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UNIVERSITY OF CALGARY

Cardiovascular and Renal Effects of ANG II and NO

in the Newborn: Roles of AT1Rs and /or AT2Rs

by

Angela Elena Vinturache

A THESIS

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Abstract

The vasoactive factors angiotensin II (ANG II) and nitric oxide (NO), and the balance between them play a major role in regulating cardiovascular and electrolyte homeostasis. During the perinatal period, both factors are elevated, yet their physiological roles have not been clearly defined. Furthermore, the temporal and spatial expression of the receptors for ANG II (AT1Rs and AT2Rs) is developmentally regulated, although their physiological roles are also unknown. The present study aimed to elucidate for the first time, the physiological effects of ANG II on the kidney during postnatal maturation, as well as the potential role of the ATRs in initiating these physiological effects. In addition, this study investigated for the first time the interaction between ANG II and NO in regulating cardiovascular and renal function during the period of adaptation of the newborn to life after birth.

Two study protocols were carried out in conscious lambs at two stages of postnatal maturation - one and six weeks. The first protocol evaluated (a) the individual functions of ATRs and (b) any possible interaction between them in mediating haemodynamic and renal physiological responses of ANG II. The second protocol investigated the roles of ATRs in modulating physiological roles of endogenoulsy produced NO during development.

Results from these experiments show for the first time, that there does not appear to be any interaction between AT1Rs and AT2Rs in mediating haemodynamic effects of endogenous ANG II early in life. In contrast, the renal effects of ANG II are mediated through AT1Rs in an age-dependent manner, whereas AT2Rs buffer these effects. My research also shows for the first time, that ANG II modulates the effects of NO on renal haemodynamics and function but not systemic haemodynamics in an age-dependent manner through activation of AT1Rs and AT2Rs. Furthermore, it appears that AT2Rs interact with AT1Rs in mediating the renal effects of NO in developing newborn animals. This is the first description of renal effects of ANG II and a new and important interaction between ANG II and NO under physiological conditions early in life.

Preface

Renin-angiotensin system (RAS) and nitric oxide (NO) system are considered factors of primary importance in modulating renal and cardiovascular function since they both influence vascular tone and, therefore, blood pressure as well as kidney function and, therefore, fluid and electrolyte homeostasis. As natural counterparts that interact via both, direct and indirect mechanisms, the balance between them is critical for cardiovascular homeostasis as well as for regulating renal haemodynamics and function as illustrated below in Figure P.1.



Figure P.1 Balance of vasoactive factors ANG II and NO in adulthood

Despite much success over the last decades in understanding their roles in cardiorenal physiology in adulthood, the functions that these two systems play in the perinatal period are poorly understood. Both systems are activated at birth, possibly contributing to the adaptation of the newborn kidney as well as the systemic circulation to life after birth. In addition, the temporal and spatial expression of both angiotensin II (ANG II) receptors (ATRs) and nitric oxide synthase (NOS) isoforms is developmentally regulated. The physiological significance of these changes during the transition to life after birth has not been elucidated. In addition, the interaction between these two vasoactive mediators within the developing kidney is not known (Figure P.2).



Figure P.2 Balance of vasoactive factors ANG II and NO in the newborn

Therefore, the studies presented in this dissertation were designed to investigate the relationship between ANG II and NO in regulating the adaptation of the newborn kidney to life after birth and to explore the possible roles of ATRs in mediating the ANG II-NO interaction during postnatal maturation, during which time there are many changes in cardiovascular and renal function.

As a result, this dissertation is developed around three core sections as follows:

(1) The <u>first core section</u> includes a literature review (Chapter 1), that forms the basis to the objective of the study. This chapter provides familiarity with some of the physical characteristics of the RAS and NO systems, including ATRs and NOS isoforms, and introduces selected cardiovascular and renal physiological effects of these two factors in adulthood as well as during the perinatal period. An overview of the known physiological interaction between ANG II and NO in adulthood as well as early in life is also provided. The Study Rationale and Hypotheses chapter (Chapter 2) describes previous experimental findings from our laboratory and the reasoning behind this study, stating as well the hypotheses and the means through which these hypotheses are tested.

(2) The <u>second core section</u> is dedicated to describing the methods used in this research study, including animals, materials, surgical procedures and experimental

protocols (Chapter 3), as well as providing technical details that facilitated the experiments (Appendices A to H).

(3) The <u>third core section</u> includes the results of the experiments (Chapter 4), with data shown in Figures and Tables, as well as discussions relating to the research findings (Chapter 5). Data obtained from the experiments that are not presented in the Figures and Tables within Chapter 5 (additional data, supportive of the main results) have been included in a separate Appendix I (Experimental data). An overview of the major research findings of my research as well as suggested future undertakings are also provided in this section (Chapter 6 Conclusions).

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ACE	angiotensin converting enzyme
ACh	acethylcholine
AVP	arginine vasopressin
AGT	angiotensinogen
ANG	angiotensin
ATR	angiotensin II receptor
AT1R	angiontensin II receptor subtype I
AT2R	angiontensin II receptor subtype II
ВК	bradykinin
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
ERK	extracellular signal-regulated kinase
FF	filtration fraction
GFR	glomerular filtration rate
GPCR	G protein coupled receptor
GTP	guanosine triphosphate
cGMP	cyclic guanosine monophospate
HR	heart rate
I.M	intramuscular
I.V	intravenous
JGA	juxtaglomerular
JAK	Janus kinases
MAP	mean arterial pressure
MAPK	mitogen activated protein kinase
MD	macula densa
MVP	mean venous pressure
NO	nitric oxide
NOS	nitric oxide synthase
PLA2	phospholipase A2
MPK-1	MAP kinase phosphatase 1
SHP-1	SH2-domain-containing phosphatase 1
РКС	protein kinase C
PLC	phospholipase C
PRA	plasma renin activity
RVR	renal vascular resistance
RAS	renin-angiotensin system
RBF	renal blood flow
sGC	soluble guanylate cyclise
STAT	signal transducers and activators of transcription
TGF	tubuloglomerular feedback
VSMC	vascular smooth muscle cell

Chapter One: LITERATURE REVIEW

1.1 RENIN-ANGIOTENSIN SYSTEM (RAS)

1.1.1 Introduction

The renin-angiotensin system (RAS) bioenzymic cascade represents an integral component of cardiovascular, renal and adrenal regulation which maintains blood pressure and water and electrolyte balance in health and disease.¹ While knowledge regarding the RAS in adult mammals has substantially increased over the last three decades, the physiological effects and the levels of functioning of the system during the perinatal period have not been thoroughly characterized.

1.1.2 Historical perspective

Knowledge of the RAS began with the first insight into the mechanisms of blood pressure regulation in 1898 when Tigerstedt and Bergman initially discovered the presence of a pressor substance in renal extracts, named renin.² Goldblatt et al (1934) later observed that renal artery constriction in dogs produced hypertension, which subsequently may elicit renal lesions.³ This pioneering work was continued by two subsequent independent studies which showed that renin was an enzyme which acts on a plasma substrate to produce the pressor hormone, Angiotonin,⁴⁻⁵ isolated ~ten years later under the name Hypertensin⁶⁻⁷ and synthesised as an active octapeptide that received the name Angiotensin II (ANG II).⁸ The discovery of ANG II was followed by the characterization of various components of the RAS cascade, and launched tremendous efforts with the hope of finding the corresponding antagonists.⁹⁻¹⁰ Publication of a series of pharmacological patents by Furakawa et al (1982) led to identification of such antagonists: Losartan (DuP 753) in 1988¹¹ and PD 123177 and the related PD 123319 in 1991.¹² These compounds have been proven useful tools in identifying the ANG II receptors subtypes: type 1 (AT1R) and type 2 (AT2R),¹³⁻¹⁵ whose general properties and biological actions are detailed in subsequent sections of this dissertation. Molecular biology techniques were later employed to identify and clone AT1R and AT2R genes in

different species including the human.^{13,14-19} More recently, the RAS field has been challenged by the discovery of novel bioactive peptides, additional receptors as well as supplementary roles for the precursor components²⁰⁻²² as detailed in subsequent sections.

1.1.3 RAS overview

Classically, the RAS was viewed as a short-term regulator of fluid balance and blood pressure and originally considered to be solely a circulating endocrine system.²³⁻²⁵ The system consists of two step sequential enzymatic cascade which catalyzes the generation of a single bioactive molecule, the circulating peptide ANG II²⁶ from the substrate angiotensinogen (AGT) (Figure 1.1). Cleavage of AGT to the decapeptide angiotensin I (ANG I) by renin, a 45 kDa substrate-specific aspartylprotease, represents the rate-limiting step in ANG II formation.²⁷⁻²⁸



Figure 1.1 Classical view of the renin-angiotensin system (RAS)

Over the last two decades, the RAS system, which is well conserved throughout evolution on the phylogenetic scale, has expanded significantly in its number of components as well as the range of functions it plays.²⁰⁻²¹ There is increasing awareness

that ANG II is not the only effector of the RAS and other angiotensin peptides, such as ANG III, ANG IV, ANG 4-8, ANG 1-7,²⁹ generated from different enzymatic pathways may also have biologic activity and physiologic importance (Figure 1.2).³⁰⁻³¹



Figure 1.2 Expanded view of RAS

showing the angiotensin fragment peptides generated through multiple pathways: ANG II, ANG III, ANG IV, ANG 1-7, angiotensin fragment peptides proven to be biologically active; ANG 1-4, ANG 1-5, ANG 1-9, ANG 5-8, additional angiotensin truncated peptides with no physiological and biological effects ascribed to date; the letters in the name of peptides indicate the number of amino acids of the respective

peptide); NEP, neprilysin endopeptidase; PEP, prolyl endopeptidase;

ACE2, angiotensin converting enzyme 2 (adapted from^{20,22,25,32-33})

It is also known that there are alternative pathways which may lead to the synthesis of ANG II (Figure 1.2),^{25,34} although these appear to be rather active locally. More recently, the RAS field has been challenged by the discovery of additional specific receptors: AT4R (IRAP, insulin-regulated aminopeptidase receptor), Mas receptors

(AT7R), and (Pro) renin receptors, as well as supplementary roles for the precursor components (reviewed by Kumar *et al* (2007),²⁰ (2009),²² Paul *et al* (2006),²¹ Fyhrquist and Saijonmaa $(2008)^{32}$). The physiological importance of these alternative pathways and their significance in health and/or disease has not yet been elucidated.

