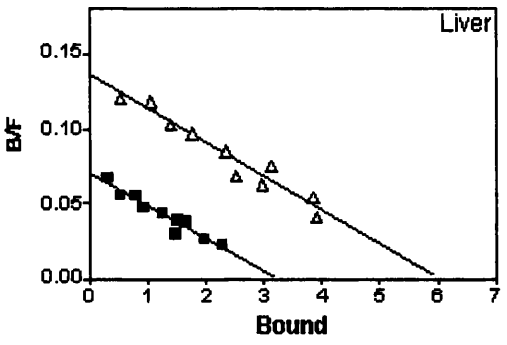


Figure 5.7. Regulation of $V_{1A}R$ mRNA

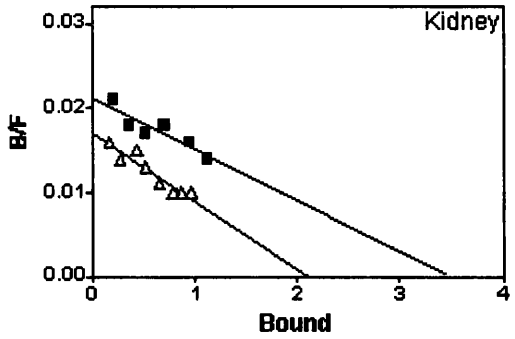
Regulation of $V_{1A}R$ mRNA in A. Liver and B. Kidney in SHR (open bars) versus DM-SHR (closed bars). Each bar represents the mean±SEM of 5 separate determinations for both SHR and DM-SHR.

GROUP		SHR (n=6-7)	DIABETIC SHR (n=7-9)	P VALUE
Liver V_{1A}	Bmax (fmol/mg)	66.7±8.23	96.1±7.60 *	0.024
	Kd (nmol/l)	0.729±0.10	0.712±0.12	0.917
Kidney V_{1A}	Bmax (fmol/mg)	8.04±0.57	11.0±1.060 *	0.038
	Kd (nmol/l)	0.888±0.108	0.971±0.093	0.573

A.



B.



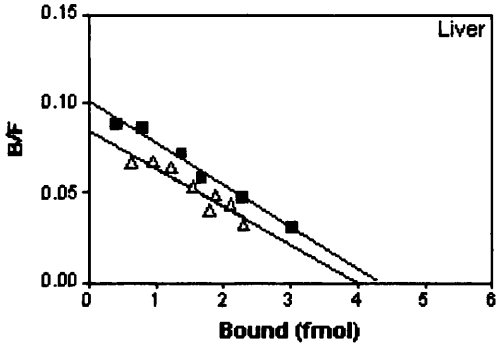
C.

Figure 5.8. Hepatic and renal Bmax and K_D in SHR and DM-SHR at the $V_{1A}R$ after 8 weeks of STZ-DM

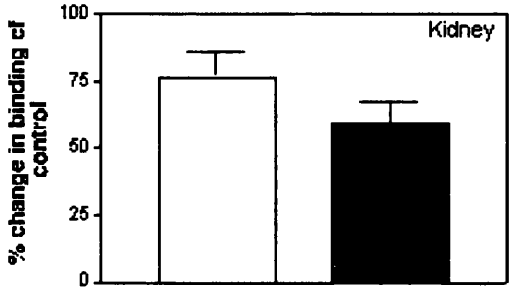
A. Summary table showing hepatic and renal Bmax and Kd in SHR and DM-SHR for the $V_{1A}R$ after 8 weeks STZ-DM. **B.** Representative hepatic $V_{1A}R$ Scatchard analyses from both SHR (△) and DM-SHR ( ). **C.** Representative renal $V_{1A}R$ Scatchard analysis from both SHR (△) and DM-SHR ( ).

GROUP		SHR (n=8)	DIABETIC SHR (n=8)	P VALUE
LiverV_{1A}	Bmax (fmol/mg)	66.2±6.7	69.0±10.7	0.083
	Kd (nmol/l)	0.641±0.08	0.712±0.12	0.34

A.



B.



C.