Furthermore, there are numerous local tissue RASs characterized by the presence of all the aforementioned components in most organs and tissues, including kidney, heart and vasculature, and brain (reviewed by Paul *et al* (2006).²¹ ANG II produced locally from these tissues is believed to act as a paracrine/autocrine factor.³⁵⁻³⁶ In addition, each local RAS appears to have a distinct enzymatic profile which may result in different²⁶ patterns of ANG fragment peptide generation within the tissues,³⁷ that can mediate various physiological effects. The presence of the RAS components have been demonstrated within the kidney,^{25,38,39} and the intra-renal RAS is thought to be of critical importance for the regulation of blood pressure and salt balance.⁴⁰⁻⁴² Tissue RASs are regulated and operated independently of the peripheral RASs (i.e. adrenal glands, brain) but can interact with it (i.e. heart, kidney) in a complementary fashion.²¹

More recently, the classical view of the RAS has been challenged by the discovery of a completely functional intracellular RAS, characterized by the presence of its components inside the cells and ANG II synthesis at intracellular sites.⁴³⁻⁴⁵ Although the exact physiological and pathophysiological role of the intracellular RAS is not yet elucidated, its presence has been proven in a variety of cells, such as heart and systemic vasculature,^{21,46-48} kidney,⁴⁹ liver,⁵⁰ pancreas,⁵¹ macrophages,⁵² adipose tissue,⁵³ and nervous system⁵⁴ suggesting a broad relevance of the intracellular system, mostly related to cell-to-cell communication, cellular growth and regulation of gene expression. It has been suggested that intracellular ANG II synthesis utilizes more diverse pathways than its extracellular synthesis, dependent upon cell type and stimulus.⁵⁵⁻⁵⁷ It is not known, however, if these intracellular RASs are independent entities or alternative forms that are only expressed under particular conditions; their separate contribution to overall fluid and electrolyte homeostasis remains to be elucidated. To date, intracellular RAS expression and function have not been investigated in the perinatal period.

1.1.4 Angiotensin II (ANG II) receptors (ATRs)

An impressive number of studies and extensive reviews address the biochemistry, molecular biology, physiological functions and potential pathophysiological implications of the ATRs.^{29,58-59} The brief overview provided, herein, emphasises the localization and physiology of the ATRs in two different circumstances, adulthood and during postnatal maturation, discussing as well the current dogma regarding the functional interactions and the molecular cross-talk between the two types of receptors.

1.1.4.1 Angiotensin receptors (ATRs) - overview

The major physiological actions of ANG II are mediated by two subtypes of G protein-coupled receptors, type 1 (AT1R) and type 2 (AT2R), which are seven transmembrane glycoproteins that share only 32–34% sequence homology⁶⁰⁻⁶² and differ in their pharmacological and biochemical properties. After ANG II binds the extracellular domain, highly complex, receptor-specific, intracellular signalling pathways are stimulated,^{60,63} reviewed by Bottari *et al* (1993)⁶⁴, de Gasparo *et al* (2000)²⁹, Jones *et al* (2008).⁶⁵ A great deal is known about these signalling events triggered after the stimulation of ATRs, that is reviewed elsewhere.^{29,65}

1.1.4.2 Angiotensin receptor type 1 - AT1R

AT1Rs properties and signal transduction. The AT1R selectively binds with high affinity antagonists such as biphenylimidazoles, including losartan, candesartan and ibesartan, and is rather insensitive to tetrahydroimidazoles pyridines such as PD 123319 and PD 123177.⁶⁶⁻⁶⁷ The binding site for ANG II, localized in the transmembrane domain and the extracellular loop, is different from the binding site for AT1R antagonists, which interact only with the transmembrane domain of the receptor.^{58,66,68} Like most G-protein-coupled receptors, AT1Rs are internalized when stimulated by ANG II, a process dependent on specific residues on the cytoplasmic tail.¹⁴ There are five classical signal transduction mechanisms for AT1R: (1) activation of phospholipase A₂ (PLA₂), (2) phospholipase C (PLC), (3) phospholipase D (PLD) and (4) L-type Ca²⁺ channels and (5) inhibition of adenylate cyclase (AC) (reviewed by Diem *et al* (2001)⁶⁷ and de Gasparo *et*

al $(2000)^{29}$) which mediate vasoconstrictor effects of ANG II. Activation of AT1Rs also stimulates tyrosine phosphorylation and phospholipase C- γ , leading to activation of downstream proteins, such as mitogenactivated protein kinases (MAPK),^{1,69} Janus kinases (JAK), and the signal transducers and activators of transcription (STAT) proteins,^{60,63} pathways that stimulate cellular growth and proliferation. Except for rodents in which two AT1R subtype exist (AT_{1a} and AT_{1b}) in all mammalian species examined including rabbit, dog, pig, sheep, cow and human only one AT1R type is present.²⁹

ATIRs distribution and localization. ATIRs are widely distributed within tissues with a high density in the central nervous systems (CNS) of several species including rat, rabbit and humans in areas involved in cardiovascular regulation. High levels of AT1Rs occur in circumventricular organs, including the subfornical organ, organum vasculosum of the lamina terminalis, anterior pituitary, and area postrema in the hindbrain,⁷⁰⁻⁷³ which are situated outside of the brain-blood barrier and, therefore, exposed to circulating ANG II. AT1Rs are also distributed within regions inside of the brain-blood barrier that include nucleus tractus solitarius, dorsal motor nucleus of the vagus, ventrolateral medulla, regions involved in the regulation of autonomic activity and cardiovascular reflexes.⁷⁴⁻⁷⁵ In the vasculature, high levels of AT1Rs are present in vascular smooth muscle cells (VSMC) of aorta, pulmonary and mesenteric arteries, whereas lower levels are present in the adventitia of these vessels.^{29,76} In the heart, AT1R levels are higher in the conducting system as compared with the myocardium of ventricles and atria.⁷⁷⁻⁷⁹ Within the kidney of a variety of mammalian species, AT1Rs are abundantly expressed in the renal microvasculature of both, cortex and medulla, including afferent and efferent arteriole and vasa recta, in mesangial cells and podocytes of the glomerulus and renal interstitial cells of the outer renal medulla.⁸⁰⁻⁸² Prominent AT1R immunostaining is also present in the brush border and basolateral membranes of proximal tubule epithelial cells, in macula densa cells of the thick ascending limb, and in the distal tubule and cortical collecting duct, while the inner medulla towards the tip of the papilla elicits lower levels (Figure 1.3).83,84



Figure 1.3 Distribution of AT1Rs and AT2Rs in the adult kidney Data compiled from studies in adult mammallian kidney^{29,76,79,83,85,86}

1.1.4.3 Angiotensin receptor type 2 – AT2R

AT2Rs properties and signal transduction. AT2Rs are characterized by high affinity for the specific receptor antagonists PD 123319, PD 123177 and CGP 42112.66 ANG II binds to AT2Rs with similar affinity as to AT1Rs. It is still unclear whether the AT2R is coupled to a G-protein since it shares only partial homology with the AT1R.⁸⁷ Various intracellular signalling pathways, G-protein dependent and independent, have been assigned to the AT2R.⁸⁸⁻⁸⁹ Stimulation of AT2Rs leads to activation of protein tyrosine phosphatase, SH2-domain-containing phosphatase 1 (SHP-1) and serine/threonine phosphatase 2A,90-91 resulting in opening of delayed rectifier K⁺ channels and inhibition of T-type Ca²⁺ channels.^{62,91} Inhibition of MAP kinase enzymes and inactivation of extracellular signal-regulated kinase (ERK1 and ERK2) mediates proapoptotic effects of AT2R, signalling activity that opposes AT1R activation.^{67,89,92} In

particular, indirect negative coupling to guanylate cyclase (GC) inhibits cGMP production,^{64,93} whereas stimulation of AT2Rs enhances the bradykinin (BK) - nitric oxide (NO) - cGMP (cyclic guanosine monophosphate) vasodilatory cascade.⁹⁴⁻⁹⁶ Besides the well known ligand-mediated activity, constitutive activation of AT2Rs has also been described.^{93,97}

AT2Rs distribution and localization. Although AT2R is the predominant receptor subtype in embryonic and fetal tissues, its expression declines with age to undetectable levels in adulthood.^{29,98} AT2R expression does, however, persist, albeit at considerably lower levels, in several tissues in adult mammals such as the adrenal medulla, brainstem nuclei, heart, kidney, ovary, pancreas, retina, skin, and uterus, as well as endothelial and VSM cells.^{65,87,99-100} The distribution of AT2Rs is very restricted in the human brain and shows a high degree of variability across species.⁷⁵ Within the CNS, AT2R expression is higher in the cerebellar nuclei and locus coeruleus of the brainstem.¹⁰¹⁻¹⁰² In the heart, AT2Rs are restricted mostly to the cardiac fibroblasts and to a lower degree to the cardiomyocytes and coronary vessels.^{78,103-104} Within the pre-glomerular vasculature of the kidney, AT2Rs are localized in the interlobular and arcuate arteries, afferent arterioles, and as well in the outer medullary descending *vasa recta*.¹⁰⁵⁻¹⁰⁷ AT2R expression has also been detected in the proximal tubule, collecting ducts and renal interstitial cells (Figure 1.3).^{58,83,87,107}