Figure 5.9. Hepatic Bmax and K_D in SHR and DM-SHR at the $V_{1A}R$ after 8 months of STZ-DM

A. Summary table showing liver Bmax and Kd in SHR (△) and DM-SHR( ) for the $V_{1A}R$ after 8 months STZ-DM. **B.** Representative liver $V_{1A}R$ Scatchard analysis from both SHR (△) and DM-SHR ( ). **C.** $V_{1A}R$ *in vitro* autoradiography in kidney sections from SHR (open bars) and DM-SHR (closed bars). Results are expressed as mean±SEM of 6-8 separate determinations and expressed as % change in binding as compared to SHR.

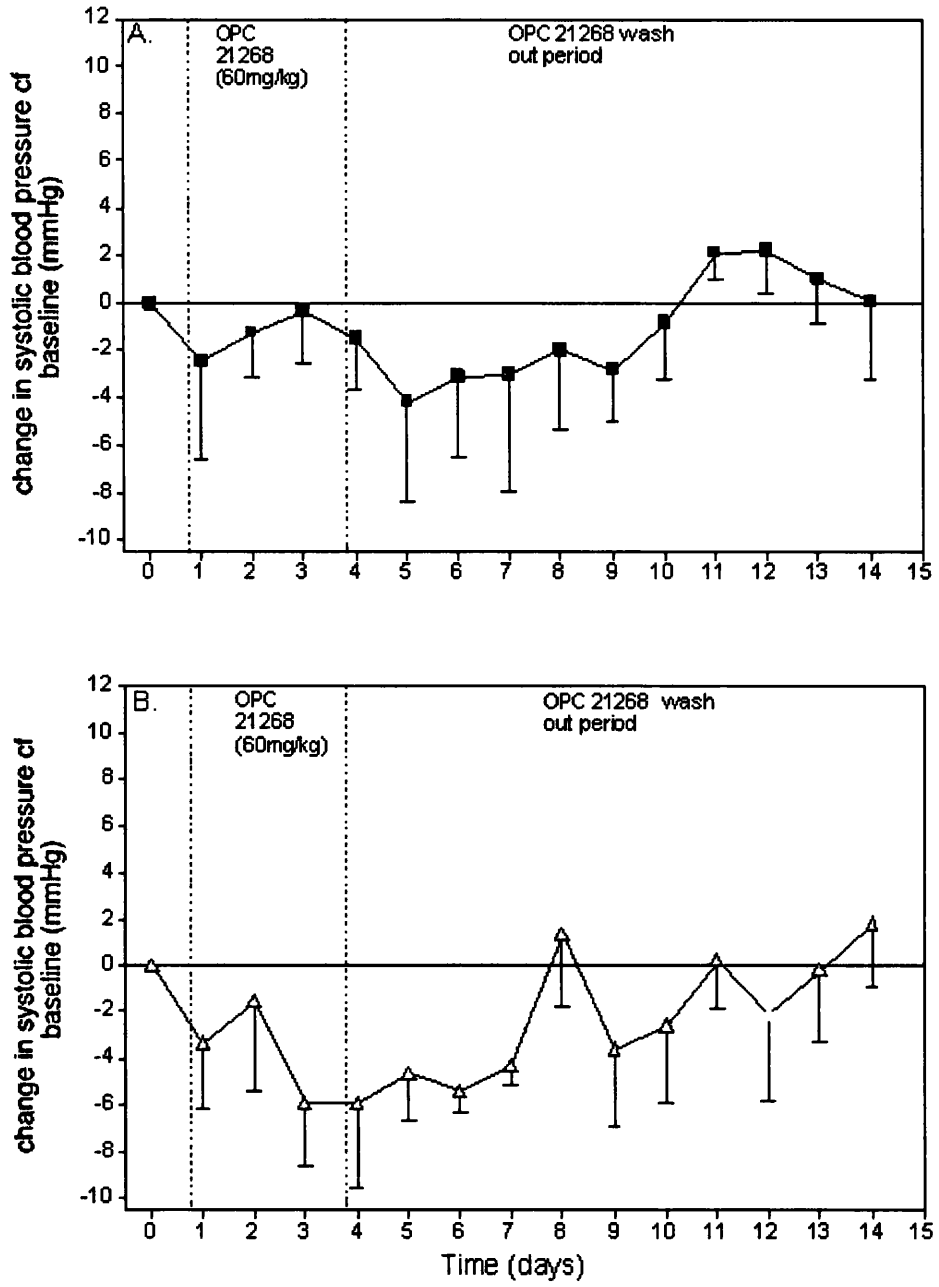


Figure 5.10. Representation of BP changes in untreated DM-SHR given OPC 21268 versus DM-SHR given OPC 21268 and perindopril