1.1.5 Physiological roles of ATRs in adulthood

The RAS plays an important modulatory role in blood pressure control and in water and salt homeostasis. The majority of the central, cardiovascular and renal physiological effects of ANG II appear to be mediated through activation of AT1Rs, while the effects mediated by AT2Rs are less understood.^{75,79,108}

Central actions of ANG II include regulation of arterial pressure, drinking and salt appetite, sympathetic nerve activity and pituitary hormone secretion and are induced through predominant activation of AT1Rs, whereas the physiological role of AT2Rs in the CNS is less clear.^{75,109} Stimulation of AT1Rs in neurons from periventricular organs mediates potent systemic vasoconstriction and a characteristic osmoregulatory response,

critically controlling the release of arginine-vasopressin (AVP) into the circulation, thirst and drinking behavior,^{72,110-111} while the AT1Rs within the *area postrema* are responsible for the central modulation of the arterial baroreflex control of the heart rate.¹¹²

AT1Rs also mediate aldosterone release, renal sodium and water retention and sympathetic facilitation,¹¹³ in addition to stimulation of fibrosis, cell growth and proliferation (extensively reviewed by de Gasparo *et al* (2000),²⁹ Dihn *et at* (2001),⁶⁷ Fyhrquist and Sainjonmaa (2008)).³² As described above, AT2R activation mediates a vasodilation through an increase in production of NO,⁹⁶ cGMP,⁹⁵ and prostaglandin F2 α ,¹¹⁴ therefore counterbalancing the vasoconstrictor and pressor effects of AT1R activation (reviewed by de Gasparo *et al* (2000),^{98,29} and Carey *et al* (2005)¹¹⁵) (Figure 1.4). In addition, ATRs play important roles in cardiovascular remodelling, inflamatory and metabolic processes (reviewed by Baker et al (1992),¹¹⁶ de Gasparo *et al* (2000),²⁹ Dihn *et at* (2001),⁶⁷ Fyhrquist and Sainjonmaa (2008)),³² the description of which is beyond the scope of this dissertation.



Figure 1.4 Physiological effects of AT1Rs and AT2Rs in adulthood

Multiple roles are described for ATRs within the kidney. ANG II and other active fragment peptides of the RAS regulate renal blood flow (RBF) and control the perfusion of both cortex and medulla through activation of AT1Rs and AT2Rs (reviewed by Navar

et al (2004),¹¹⁷ Ichihara *et al* (2004)).¹¹⁸ It is considered that, in general, ANG II decreases total RBF and cortical blood flow through vasoconstriction mediated by activation of AT1Rs, while eliciting various effects (increase, decrease or no effect) on the regulation of medullary blood flow (reviewed by Evans *et al* (2010)),¹¹⁹ effects mediated through both, AT1Rs and AT2Rs. ANG II is also involved in the autoregulatory mechanisms controlling RBF and glomerular filtration rate (GFR) modulating myogenic and tubuloglomerular feedback (TGF) mechanisms within the kidney, probably through activation of AT1Rs, which are abundantly expressed on both, apical and basolateral membranes of *macula densa* cells (See also Figure 1.3).¹²⁰⁻¹²²

In addition to regulating renal haemodynamics, the RAS is a major regulator of renal function. ANG II regulates pre- and post- glomerular vascular tone and mesangial cells contractility and, therefore, GFR, through activation of both ATRs.¹²³ While on efferent arterioles the ANG II vasoconstrictor effect is modulated by AT1R-induced prostaglandins (PGs) released upstream in the glomerulus, NO and PGs release through AT2R activation modulates ANG II actions in the afferent arteriole.¹²³ ANG II regulates renal sodium (Na⁺) and water handling, through direct effects on renal tubular transport as well as extrarenal effects, via regulation of renal haemodynamics, GFR, and aldosterone secretion.^{35,49,124} ANG II effects on tubular transport appear to be mediated through both, AT1Rs and AT2Rs.¹²⁵⁻¹²⁶ The effects of ANG II in increasing Na⁺, bicarbonate, and fluid reabsorption along the nephron are predominantly mediated through activation of AT1Rs from the brush border and basolateral membranes of the proximal and distal tubule.^{29,127-128} AT1Rs also mediate regulatory effects on the expression and/or activity of various Na^+ transporters, such as Na^+ -PO₄⁻ co-transporter type IIa (NaPi-IIa)¹²⁹ and apical Na⁺-H⁺ exchanger (NHE3) in the proximal tubule,¹³⁰ thiazide-sensitive Na⁺-Cl⁻ co-transporter (NCC) and Na⁺-K⁺-ATPase pump in the distal tubule¹³¹ as well as aquaporins.¹²⁷⁻¹²⁸ Involvement of AT2Rs on Na⁺ handling along the nephron has also been suggested,¹²⁵ although the specific roles and signalling transduction mechanisms are not well understood.¹³² It has, however, been shown that AT2Rs stimulates Na⁺ excretion, possible via a BK-NO-cGMP signaling pathway¹³³ and, therefore, appears to counterbalance the effects of AT1Rs on electrolyte and water

transport¹³⁴⁻¹³⁵ (reviewed by Carey and Padia (2008).¹²⁵ These findings provide evidence for an interaction between ATR subtypes in mediating ANG II effects on glomerular and tubular function in adulthood, as described in the following section (see also Figure 1.4).

1.1.6 Interactions between AT1Rs and AT2Rs

An increasing body of evidence shows that there are different levels of functional interaction between the AT1Rs and AT2Rs that may contribute to the regulation of the net physiologic responses to ANG II.¹³⁶ In fact, it is considered that the predominant physiologic effects of AT2R are to counterbalance the AT1R-mediated responses to ANG II, although synergistic effects of the ATRs have been also reported.¹³⁷ A cross-talk between AT1Rs and AT2Rs in the brain may be involved in the regulation of various processes, for example the release of AVP.¹³⁸ As described in the previous section (Section 1.1.5.), ANG II binding to AT2Rs activates a counter regulatory pathway through which vasoconstriction mediated by activation of AT1Rs is opposed by the AT2R induced vasodilation through activation of the BK-NO-cGMP autacoids cascade.^{115,139-141} The opposing effects of AT2R activation demonstrated in a large variety of resistance vessels including renal, mesenteric, uterine, coronary and cerebral beds were unmasked, however, in normo- and hypertensive animals in which AT1R activity was inhibited.¹⁴²⁻¹⁴⁴ More recently it has been shown that AT2R-induced natriuresis counteracts the anti-natriuresis initiated by AT1R activation.¹³³ Furthermore, while classically the ANG II negative feedback on renin secretion was considered to be mediated through exclusive AT1R activation, emerging data suggests that the AT2R may contribute as well to the regulation of renin release.^{124,145} Therefore, the functional interaction between the two ATRs appears to be more complex than initially considered.

The presence of a reciprocal cross-talk between AT1R and AT2R has more recently been demonstrated at the molecular level. An interaction at the level of signal transduction pathways has been described in primary hypothalamic neurons in culture, in which ANG II binding to AT1Rs activates ERK 1 and 2 protein kinases, responses augmented by inhibition of AT2Rs.¹⁴⁶ Several studies have provided evidence that inhibition of AT1Rs increases AT2R gene expression and activity in cultured rat

endothelial cells¹⁴⁷ thus providing evidence for a dynamic cross-talk between AT1R and AT2R within cells.⁵⁹ Conversely, AT2R activation in the brain increases AT1R mRNA transcription in rat cortical cells,¹⁴⁸ and AT2Rs over-expression down-regulates AT_{1a}R mRNA and protein levels through a BK-NO pathway in rat cultured VSMC.¹⁴⁹⁻¹⁵⁰ AT2R knockout mice elicit an increased expression of vascular AT1R mRNA and protein levels.¹⁵¹ Furthermore, in addition to traditionally functioning as monomers, ATRs form dimers as part of their normal functioning and trafficking.¹⁵² Hetero-dimerization through direct binding of AT2R to AT1R inhibits AT1R activation and antagonizes AT1R function in fetal fibroblasts and human myometrial biopsies.¹⁵³ Since AT1R activates predominantly kinase pathways and AT2R signals through phosphatases, this may provide, as well, a general indication that the two receptors are antagonistic.¹⁵⁴

In conclusion, according to the cross-talk theory, which is becoming the accepted dogma for the ATRs at least in adulthood, the AT1Rs and AT2Rs reciprocally affect their expression at transcriptional and protein levels. There is also an increasing body of evidence showing an interaction between AT1Rs and AT2Rs in mediating cardiovascular and renal effects of ANG II. While the presence of an interaction between AT1Rs and AT2Rs has been described in adult animals, any receptor cross-talk in developing organisms had not been investigated to date. This will be explored for the first time in my dissertation by measuring the cardiovascular and renal responses to inhibition of both ATRs separately and together in conscious developing animals. An overview of structural and functional maturational changes of the RAS, including ATRs, during postnatal development is provided below.