Change in average 24 hour SBPs relative to a 4 day pre-treatment baseline in A. Untreated DM-SHR given OPC 21268 for 3 days ( ) and B. DM-SHR given OPC 21268 for 3 days on a background of ongoing chronic perindopril therapy (Δ). Each point represents the mean±SEM of 3 separate determinations.

5.6 DISCUSSION

The regulation of AVP and its receptors in DM has thus far been little researched. This 8 week diabetic study confirms previous findings of elevated plasma AVP concentrations in insulin-deficient DM and interestingly it demonstrates for the first time up-regulation of hepatic and renal $V_{1A}R$ numbers in a long-term and hypertensive model of this disease. The results of this study also show that changes in AVP receptor numbers were not accompanied by concomitant changes in liver or kidney $V_{1A}R$ mRNA.

Characterisation of this 8 week, STZ-induced model of DM was largely as expected when compared to that found previously by other groups. Weight gain is significantly less in the diabetic group as compared to control while HBA1c and plasma glucose are elevated to predictable values. Plasmas AVP and ACE are higher in DM-SHR as compared to control, while PRA remains unaltered. It is well known that AVP, in a bid to combat excessive dehydration, is raised in the diabetic state. Likewise, it has been shown that serum ACE is higher in the diabetic compared to control animal (411) and that this tends to be inversely proportional to the degree of diabetic control (412). PRA levels in DM appear to be more variable. A previous study within our group demonstrated no change in plasma PRA (413) while others have found this hormone to be reduced (411). In this study, PRA levels tended to be higher in the DM-SHR as compared to SHR although this was not statistically significant. This may reflect a slightly higher degree of dehydration within these diabetic animals as metabolic studies revealed the DM-SHR to be sodium excretors as compared to the more common finding of urinary sodium retention (375). Plasma osmolality is significantly higher in the DM-SHR as compared to SHR. The lower plasma sodium in the diabetics reflects this high osmolality as the concentrations of plasma solutes such as

glucose and triglycerides seen in this disease give rise to a state of pseudohyponatraemia. All other aspects of the metabolic study were as would be expected in that the DM-SHR drank more, ate more and passed more urine of a lower osmolality than their non-diabetic counterparts. In terms of SBP, the development of hypertension was not as great in DM-SHR compared with controls. This finding agrees with that of previous studies (414). In terms of organ weights, these were adjusted for body weight in order to account for diabetic growth retardation. With this taken into account cardiac weights were not significantly different between the 2 groups. In view of the finding that SBP in the DM-SHR was significantly lower than in controls it may be that after adjustment for this variable then DM per se may cause cardiac hypertrophy, but this was not undertaken here. Indeed, Black *et al* looked at the hearts from diabetic SHR, 12 weeks post induction of STZ-DM, and found significant increases in cardiac sizes at this time point despite there being lower BPs in this group (414). As expected kidney and liver sizes were significantly greater in the DM-SHR compared to SHR secondary to hyperfiltration and fatty changes respectively.