1.1.7 Renin-angiotensin system during postnatal maturation

1.1.7.1 RAS maturational changes at birth

The activity of the RAS undergoes remarkable developmental changes. Studies conducted in different mammalian species including the human, have demonstrated that the RAS is activated perinatally, all system components being highly expressed during this period in a pattern suggesting a relevant physiological role. Genetic and pharmacological interruption at the various levels of the RAS suggests that an intact system is a prerequisite for normal development, providing evidence that the genetic expression of the RAS components is also developmentally regulated.¹⁵⁵⁻¹⁵⁶ Furthermore, the spatial and temporal expression of the RAS components changes within the kidney as maturation proceeds. For example, angiotensinogen, renin and ACE mRNAs are highly expressed in the fetal and newborn rat kidney as compared with the adult of the same species.¹⁵⁷⁻¹⁵⁹

Circulating levels of the RAS enzymes and effector peptides follow as well a developmentally regulated pattern. The levels of renin, ACE and ANG II rise as early as day 12 of gestation in fetal rats and increase thereafter¹⁶⁰. During fetal life, the RAS is activated, levels of renin and ANG II being highly elevated near-term,¹⁶¹⁻¹⁶² and further activatated at birth.¹⁶³ Plasma renin activity (PRA) and circulating ANG II levels are elevated in newborns of several species including rat, sheep, dogs and humans¹⁶¹⁻¹⁶⁸ and progressively decline in an age-dependent manner after birth.^{166,169-170} To date, however, the functional significance of activation of the RAS during the perinatal period is poorly understood. The research described in this dissertation aims to explore the functional roles that endogenous ANG II, through activation of ATRs, may play in modulating haemodynamics and renal function during the transition to life after birth and as postnatal maturation proceeds.

1.1.7.2 Maturational changes in ATRs distribution

The density of AT1Rs and AT2Rs is developmentally regulated, a reciprocal developmental pattern of systemic vascular and intrarenal distribution being observed for both ATRs in most species.¹⁷¹⁻¹⁷⁶ As a general trend, AT2Rs are highly expressed early during embryonic life and in the fetus in all species studied to date, including mice, rat, pig, sheep and humans,^{83,177-179,180-182} after which AT2Rs decline with age. Conversely, AT1Rs are expressed later during fetal development, their expression increasing progressively postnatally to adult levels.^{83,179,182-183}

The AT2R is the predominant receptor present in the cardiopulmonary system of several species in early development. In the heart, AT2Rs levels are high during the late

fetal and immediate neonatal period and decrease rapidly after birth to adult levels within two days in valvular tissues of the heart in the rat^{77,86} and within one week in all four heart chambers in sheep.¹⁸⁴ Within the vasculature, AT2Rs are highly expressed in all wall layers of major vessels of the developing rat,¹⁷² swine¹⁸³ and sheep,¹⁷⁵ the vascular expression decreasing significantly soon after birth, being barely detectable in newborn and adult animals.¹⁷⁵ In contrast, cardiac expression of AT1Rs is relatively unchanged during late fetal and newborn life in sheep,¹⁷⁶ and persist to adulthood in rodent.^{86,185-186} AT1R binding is up to three fold higher in the newborn piglet aorta as compared with the fetus,¹⁸³ while transitioning from the fetal umbilical vessels to systemic vasculature by three months after birth in sheep.¹⁷⁵ Taken together it is plausible to suggest that ATRs mediate different physiological responses in the cardiovascular system at different stages of maturation.

Several studies have also shown that ATR expression within the kidney is developmentally regulated, although the role and significance of the spatiotemporal pattern of ATRs distribution during renal ontogenesis is unknown. In early embryonic development AT2Rs are localized primarily to undifferentiated mesenchyme and advancing tubule of the ureteric bud,¹⁸⁷ while after birth their expression is associated with the nephrogenic cortex in the rat,¹⁷¹ and confined to discrete cortical pre- and juxtaglomerular areas and the vascular elements of the medulla in swine, simian and human.¹⁸¹ Recent studies have reported AT2R mRNA levels considerably higher in afferent, interlobular and arcuate arteries of newborn piglets as compared with adult pig (Figure 1.5).¹⁸⁸⁻¹⁸⁹ Within the rat kidney, AT1Rs expression is detected later in gestation than AT2Rs, remaining constant throughout fetal life and progressively increasing after birth to adult levels.^{172,187} AT1Rs exhibit maximal postnatal density in the glomeruli from the renal cortex in swine,¹⁸¹ in the inner stripe of the outer medulla in rodents,^{171, 185,187,190} and lower levels in cortical extraglomerular sites in both species.^{171,181} The expression of AT1Rs is lower in all preglomerular vessels in newborn piglets as compared to older swine (Figure 1.5).¹⁸⁸⁻¹⁸⁹





In sheep, similar with the findings in rat,¹⁷⁷ mouse,¹⁹² and swine,¹⁸¹ AT2Rs are highly expressed at the onset of kidney development in the undifferentiated mesenchymal tissue of the nephrogenic area, and confined to the distal tubule and ascending limb of loop of Henle, including *macula densa* later in gestation.^{173,180} After nephrogenesis is complete, AT2Rs are predominantly expressed in differentiated epithelial cells of the *macula densa*.^{173,182} In contrast, AT1Rs mRNA expression increases progressively during gestation in cortex and outer medulla of fetal sheep with abundant labeling in more differentiated structures, such as mesangial and endothelial cells of mature glomeruli around term,^{173,180,191} and decreasing to lower levels ten days after birth.¹⁹¹ Previous studies in our laboratory have shown a spatial and temporal intra-renal expression of ATRs during the first three months of post natal life in sheep: AT1R mRNA expression is

higher in the medulla than cortex before six weeks and at twelve weeks is higher in the cortex, whereas AT2R mRNA expression is predominately expressed in the cortex in all age groups studied (Figure 1.6).¹⁹³

The changes in temporal and spatial pattern of expression in cardiovascular and renal structures of both ATRs may suggest specific roles in modulating physiological effects of ANG II during adaptation to life after birth. AT2Rs appear to be rather involved in cell proliferation and early differentiation of the nephron, although their persistence in discrete locations later in development as shown by us¹⁹³ and others,^{188-189,194} may also suggest their possible involvement in cardiovascular and renal physiological function during maturation. Similarly, AT1Rs may play a dual role, early in nephron differentiation and later in development, in modulating cardiovascular and renal function. To date, however, the physiological roles of AT1Rs and AT2Rs and the presence of any potential interaction between these receptors in regulating cardiovascular and renal homeostasis during postnatal development have not been investigated. This forms the basis to my doctoral studies on haemodynamic and renal effects of ATRs during postnatal maturation in conscious lambs.



Figure 1.6 Age-dependent mRNA expression of ATRs in developing sheep kidney Values are mean±SD; *p<0.05 as compared to 24h; †p<0.001 as compared to medulla (Vinturache, Qi & Smith, *FASEB J*, 23:606.2, 2009¹⁹³).

1.1.7.3 RAS regulation of haemodynamics during postnatal maturation

The mechanisms regulating cardiovascular homeostasis at birth are not entirely understood, altough several studies provide evidence of regulatory functions of the RAS on the systemic and renal haemodynamics during development as follows: A dose-dependent rise in arterial pressure in newborn sheep was observed after systemic ANG II infusion,¹⁹⁵⁻¹⁹⁸ while arterial pressure decreased after inhibition of ANG II,¹⁹⁹⁻²⁰⁰ ACE,^{166, 201-207} or AT1Rs,^{198,208-210} in newborn anesthetized piglet, rat, rabbit and newborn conscious lamb. (Figure 1.7)



Figure 1.7 Physiological effects of AT1Rs and AT2Rs in the newborn

The effects on renal haemodynamics were more variable, depending upon species, degree of maturation of the urinary system, and different experimental conditions: conscious vs. anesthetized, type of drug and dose used.²⁰⁸⁻²¹⁰ In previous experiments in conscious newborn lambs in our laboratory administration of the AT1R selective antagonist, ZD 7155, but not the AT2R selective antagonist, PD 123319, was associated with a dose-dependent reduction in arterial pressure and RVR.^{198,211} AT2Rs have, however, been shown to mediate vasodilation in *in vitro* experiments from isolated afferent arterioles,²¹² and mesenteric arteries²¹³ of newborn rats, counterbalancing the

effects mediated by AT1Rs, and, therefore, providing evidence for AT2Rs in modulating vascular tone early in life. It is not known, however, if AT2Rs influence the aforementioned effects of AT1Rs during the postnatal period *in vivo* or if there is a possible interaction between ATRs in mediating ANG II effects on cardiovascular adaptation to life after birth (Figure 1.7). This will also be explored for the first time in my dissertation in experiments in which haemodynamic responses to inhibition of both AT1Rs and AT2Rs separately and together will be measured in conscious developing animals.

1.1.7.4 RAS regulation of renal function during postnatal maturation

Because all of the RAS components are present within the kidney from early nephrogenesis, it has been suggested that ANG II contributes to the growth and structural as well as functional development of the immature mammalian kidney.^{160,214-218} This hypothesis is supported by the observations that treatment of immature rats with either an ACE inhibitor or an AT1R antagonist leads to abnormality of growth and function.¹⁹⁰ Studies in AT1R or AT2R knockout mice have yielded similar findings.^{156,219-221} Furthermore, newborns from mothers who received an ACEI or AT1R antagonist²²²⁻²²³ during gestation elicit persistent anuria and impaired renal function. This suggests that preventing the normal action of ANG II during fetal life can be detrimental to the developing kidney, although the underlying physiological mechanisms for this have not been determined.

There is limited experimental evidence regarding the RAS roles in regulation of kidney function during postnatal maturation. Furthermore, variable effects on glomerular and tubular function have been described, that appear to be dependent upon species, experimental conditions and means of RAS inhibition (reviewed by Chen et al (2004)).¹⁵⁶

To date, roles played by various components of RAS in the adaptation of the newborn kidney to life after birth are still not entirely elucidated. This will be explored in my dissertation by providing the renal responses to inhibition of both AT1Rs and AT2Rs, separately and together, in conscious lambs.

1.2 NITRIC OXIDE

1.2.1 Introduction

Since its discovery, NO, a ubiquitous molecule present in all living cells has been shown to be involved in multiple processes throughout the entire body, including a pivotal role in regulating systemic and renal haemodynamics and renal function. This section provides a short overview of the information available to date regarding selected physiological effects of NO on cardiovascular and renal systems in mature and developing organisms.

1.2.2 Historical perspective

Biomedical research was challenged in 1970 by the first observations of NO inducing smooth muscle relaxation²²⁵⁻²²⁷ and a decade later (in the 1980s), independent work from Furchgott,²²⁸ Ignarro^{229,230} and Moncada and colleagues²³¹ identified endothelium derived relaxing factor (EDRF) released from arteries and veins as NO. Four different streams of biomedical research converged in 1987 to 1988 and triggered a torrent of interdisciplinary research on NO that culminated in the publication of entire journals and books dedicated to this major secretory molecule from mammalian cells.²³²⁻²³³ This pioneering work led to this group (Furchgott, Murad, Ignarro) receiving the Nobel Prize in Physiology and Medicine in 1998.²³⁴ Since its discovery, an avalanche of research has shown extensive and multifaceted roles of NO in physiology and pathophysiology, including regulation of vascular resistance and cardiovascular homeostasis, renal function, fibrinolysis and hemostasis, as well as a wide range of immunological and cytotoxic processes.^{227,235-236} Detailed descriptions of these processes are beyond the scope of this dissertation.

1.2.3 NO signalling pathways

NO, a simple gaseous molecule, albeit highly reactive, is released as a by-product in the process of amino acid substrate L-arginine oxidation to L-citrulline by a family of NO synthase (NOS) enzymes (Figure 1.8).²³⁷ As a gas, NO has a very short life in
biological systems (<2 ms in blood²³⁸ and <0.1s in normoxic tissue²³⁹) because either it binds to heme molecules or Fe-S groups present in proteins such as oxyhemoglobin,²³⁸ or interacts with reactive oxygen to form nitrate and nitrite end products²³⁹ which are then and eliminated in the urine,²³⁶ or reacts with superoxide anion (O₂⁻) to form peroxynitrite.²³⁶ The chemical and biologic properties of NO confer to this labile molecule vasodilator properties and the capacity to act as a local modulator of blood flow through action on the underlying VSMC.²⁴⁰ NO binds soluble guanylate cyclase (sGC), initiating guanosine-5'-triphosphate (GTP) transformation in cGMP, which further mediates vasorelaxation.^{237,241} Although NO easily diffuses through the cell membrane to adjacent cells, it has a relatively limited diffusion distance in tissues. Therefore, NO is generally viewed as an autocrine or paracrine messenger, mostly regulating local intracellular processes or acting on cells in its near vicinity. It has been proposed, however, that NO may play some endocrine systemic functions,²⁴²⁻²⁴³ modulating remotely vascular resistance and tissue perfusion, albeit in certain conditions.²⁴³⁻²⁴⁵

The intrinsic stimulus for basal generation of NO from vascular endothelial cells is the shear stress or tangential shear force generated by blood flowing against the endothelial cells surface that triggers the opening of Ca²⁺ channels on the endothelial cells, thereby leading to the Ca²⁺-dependent activation of nitric oxide synthases (NOS) and local release of NO. In addition, NO may be released in response to endotheliumdependent vasodilators such as acetylcholine (ACh), platelet-derived products (thrombin, serotonin), and vasoactive agents (bradykinin, histamine, norepinephrine, substance P, vasopressin) as illustrated in Figure 1.8. Increasing evidence indicates that, in addition to vasodilation NO mediates a plethora of other physiological actions through cGMPdependent and independent mechanisms.^{237, 246-248}



Figure 1.8 Pathways of nitric oxide (NO) synthesis

NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; R, receptor; ACh, acetylcholine; BK, bradykinin; GTP, guanosine-5'-triphosphate; cGMP, cyclic guanosine monophosphate; GC, guanylate cyclase; NOS, nitric oxide synthase (adapted from Klabunde R (2011)²⁴⁹)

1.2.4 NOS isoforms

In the early 1990s three distinct isoforms of NOS enzymes were identified.²⁴⁶ Two isoforms, neuronal NOS (nNOS or NOS I) and endothelial (eNOS or NOS III) are constitutively expressed.²⁵⁰ Both isoforms are tonically active and readily available since the process does not require synthesis of new proteins, but releases low levels of NO in the picomolar range.²⁵¹ In contrast, the third isoform, inducible NOS (iNOS or NOS II), is not normally expressed within cells under basal conditions, its activity being regulated by cytokine stimulation. The activation of iNOS releases quantities of NO far exceeding those produced by the other two isoforms,²⁵² in the nanomolar levels and for longer

period of times (days).²⁵¹ nNOS and eNOS are dependent on Ca²⁺ and calmodulin for activation²⁵⁰ and all NOS isoforms require several cofactors for proper functioning (i.e. tetrahydrobiopterin (BH4)). Although these enzymes share a common lineage and a high degree of genetic homology, their tissue distribution and expression indicate that they are evolutionarily specialized.²⁵¹

1.2.5 NOS isoforms distribution and expression in adult

The expression of the three NOS isoforms within tissues is diverse and subject to complex and distinct control mechanisms.^{253,255}

The mRNA transcript for nNOS is detected in the central and peripheral nervous system, adrenal medulla, pancreatic β -cells and skeletal muscle among other tissues.²⁵¹ Expression of eNOS is largely restricted to vascular endothelial cells, while iNOS is expressed in multiple cell types, including macrophages, megakaryocytes, and cardiac myocytes.²⁵¹

All three NOS isoforms are present within the kidney, suggesting that their cellular localization may be important for NO-mediated kidney functions. Several studies consistently documented the nNOS isoform confined to *macula densa*.^{255,257} nNOS transcript was also found in the cytosol of afferent and efferent arterioles, glomerular tuft of Bowman's capsule, thick ascending limb, distal tubule and collecting duct cells and renal sympathetic nerves from the perivascular connective tissue and near pelvic epithelium in rat, mouse, guinea pig, rabbit, pig, and human kidney (Figure 1.9).^{253,258-260}

Both eNOS mRNA and protein are strongly expressed in the endothelium of cortical and medullary vessels: arcuate and interlobular arteries, afferent arterioles and endothelium of glomerular capillaries and *vasa recta* of rat, mouse, guinea pig, rabbit, pig and human.^{253,258,261,263} In addition, eNOS has been detected in the proximal convoluted tubule,²⁵⁹ thick ascending limb of the loop of Henle,²⁶² and the inner medullary collecting duct²⁶⁴ (as illustrated in Figure 1.9).



Figure 1.9 Distribution of NOS isoforms in the adult kidney Data compiled from^{253,257-262}

There are reports regarding constitutive expression of iNOS mRNA in healthy mouse and rat kidney,^{260,264-266} with the highest basal expression in the medullary thick ascending limb,²⁶⁵ (see also Figure 1.9) although this is controversial.²⁶⁷ Nevertheless, the expression of both protein and mRNA iNOS is dramatically upregulated following inflammation and in pathophysiological processes (i.e. ischemia-reperfusion), when NO may play either beneficial or detrimental roles for disease progression.^{265,267-268}

1.2.6 Physiological roles of NO in adult

The diversity and complexity of expression and spatial-temporal distribution of NOS isoforms in tissues reflects the diverse roles NO plays in physiological and pathophysiological processes within the body. NO versatile actions range from roles in different defense mechanisms such as protection against infections through recruitment of

neutrophils, to functions in cardiovascular regulation and platelet aggregation, influencing as well cellular proliferation, differentiation, and apoptosis.²⁶⁹⁻²⁷⁰ NO also has numerous physiological roles in the kidney, including modulation of RVR and glomerular haemodynamics,²⁷¹ maintenance of medullary perfusion,²⁷² modulation of TGF,²⁷³⁻²⁷⁴ inhibition of tubular Na⁺ reabsorption²⁷⁵ and modulation of sympathetic activity.²⁷⁶

Inhibition of NO synthases with L-arginine analogues such as N^{G} -nitro-L-arginine methyl ester (L-NAME), is associated with a robust increase in systemic vascular resistance and mean arterial pressure and a decrease in heart rate in adult anesthetized dogs, rabbits and guinea pigs²⁷⁷⁻²⁷⁹ and conscious dogs²⁸⁰⁻²⁸¹ and rats.²⁸² All the aforementioned cardiovascular responses were reversed by the addition of the NO substrate, L-arginine, supporting the role of endogeneous NO in cardiovascular regulation.^{280,283} Inhibition of NO production is also associated with a decrease in regional blood flows and increase in vascular resistance in a variety of vascular beds including kidney, pulmonary, coronary, mesentery and hindquarters.^{282,284} Furthermore, tonic intra-renal release of NO contributes to maintaining lower vascular resistance in the kidney than in most other vascular beds (i.e. brain, femoral, coronary), providing evidence that the renal vasculature is more dependent on endogenous NO than other organs to maintain normal perfusion.²⁷¹ Therefore, NO appears as a major regulator of renal haemodynamics. NO synthesis inhibition effects on renal haemodynamic may be facilitated, at least in part, via activation of the sympathetic nervous system,²⁸⁵⁻²⁸⁶ and/or may result from the disturbance of the intrarenal balance between vasoconstrictor effects of ANG II, norepinephrine and endothelin and the vasodilatory effects of NO²⁸⁷⁻²⁸⁸ as illustrated in Figure P.1 (Preface). As shown by elegant micropuncture studies in isolated rabbit and rat afferent arteriole and glomeruli preparations the tonically intrarenal release of NO regulates both, pre- and post-glomerular vascular resistance.²⁸⁹⁻²⁹³ It has also been suggested that NO generated by eNOS regulates predominantly the tone of the efferent arteriole and mesangial cells, while NO generated by nNOS from macula densa cells modulates afferent arteriole resistance and controls glomerular haemodynamics via modulation of TGF and renin release.²³⁵⁻²³⁶ Furthermore, NO appears as a physiologic

regulator of the glomerular ultra-filtration coefficient (Kf),²⁹⁴⁻²⁹⁵ glomerular capillary pressure and plasma flow.²³⁵ Considerable evidence also supports an important role for NO in the regulation of water, Na⁺ and K⁺ excretion^{271,296} through either direct effects on tubular epithelial transport through the Na⁺/H⁺ exchanger, Na⁺-K⁺-ATPase and amiloride-sensitive Na⁺ channels²⁵³ in cells of the proximal tubule, thick ascending limb of loop of Henle, and collecting tubule²⁶² or indirectly through changes in peritubular haemodynamics and renal interstitial pressure.²⁹⁷⁻²⁹⁸