It is well known that for the vast majority of biological systems an elevation in circulating hormone levels results in the down-regulation of that particular hormones biological response and that this is usually through a reduction in its receptor number. Certainly, in the healthy SD rat, it has been shown that there is a reduced response to elevated AVP at the $V_{1A}R$ and that this $V_{1A}R$ desensitisation is fast and accompanied by sequestration of receptors inside the cell (403). Previous studies have demonstrated, however, that in disease states an elevation in circulating AVP concentrations have variable and unpredictable effects on receptor regulation. In dehydration an elevated AVP is associated with down-regulation of renal V_2Rs. (415). In DOCA-salt hypertension, an animal model

of mineralocorticoid hypertension, increased circulating AVP levels are associated with down-regulation of liver V_{1A} and kidney V_2Rs and up-regulation of kidney V_{1A}Rs with interestingly no changes in mRNA levels seen. (120) In a 2 week normotensive model of DM an elevated plasma AVP was associated with down-regulation of both hepatic and renal V_{1A}Rs with no alteration to the renal V_2R (51). Many studies, however, looking at AVP receptor regulation, have used the AVP receptor antagonist ^{3}H[AVP] that binds non-selectively to both the V_{1A} and V_2Rs. Caution should thus be exercised when interpreting these studies. This problem highlights the necessity for studying individual receptor subtypes with selective and specific radioligands and for studying these receptors in specific animal models and individual tissues. Our group has available to us the selective V_{1A}R antagonist, radiolabelled ^{125}I[d$(CH_2)_5$, sarcosine^7]-AVP, and it was used throughout this study.

AVP receptors may show differential regulation in the more severe hypertensive 8 week diabetic model as compared to the previously studied 2 week normotensive model for a number of reasons. Initially down-regulation of receptors may simply be a regulatory negative feedback type response to high circulating AVP levels. However, as the disease progresses, the kidney sustains some damage, as evidenced by the development of microalbuminuria, and these regulatory responses are therefore likely to alter. Structural changes to the kidney may result in the cell surface V_{1A}R ceasing to function as efficiently and consequently more receptor numbers being placed on the cell surface in a bid to compensate for this. The actual receptor binding domains remain unaltered and therefore Kd is unchanged whilst Bmax is increased.

The increase in $V_{1A}R$ number could also serve a physiological purpose and thus be a "normal" regulatory response in trying to limit the excessive diuresis associated with DM. The antidiuretic action of AVP mainly depends on V_2R-mediated effects in the renal collecting duct. Other actions mediated by the renal $V_{1A}R$ may also contribute either to reinforce or to blunt this antidiuretic action (160). A high level of AVP is sufficient to induce general $V_{1A}R$-mediated vasoconstriction and could thus limit any excessive diuresis associated with DM, helping prevent dehydration (416). At lower AVP levels, selective vasoconstriction of central vasa recta causes a reduction in intramedullary blood flow, and thus also has a water sparing effect (135-137). On the other hand, studies have shown that $V_{1A}R$ stimulation in collecting duct activates prostaglandin synthesis and that these in turn reduce the V_2R-dependent stimulation of adenylate cyclase reducing the intensity of V_2R-mediated cellular effects (417). Physiologically, the activation of these collecting duct $V_{1A}Rs$ may not play a role until higher plasma AVP levels and thus an antidiureses that is too intense may be avoided.

The up-regulation of the hepatic $V_{1A}R$ seen in this study may also have advantages to the diabetic patient. AVP stimulates hepatic urea synthesis and this effect is mediated through the liver $V_{1A}R$ (418). Such an up-regulation could improve urinary concentrating capacity by helping to provide more urea to the kidney. *In vitro* studies of perfused rat liver or isolated hepatocytes have however shown that AVP stimulates glycogenolysis and gluconeogenesis and with this up-regulation of hepatic $V_{1A}Rs$ the metabolic effects of DM are therefore potentially accelerated (418).

Several explanations exist for the dissociation between changes in receptor numbers and mRNA expression in the 8 week, DM-SHR although all remain speculative. Firstly

alterations in post-translational mechanisms such as internalisation and recycling may occur. Receptor-mediated endocytosis, by removing receptors from the cell surface, plays a role in desensitising target cells to subsequent exposure to hormone (419). Cell culture studies using Chinese hamster ovary cells and A-9 lung fibroblast cells transfected with the $V_{1A}R$ (420) or A-10 vascular smooth muscle cells expressing $V_{1A}Rs$ (421), have shown that the AVP receptor-ligand complex is internalised into cells by receptor-mediated endocytosis. This process can be inhibited by AVP and also by hypertonic sucrose (420, 421).