1.2.7 NOS distribution and expression during ontogeny

nNOS and eNOS mRNA expression is developmentally regulated in the kidney, lungs, choroid and blood vessels.²⁹⁹⁻³⁰³ Localization studies have shown a species-dependent pattern of NOS isoforms expression within the kidney during postnatal maturation, as detailed in the following paragraphs.

In neonatal rat kidney, Fisher *et al* (1995)³⁰⁴ found the strongest nNOS signal in afferent arteriole by day two postnatally. In newborn piglet, nNOS expression was enhanced in interlobular, arcuate and afferent arteriole during first week after birth, after which progressively decreased by three weeks to the adult levels.¹⁸⁸ eNOS expression followed an opposite pattern at these vascular sites, increasing progressively to adult levels by three weeks after birth in swine (Figure 1.10).^{189,305} There are no studies regarding distribution of NOS isoforms in the renal vasculture of the newborn sheep.

In the developing rat kidney, nNOS transcript and protein are highly expressed in epithelial cells of *macula densa* and distal tubule, suggesting an interdependent relation with the renin-producing cells and possible involvement in the organization of the juxtaglomerular apparatus during early postnatal nephrogenesis.³⁰⁴ In sheep and swine, NOS isoforms follow a cortico-medullary pattern of expression during post natal development.³⁰⁶⁻³⁰⁷ For example, whole kidney nNOS mRNA and protein were upregulated at birth in piglets decreasing progressively during renal maturation to adult levels, with levels higher in medulla than in cortex.³⁰⁶ In contrast, previous studies in our laboratory have shown that nNOS expression was higher in cortex than medulla in lambs over the first three months of postnatal life (see also Figure 1.10).³⁰⁷



Figure 1.10 Distribution of NOS isoforms in postnatal developing kidney Data compiled from^{178,305-306,308-309}

Similar to nNOS, the eNOS isoform also undergoes a cortico-medullary regulation during renal maturation in a pattern distinct from that found in adulthood. In rat pups, Han *et al.* (2005)³⁰⁸ observed eNOS-positive cells present in the immature glomeruli of the nephrogenic zone until seven days postnatally. eNOS gradually increased to the highest levels at three weeks postnatal in the developing vascular bundles and peritubular capillaries of the rat medulla (Figure 1.10). A similar cortico-medullary pattern of expression was described from experiments in developing piglets in which whole kidney eNOS expression decreased to the lowest levels at seven day postnatally and returned by day 14 to adult levels.¹⁷⁸ In sheep, eNOS mRNA levels were higher in cortex than in medulla immediately after birth and during the first three weeks post natally, decreasing thereafter.³⁰⁷

There is little information, however, regarding the developmental expression of the iNOS isoform. Studies in developing ovine kidney from our laboratory have shown that iNOS mRNA levels in the cortex were higher than in the medulla until three weeks after birth,³¹⁰ while the iNOS protein levels were similar over the first three months after birth (Figure 1.10).³¹¹

The distinct spatiotemporal pattern of NOS isoform distribution in developing kidney supports the hypothesis of an enhanced functional role of NO in the newborn kidney.^{305-306, 312} It is also possible that during postnatal development, NO contributes to the regulation of renal function and haemodynamics in a step-wise fashion that facilitates the transition to extrauterine life and adaptation of the newborn kidney to life after birth, a phenomen which may be species- and age-dependent.

1.2.8 Physiological roles of NO during postnatal maturation

Increasing evidence shows that, similar to the adult, NO is tonically synthesized within the developing kidney; however, the immature vasculature of the developing organisms is more sensitive and highly responsive to alteration of NO.^{257,312-313} In addition, the newborn vasculature has a greater capacity to release NO in response to a given stimulus compared with later in life. For example, an age-dependent decrease in endothelium-dependent relaxation in response to ACh was observed in aortic rings and carotid arteries of newborn dogs³¹⁴ and femoral artery rings of newborn piglet.³¹⁵ ACh infusion altered to a greater extent MAP and RVR in one than six weeks old conscious lambs^{312,316} and was associated with more profound effects on renal haemodynamics in developing anaesthetized piglet than in their adult counterparts.³¹⁷ Conversely, infusion of the L-arginine analogue, L-NAME, produced a greater increase in RVR in one week as compared with three and six weeks old conscious lambs³¹⁶ and in anaesthetized developing piglet than in adult pigs.³¹³ These studies demonstrate an age-dependent basal release of NO that mediates developmentally regulated physiological effects on the systemic and renal haemodynamics during postnatal maturation. In addition to the profound haemodynamic changes, NO influences on renal function of the immature kidney are also developmentally regulated. It has been suggested that NO regulates glomerular function in the immature kidney through direct vasoactive effects on the capillary beds within the glomeruli and on the post-glomerular arteriole and also through direct modulation of the ultrafiltration coefficient.^{313,318} In conscious lambs in our laboratory, inhibition of endogenous NO production elicited a significant decrease in GFR and FF only in newborn animals, whereas there was prompt diuresis and natriuresis in six but not in one week old lambs.³¹⁹ Similar effects on GFR were described in anesthetized newborn piglets³¹⁷ and rabbits.³²⁰ These findings are consistent with observation in fetal sheep and human infants that newborns generate more endogenous nitrate as a marker of NO production than adults (measured by nitrate levels in urine), and therefore, may contribute to explain the haemodynamic and renal changes that occur with maturation³²¹⁻³²² (reviewed by Baylis and Vallance (1998)³²³ and Honold et al $(2000)^{324}$). The difference in the effects observed in newborn as compared with later in life may reside in the difference in intrarenal distribution of the synthesizing NOS enzymes in newborns as compared to the adult animals³¹³ (as illustrated in Figures 1.9 – 1.10) It is also possible that these different renal effects result from alterations in the circulating levels of other vasoactive factors such as ANG II, since PRA activity decreased age-dependently after L-NAME administration.³¹⁹ This provides evidence that NO and ANG II may work synergistically in modulating renal function early in life. This is detailed below, and forms the basis to my dissertation studies.

1.3 ANGIOTENSIN II AND NITRIC OXIDE INTERACTION

1.3.1 Overview

An increasing body of evidence shows that a delicate balance exists between the two vasoactive factors, the vasoconstrictor peptide ANG II, and the vasodilator NO, in the physiological regulation of cardiovascular and renal function (Figure P.1). Multiple and complex levels of interaction between ANG II and NO have been revealed from experiments *in vitro* and whole organisms, including gene knockout models. Firstly, it is known that synthesis of both ANG II and NO occurs in endothelial cells of the vasculature as well as various cells within the kidney, while VSMC as well as glomerular and tubular cells are important targets of both factors. Secondly, within these structures, RAS and NO systems components are ideally situated in the near proximity to influence

each other's functioning and production.³²⁵ In the short term, through activation of AT1Rs or AT2Rs, ANG II stimulates NO release, while in the long term ANG II regulates the expression of NOS isoforms, and therefore, NO production.³²⁵⁻³²⁶ On the other hand, NO down-regulates the expression of AT1Rs, an effect that contributes to the protective roles of NO on the vasculature.³²⁷ Although the importance of the cross-talk between ANG II and NO in the regulating of systemic and renal haemodynamics and kidney function has recently been explored in adult mammals, this interaction has not yet been established during postnatal development. Therefore, my dissertation experiments were designed to explore whether there is such an interaction between NO and ANG II in regulating kidney haemodynamics and function early in life, under physiological conditions, in awake animals, in order to elucidate if the pathway of such an interaction involves activation of AT1Rs, AT2Rs, or both receptors.

The following section summarizes recent research advances concerning the interaction between NO and ANG II in regulating cardiovascular haemodynamics and renal function in adult and developing mammals.

1.3.2 Interaction between ANG II and NO in adult: roles of AT1Rs and AT2Rs

1.3.2.1 ANG II -NO interaction: cardiovascular homeostasis

The cross-talk between RAS and NO systems plays a crucial role in the regulation of arterial pressure and regional blood flow. It has been suggested that generation of NO in the vasculature may modulate the effects of ANG II in order to protect the circulation from excessive ANG II-induced vasoconstriction.³²⁸ However, the data available are not consistent in regard to the contribution of the ATRs in mediating ANG II-induced NO release. Findings from experimental animals and human subjects point to an AT1R-dependent endogenous NO-mediated beneficial effects on cardiovascular homeostasis. For example, AT1Rs mediate ANG II-induced NO production in aortic rat endothelial cells³²⁹ and coronary vessels from dogs,³³⁰ effects that occur via increased intracellular Ca²⁺ and/ or through a kinin-mediated mechanism. Endothelium-derived NO mediates the vasodilatation induced by the selective AT1R antagonist, losartan, in the coronary artery of anesthetized dogs³³¹ and pulmonary and renal vasculature of anesthetized and

conscious rats.³³²⁻³³³ Conversely, the L-NAME-induced increase in arterial pressure appears to be mediated, at least partially, through activation of AT1Rs in rats.³³⁴ In adult animals, the renal but not the systemic haemodynamic responses to NO synthesis inhibition appear to be mediated by ANG II, predominantly through AT1Rs.³³² In addition, the balance between ANG II and NO appears to regulate the renal circulation with a greater influence on medullary blood flow as compared to cortical flow³³⁵⁻³³⁶ (Figure P.1, Preface).