Alternatively, post-translational processing of AVP receptors may be responsible for the disparity between receptor numbers and mRNA expression in the hypertensive, diabetic model. Certainly glycosylation appears to be important for receptor trafficking of the V_2R in LLC-PK-1 cells where inhibition of specific intracellular carbohydrate processing enzymes inhibits V_2R biosynthesis and internalisation (422). To date the role of post-translational processing in the function or expression of the $V_{1A}R$ has not been demonstrated.

After 8 months of combined DM and hypertension the data available from study 2 demonstrates an unaltered number and affinity of liver $V_{1A}Rs$ while those in the kidney were significantly down-regulated. These differences in hepatic and renal gene expression over time most likely relate to the duration of the diabetic process although one cannot exclude that other factors, such as the severity of DM or low-dose insulin treatment, may also influence $V_{1A}R$ number and affinity. As described above, physiologically water conservation should take precedence early in the diabetic process. With more prolonged disease however, structural damage such as basement membrane thickening, glomerular

hypertrophy and mesangial expansion (286) becomes increasingly severe and it makes sense that preservation of renal function and anatomy would become of higher priority. By analogy with angiotensin II and from the limited studies available using the non-peptide AVP receptor antagonists it can be inferred that AVP is likely to be deleterious when present long-term and in excess. For instance, a recent study in Brattleboro and control rats with STZ-DM demonstrated that urinary albumin excretion more than doubled in control animals but rose by only 34% in the Brattleboro-DM group (312). IV AVP has been shown to result in glomerular cell expansion via the over-expression of transforming growth factor beta-1 (TGF-β1) and that this proliferative response is much enhanced in SHR as compared with normotensive controls (423). If an elevated plasma AVP results in the increased expression of cytokines then again by analogy to other systems such as the vascular endothelial growth factor (VEGF) and its receptor VEGF receptor 2, it is possible that these cytokines serve to down-regulate the expression of the renal $V_{1A}R$ (424). As TGF-β expression has been reported to increase in the kidney with duration of DM this would represent a likely candidate (425, 426). Indeed, a negative feedback role for increase TGF-β on the AVP system has been demonstrated in a couple of studies in rat. TGF-β has been show to block the acetyl choline stimulated release of AVP from the hypothalamus and amygdala where a variety of other cytokines failed to do so (427). In a separate study a 4 fold increase in mesangial cell TGF-β was seen with AVP stimulation and this increased concentration of growth factor in turn inhibited the cellular signalling of AVP at a stage prior to phospholipase C activation. Whether this inhibition was effected at the $V_{1A}R$ level or at a more distal point in the signalling pathway was however not clear as, although receptor numbers appeared unchanged, their number was determined using the non selective V_1R agonist [^{3}H]AVP (428).

In long term DM, as structural damage in the diabetic liver is limited primarily to fatty change, it makes sense that no significant differences in the $V_{1A}R$ over control SHR was seen in this organ.

The preliminary data presented from study 3 would suggest that, as compared to the findings presented in chapter 4 in non-diabetic SHR, dual ACE and $V_{1A}R$ antagonism does have a role to play in lowering BP in the diabetic hypertensive animal. This would make sense in light of the findings in this chapter of an up-regulated $V_{1A}R$ system in DM.

In conclusion, in a rat model that combines DM and hypertension, increased levels of plasma AVP are associated with up-regulation of the hepatic and renal $V_{1A}Rs$ with no significant change in affinity of the hepatic and renal $V_{1A}Rs$ for AVP. This study is the first to show this and suggests a rationale for the use of $V_{1A}R$ blockade in DM.

Chapter 6

General discussion and future directions

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Contents

In this concluding chapter the major findings of this thesis will be drawn together and the broader applications of these, in terms of the place of vasopressin (AVP) receptor blockade and further study of the AVP system in cardiovascular disease (CVD), discussed.

6.1 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The characterisation of an orally effective dual V_{1A} and V_2 receptor (V_{1A} and V_2R) antagonist, drug A, was described in chapter 3. This drug had undergone little previous research and was demonstrated here to interact reversibly and competitively at the hepatic $V_{1A}R$ and renal V_{1A} and V_2R sites in Sprague Dawley rat. Drug A remained metabolically active after 5 days of continual dosing but its aquaretic response was diminished.