The pathways that mediate the NO-mediated vasodilatation elicited by AT2Rs are not well understood (reviewed by Toda *et al* (2007)³²⁵). Numerous studies have shown that predominant activation of AT2Rs elicits vasodilation through the cascade of autacoids bradykinin/NO/cGMP pathway *in vitro*,^{142,337-338} and in adult anaesthetized and conscious animals(Figure 1.11).^{94,96,339-340}



Figure 1.11 Pathways of ANG II-NO in adult: roles of AT1R and AT2R

NOS, NO synthase isoforms; BK, bradykinin

ANG II may also be a mediator of the pressure response to decreased NO levels, since L-NAME administration is associated with changes in PRA,^{341,342} elevated vascular and myocardial levels of ACE,³⁴³ and up regulation of the cardiac ATRs.³⁴⁴ These studies demonstrate that, within the adult vasculature, a reciprocal buffering effect between ANG II and NO contribute to preserving the perfusion of the tissues,³⁴⁵ revealing the crucial role of the NO and ANG II balance in maintaining vascular homeostasis (reviewed by Gibbons *et al* (1996)³⁴⁶ and Toda *et al* (2007)³²⁵). Although the pathways of the reciprocal changes are not known, these findings suggest a supportive role for ANG II in maintaining NO activity,³⁴⁷ an effect that could be mediated through AT1Rs and/or AT2Rs.

1.3.2.2 ANG II - NO interaction: renal effects

The balance of ANG II and NO also plays an important role in influencing RVR, GFR and TGF.^{91,333} The distribution of the NOS isoforms in the proximity of RAS components may explain the complex interaction between ANG II and NO within the kidney. For example, NO may function as a paracrine stimulatory mechanism for the local release of renin within the kidney. NO released from nNOS in the *macula densa* cells influences renin secretion from neighbouring granular cells, an effect that is predominantly mediated through activation of AT1Rs.³⁴⁸

Another area of interaction between ANG II and NO in the kidney is the control of renal arteriolar tone and GFR. Several studies in mouse³⁴⁹ and rat³⁵⁰⁻³⁵¹provided evidence to suggest that NO released by activation of AT1Rs may protect pre-glomerular vessels and to a lesser extent post-glomerular vessels from ANG II-induced renal vasoconstriction,³⁵² therefore, contributing to maintaining renal perfusion and glomerular filtration.³⁵³ In addition to influencing net filtration pressure, both vasoactive factors modulate the permeability of the filtration membrane and ultrafiltration coefficient Kf,^{287,291,354} through AT1Rs (Figure 1.11). Renal effects of NO may also be modulated by ANG II through possible activation of AT2Rs. Several studies provide evidence for involvement of AT2R in mediating the interaction between ANG II and NO in regulating reabsorptive and secretory processes at the proximal and distal tubule³⁵⁵⁻³⁵⁷ suggesting

that intrarenal NO may serve as homeostatic mediator of ANG II effects, especially when intrarenal RAS is up-regulated (Figure 1.11). Since the newborn period is characterized as well by elevated levels of RAS it is possible that NO may counterbalance the physiological effects of ANG II in the newborn period. My dissertation explores the presence of such an interaction between ANG II and NO early in life and investigates for the first time the mediating roles that ATRs may play under physiological conditions in the newborn.

1.3.3 ANG II-NO interaction during postnatal development

One of the most interesting features common to these two systems (RAS and NO) is that the components of both systems are up-regulated in the perinatal period. As previously mentioned in this dissertation, RAS is highly activated at birth.^{161,358} In addition, NO system is also activated at birth and the immature renal vasculature is highly responsive to alteration in NO synthesis as reflected by the age-dependent changes in renal haemodynamics and function associated with NOS inhibition.³¹⁶⁻³¹⁷ Several lines of evidence support the presence of an interaction beween ANG II and NO during postnatal development (Figure 1.12).^{209,304,359-360}

In previous studies in conscious lambs in our laboratory, similar to findings in conscious adult rabbits³⁶¹ and primary cultures of mouse juxtaglomerular cells *in vitro*³⁶² it was found that systemic administration of the L-arginine analogue, L-NAME elicited a decrease in PRA. This observation suggests that RAS may modulate, at least in part, the cardiovascular and renal responses to NO inhibition.³¹⁹ In support of this theory, elegant co-localization studies have suggested an interactive relationship between nNOS containing cells and renin producing cells in the developing rat kidney.³⁰⁴ Furthermore, changes in NOS protein and gene expression parallel changes in renin protein and gene expression in the developing piglet kidney.¹⁸⁹



Figure 1.12 Pathways of ANG II –NO interaction during postnatal development

Several studies provide evidence for a functional interaction between ANG II and NO in the developing kidney suggesting that NO may be an important regulator of renal function in the postnatal kidney through modulating RAS activity soon after birth. In the isolated perfused kidney of the newborn rabbit, the renal vasoconstrictor responses to exogenous ANG II were buffered by NO.³⁵⁹ Recent studies in developing porcine kidneys have shown that eNOS and nNOS gene expression and activity is modulated, at least in part, by ANG II through activation of the AT1Rs.³⁰⁵ Solhaug *et al* (1996)²⁰⁹ observed that, in anaesthetized piglets after pre-treatment with an AT1R antagonist, the renal haemodynamic response to L-NAME was attenuated to a greater degree in the immature piglet than in the adult pig. Recent experiments in our laboratory in conscious lambs have shown that L-NAME has no apparent effects being buffered instead by endogenously produced PGs.³⁶³ In addition, Wehlage and Smith (2012)³⁶⁰ demonstrated

that endogenously produced NO and ANG II may work synergistically to modulate cardiovascular homeostasis as well as the arterial baroreflex control of heart rate in the newborn period, effects mediated through activation of AT1Rs. Taken together, these studies demonstrate the complex interaction between the vasoconstrictor and vasodilator factors in regulating cardiovascular homeostasis during postnatal maturation.

Vasoactive factors such as ANG II and NO may also play a critical role in governing cardiovascular as well as fluid and electrolyte homeostasis in the critical period of adaptation to life after birth and modulate the adaptive haemodynamic and renal changes occurring during early post natal life. Nevertheless, our understanding of the interaction between these two systems during development is limited. For example, the roles ATRs play in mediating the renal responses of ANG II in the newborn are not known. Also the presence of an interaction between ANG II and NO in regulating kidney function and the potential roles of AT2Rs in mediating this interaction have not been investigated to date (Figure 1.12).

Therefore, as an extension of our earlier investigations, my dissertation studies were designed to explore whether endogenously produced ANG II regulates haemodynamics and renal function early in life and if these responses involve activation of AT1Rs or AT2Rs or both receptors. Secondly, my studies investigated whether AT1Rs and AT2Rs act individually or are additive in mediating the physiological effects of ANG II during postnatal development. Further, these studies explored whether ANG II is involved in the age-dependent physiological effects of L-NAME through activation of AT1Rs or AT2Rs or a combination of both receptors. It is important to note that all of the experiments described in this dissertation were carried out in conscious lambs under physiological, undisturbed conditions, therefore providing a more relevant insight into the roles of the vasoactive factors ANG II and NO in modulating cardiovascular haemodynamics and renal function during ontogeny.

Chapter Two: STUDY RATIONALE AND HYPOTHESIS

2.1 Rationale of the project

The RAS and NO systems are of primary importance in modulating renal and cardiovascular function. Both systems are activated at birth, possibly contributing to the adaptation of the systemic circulation as well as the newborn kidney to life after birth.^{161,364} There is little information, however, about why this is so and also about the roles that the ATRs may play during postnatal maturation. While the majority of known RAS functions are mediated through AT1Rs, the function(s) of AT2Rs is still under investigation. As previously mentioned, studies in mouse, rat, swine and sheep have shown a developmentally regulated spatial and temporal expression of AT1Rs and AT2Rs^{79,83} within kidney and vasculature: in the developing fetus AT2Rs predominate, whereas AT1Rs expression is low in early gestation and increases with age (detailed in section 1.1.7.2) We have also shown that in the developing ovine kidney, the expression of both ATRs is developmentally regulated over the first twelve weeks of post natal life¹⁹³ (see also Figure 1.6).

Previous studies in our laboratory showed that the effects of ANG II administration to conscious lambs results in a dose-dependent increase in MAP and decrease in RBF, with effects greater at six weeks than at one week of age;¹⁹⁸ these effects were mediated by AT1R but not AT2R. In another study, it was shown that the haemodynamic effects of endogenously produced ANG II occur through activation of AT1Rs, but not AT2Rs²¹¹ (detailed in section 1.1.7.3). The aforementioned studies, however, did not measure any parameters of renal function, including the effects of ANG II on glomerular and tubular function during development or the type of receptor through which these renal effects may be exerted. Therefore, we hypothesized that the post natal changes in intrarenal AT1R and AT2R mRNA expression may reflect age-dependent alterations in physiological effects of ANG II on renal haemodynamics and function. For this purpose, experiments were carried out under selective, individual inhibition of AT1Rs and AT2Rs to reveal the physiological roles each receptor may play in mediating

the cardiovascular and renal effects of endogenously produced ANG II in awake, developing animals.