To assess the potential clinical usefulness of this drug a longer-term metabolic study should be undertaken to look into the possibility of continuing drug tachyphylaxis and ultimately lack of any clinical effect of drug A. Currently one of the most promising clinical indications for the prescription of a mixed AVP receptor antagonist is in the treatment of heart failure where elevated plasma AVP contributes to increased vascular resistance through its $V_{1A}R$ and to fluid retention via its effects at the V_2R. As described previously, YM087 or conivaptan is a mixed non-peptide V_{1A}/V_2R antagonist that has shown great promise thus far as a treatment for congestive cardiac failure in animal models and humans (265, 266). Our group has the experimental expertise to induce myocardial infarction in rats (41, 429) and a 4 week study to look at the effect of oral administration of drug A on cardiovascular end points within this model will shortly be undertaken. It must be remembered that in order to put into perspective any findings from the study of AVP receptor antagonists, there is considerable inter-species variation in drug responses (249). YM087 is not however subject to the same species heterogeneity that affects these other

AVP receptor antagonists (383). Drug A has thus far undergone little research in any species other than rat and caution should be exercised when trying to interpret findings from any such future studies.

Chapter 4 reported on the combination treatment of ACE inhibitor ramipril with $V_{1A}R$ blocker OPC 21268 in the adult spontaneously hypertensive rat (SHR). No additive hypotensive effects were seen with the combination treatment and OPC 21268 alone did not lower blood pressure (BP) over hypertensive control. This was the first study to look at combination ACE and $V_{1A}R$ antagonist treatment in an adult model of essential hypertension where plasma AVP was not elevated. Although a negative study it is important to understand that the addition of $V_{1A}R$ antagonism to ACE inhibition does not have a therapeutic role to play in this particular clinical setting. This is unlikely to be the case, however, in hypertension associated with low plasma renin levels, such as is seen in African-Americans, renal failure patients, the elderly and diabetics. Indeed a pilot study in a rat model combining hypertension with diabetes mellitus (DM) did show additive BP lowering effect with both treatments. This is described in detail in chapter 5.

Future studies should aim to look for any additive effects of these treatments in those cases of low renin hypertension, for instance in renal failure, a rat model that has been used with success in our group (267).

The effect on the AVP system of 8 weeks of streptozotocin induced DM (STZ-DM) in SHR was reported in chapter 5. In the context of an elevated AVP, a normal finding in DM, an up-regulation of the $V_{1A}R$, both in liver and kidney, was seen. This was manifest as an increase in receptor density with no associated increase in affinity of the receptor for

the $V_{1A}R$ specific radioligand ^{125}I-labelled [d$(CH_2)_5$,sarcosine^7]AVP being seen. This up-regulation of receptor protein density was observed despite normal renal and hepatic $V_{1A}R$ mRNA levels. This study was the first to describe the up-regulation of the $V_{1A}R$ in DM and contrasts with a previous study from our group that demonstrated hepatic and renal $V_{1A}R$ down-regulation using a 2 week normotensive model of STZ-DM (51). Normally in biological systems when the concentration of a receptor ligand is increased then the cell surface receptors for that particular ligand are subsequently down-regulated in a bid to "keep things in balance". A number of possible explanations exist for the apparent disregulation of the AVP system seen in this hypertensive model of DM but all of them remain speculative at this time. These are discussed in more detail in chapter 5. Briefly, in the relatively severe model of 8 weeks of DM combined with hypertension, where renal damage has been sustained (in this case as evidenced by albuminuria), then secondary to structural and functional damage, normal regulatory responses are no longer seen. In the previous study, using the 2 week normotensive rat model of DM, very little structural or functional damage, if any, would be expected. In humans, the prevalence of hypertension is 1.5 to 2 times greater in people with DM as compared to matched non-diabetic individuals (12). An animal model, therefore, that incorporates both could be seen as more relevant to the clinical situation. This observed disregulation may serve a physiological purpose and thus be a "normal" regulatory response in trying to limit the excessive diuresis associated with DM. Many of the actions of AVP at the $V_{1A}R$, both in the kidney and to a lesser extent perhaps in the liver, are to reinforce or blunt the antidiuretic action mediated via the V_2R. It is known that the poorer the diabetic control then the higher the plasma AVP concentration (321) and I propose that at lower AVP concentrations more $V_{1A}Rs$ are recruited to assist in water retention. These receptors are located in the vasa recta and selective vasoconstriction of these arteries causes a reduction in intramedullary blood flow

and thus a water sparing effect (135-137). Higher AVP concentrations, associated with poorly controlled DM, may see the physiological activation of collecting duct V_{1A}Rs (117). These are known to blunt the V_2R dependent production of cyclic adenosine monophosphate and would thus help limit any excessive water retention that might be expected as AVP levels climb and its antidiuretic action continues unchecked (417, 430).

Several explanations for the dissociation between changes in receptor number and mRNA expression in DM exist. These include alterations in post-translational mechanisms such as internalisation and recycling via receptor-mediated endocytosis (419). Alternatively, post-translational processing of AVP receptors has been proposed to explain the disparity between the receptor number and mRNA expression observed in chapter 5. Some previous studies have indicated that post-translational events such as glycosylation (422, 431) may be important for receptor trafficking and /or biosynthesis and internalisation and this may be particularly relevant here.

The limited information gained from an 8 month pilot study of STZ-DM in SHR would suggest that after a more prolonged period of DM, combined with hypertension, then the V_{1A}R numbers in liver and kidney are no longer up-regulated but are equivalent to that of hypertensive control rats. Therefore it may be that the up-regulation of the V_{1A}R system has a role to play early on in the natural history of this disease. Unusually, however, in this study the plasma AVP levels were not elevated in the DM-SHR as compared to SHR.

A second pilot study looked at the effect of combination ACE inhibition with V_{1A}R antagonism on BP and demonstrated an additive hypotensive effect, a finding not previously seen with non-diabetic SHR (as described in chapter 4). This would make

sense in light of the findings from chapter 5 where elevated plasma AVP is associated with up-regulation of the $V_{1A}R$ in the DM-SHR as compared to the non-diabetic SHR and thus the blocking of an up-regulated system provides clinical benefit.

Future studies would aim to look at the V_2R and whether it too is up-regulated in this particular animal model.

It would be beneficial to study the second messenger systems for both receptors as it is feasible that while receptor numbers are up-regulated their cell signalling pathways are not. Localisation studies to determine where in the kidney ultrastructure these up-regulated $V_{1A}Rs$ can be found would aid our understanding of the purpose these receptors might serve in this disease model.

A time course experiment over at least 8 months with animals being sacrificed at various stages would provide information on the regulation of the AVP system over time in DM and hypertension. This would guide our understanding as to when AVP receptor blockade would be of most benefit in the treatment of this disease. Information gained so far would suggest that early blockade of an up-regulated $V_{1A}R$ system would provide greatest clinical benefit.

The most important study to be undertaken is an intervention study and this is currently planned for within our laboratory. This will be an 8 week study. SHR are to be made diabetic at 8 weeks of age and randomised to receive vehicle, OPC 21268, ramipril or combination ramipril and OPC 21268. BP will be measured every 2 weeks, urinary albumin excretion every 4 weeks and all metabolic, hormonal and biochemical parameters,

as well as tissue weights, will be determined at sacrifice. It is hoped that the treated DM-SHR will, most importantly, demonstrate a reduction in urinary albumin excretion, BP and tissue weights and that the combination of ramipril and OPC 21268 will be additive in this regard.

6.2 GENERAL CONCLUSION

In conclusion, the studies reported in this thesis have attempted to enhance our understanding of the role of AVP and its $V_{1A}R$ system in the CVDs of hypertension and DM. New treatments are continually being sought in a bid to improve morbidity and mortality in people with these conditions. Non-peptide AVP receptor blockers are looking increasingly promising as an additive treatment to existing drugs such as ACE inhibitors. From this thesis it seems likely that they will have a particularly exciting future in those CVDs associated with an elevated AVP such as DM.

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