Studies in adult animals have also provided evidence that AT2Rs play important roles in antagonizing the physiological responses to activation of AT1Rs, especially in conditions of where AT1Rs are inhibited and where ANG II levels are increased (detailed in section 1.1.5). Since in newborn animals, ANG II levels are increased and AT2R expression is up-regulated, we aimed to study the effects of ANG II mediated through AT2R in conditions similar to those described in the literature, after pharmacological inhibition of AT1Rs. This may better reveal AT2R activity in order to investigate the effects of RAS on renal function during early postnatal period. To this end, experiments were also carried out under the consecutive inhibition of both receptors (AT1Rs first, followed by AT2Rs) in order to uncover any possible roles of the AT2Rs in modulating AT1R-mediated effects.

To understand the putative mechanisms that regulate vascular tone and body fluids homeostasis during perinatal period, our laboratory explores a wide array of neurohumoral factors that act in concert to modulate kidney function during the critical adaptation to life after birth. In addition to ANG II, previous experiments conducted in our laboratory have also addressed the role of endogenously produced NO in modulating cardiovascular and renal function during postnatal maturation under physiological conditions. For example, inhibition of endogenous NO production by administration of L-NAME altered cardiovascular haemodynamic and renal function in an age-dependent manner^{312,316,319} (as described in section 1.2.8). In conscious, chronically instrumented lambs, these experiments showed that L-NAME elicits a decrease by 50% in GFR and FF in lambs aged one but not six weeks and decreases proximal Na⁺ reabsorption and total fractional Na⁺ and K⁺ reabsorption that leads to a natriuresis and kaliuresis in six but not in one week old lambs.³¹⁹ Also, the diuresis following L-NAME was accompanied by an increase in free water clearance and decrease in urine osmolality at six weeks but not at one week. In another study, it was shown that cardiovascular function was altered by NO inhibition also in an age-dependent manner: there was a decrease in heart rate which was greater at one than six weeks of age and an age-dependent increase in RVR, which was

greatest at one week.³¹⁶ In addition, NO regulates the arterial baroreflex control of heart rate also in an age-dependent manner, with predominant effects occurring at one week.³¹⁶ More recently, Wehlage and Smith have also shown that these effects of NO on the arterial baroreflex control of heart rate are modulated by ANG II through AT1Rs and appear to be predominant in the newborn period thus demonstrating an interaction between these two systems in regulating the baroreflex.³⁶⁰

In other studies from our laboratory, Sener and Smith also showed a greater capacity of the newborn renal vasculature to release NO in response to a given stimulus as compared with older animals.³¹⁶ In addition, there was a decrease in PRA after L-NAME in both age groups studied, which was greatest at one as compared to six weeks old lambs.³¹⁹ This observation suggests that L-NAME effects on renal haemodynamic and function may result, at least in part, from a decreased level of ANG II, evidence that advocates for an interaction between NO and RAS in early development.³¹⁹

Nevertheless, our understanding of NO interaction with ANG II is limited especially with regard to the newborn kidney. In adult animals it was shown that, within the kidney, the balance of ANG II and NO plays an important role in influencing RVR, GFR and TGF (as described in section 1.3.2). Several studies have demonstrated in adult animals that the NO interaction with ANG II may be mediated through AT1Rs³⁶⁵ as well as AT2Rs.^{339,366} Although the interaction between NO and RAS is extensively described in adult organisms, in the newborn kidney this interaction is not well characterized. Since NO appears to be a more important intra-renal vasodilator in the developing animal compared with the adult, perhaps it functions to counterbalance the highly activated vasoconstrictor mechanisms, such as RAS. Therefore, it was hypothesised that NO effects on renal function and haemodynamics are modulated by ANG II through AT1Rs and AT2Rs.

To this end, the research described in this dissertation represents ongoing studies in our laboratory, in which the role of NO and RAS in influencing systemic and renal haemodynamics and function in conscious developing animals continues to be investigated. The protocols described herein aim to explore the mechanism(s) that underlie the effects of ANG II in the postnatal kidney as well as the possible mechanism which may influence NO actions on systemic and renal vascular resistance and renal function during postnatal maturation.

2.2 Hypotheses and objectives

My dissertation research aims to test two overall hypotheses as follows:

Hypothesis 1:

"During postnatal maturation, ANG II regulates systemic and renal haemodynamics and renal function through activation of angiotensin receptors type 1 and/or type 2 in an age-dependent manner"

Hypothesis 2:

"During postnatal maturation, ANG II modulates NO effects on systemic and renal haemodynamics and renal function through activation of angiotensin receptors type 1 and/or type 2 in an age-dependent manner"

These hypotheses address a number of questions as follows:

- Does ANG II regulate renal function during postnatal development? If so, are these effects mediated through AT1Rs, AT2Rs or both receptors in the immediate newborn period?
- Do AT2Rs modulate the AT1R-mediated ANG II effects on systemic and renal haemodynamics and renal function post natally?
- Is the greater systemic and renal haemodynamic response to NO in the post natal period mediated by ANG II?
- Are the renal effects of NO in the post natal period modulated by ANG II? If so, are these actions occurring through activation of AT1Rs, AT2Rs or both receptors?

The test the hypotheses 1 and 2 and answer the aforementioned research questions, *in vivo* experiments were carried out in conscious lambs at different stages of maturation, trained to the laboratory environment and studied in the awake, undisturbed state. Methods applied in these experiments were state-of-the-art and included measurements of systemic and renal haemodynamic as well as glomerular and tubular function. Experiments were carefully designed to evaluate effects of concomitant inhibition of NO

production as well as AT1R and AT2R on systemic and renal haemodynamics, as well as various parameters of renal function in the developing animal in two separate protocols:

Protocol 1

To investigate if the effects of endogenously produced ANG II on haemodynamics and renal function during the post natal period were mediated through angiotensin receptors AT1R, AT2R, or both, pharmacological selective antagonists directed to these receptors were used. The protocols aims were:

Aim 1: To evaluate the effects of ANG II on renal function during maturation and determine whether they are mediated through AT1Rs or AT2Rs;

Aim 2: To determine if there is cross-talk between AT1Rs and AT2Rs in mediating ANG II effects on systemic and renal haemodynamics and kidney function during the postnatal period.

Protocol 2

To investigate if the effects of endogenously produced NO on systemic and renal haemodynamics and renal function were modulated by ANG II through activation of AT1Rs, AT2Rs or both, AT1Rs and AT2Rs, inhibition of NO production was pharmacologically induced after inhibition of ATRs. The protocol aims were:

Aim 1: To determine if the aforementioned effects of NO on renal and systemic baseline haemodynamic postnatally are modulated by ANG II through activation of AT1R or AT2R or both receptors;

Aim 2: To determine if NO regulation of renal function during postnatal maturation is modulated by ANG II through activation of AT1R or AT2R or both receptors.

The methods used in protocol 1 and 2 are detailed in Chapter 3.

Chapter Three: METHODS

3.1 Overview

Although the research project that forms the basis of this dissertation comprises a unitary experimental design, in order to test the overall hypothesis of this study and to thoroughly answer the specific scientific questions, the experiments were divided into two sections (*3.4 Experimental Protocols*). To test the specific aims of this dissertation (described in Section 2.2 *Hypotheses and objectives*), the experiments employed measurements of systemic and renal haemodynamic variables in conscious chronically instrumented lambs and laboratory measurements of parameters that characterize renal function in urine and plasma collected during the experiments.

All the surgical and experimental procedures described in this dissertation were carried out with the approval of the Animal Care Committee at the University of Calgary, and in accordance with the "Guide to the Care and Use of Experimental Animals" provided by the Canadian Council on Animal Care.³⁶⁷⁻³⁶⁹

The following sections provide an in-depth description of the experiments performed to test the hypotheses of the study, and include choice of species, surgical instrumentation of animals, experimental design and procedures, drugs selection, and data handling and analyses. Details of daily animal care, *in vitro* experiments methods development and testing (electrolytes concentration measurement by ion chromatography, creatinine measurement assay, osmolality measurement) and technical information of *in vivo* haemodynamics measurements are included in Appendices A to H and referenced within the following paragraphs.

3.2 Animals

The experiments described in this dissertation were carried out in two age groups of conscious animals: group one represented by one week old lambs and group two represented by six weeks old lambs. Nine newborn sheep aged 5 to 11 days postnatally formed group one. Thirteen young sheep aged 33 to 49 days after birth were studied in group two. The demographic characteristics of the two groups of animals are presented in

Table 3.1. All lambs were obtained from a local source (Woolfitt Acres, Olds, Alberta, Canada).

	One week (N=9)	Six weeks (N=13)
Sex	3♀/6♂	9 ♀/4♂
Age (days)	8 ± 2	41 ± 4 †
Body weight (kg)	7.6 ± 1.7	15.2 ± 2.8 †
Total kidney weight (g)	62.4 ± 14.1	79.9 ± 8.7 †
Right kidney weight (g)	31.2 ± 7.5	38.5 ± 5.1 †
Left kidney weight (g)	31.5 ± 7.2	41.4 ± 4.8 †

Table 3.1 Demographics of conscious lambs

Values are mean \pm SD; \dagger p<0.05 compared to one week

3.2.1 Justification of species selection

Animals as experimental models have been an essential component of medical research for centuries. Studies in various species have made a tremendous contribution to our understanding of integrative physiology and pathophysiology with respect to the perinatal period. Significant insight into the molecular and cellular basis of cardiovascular and renal physiology has come from the small animal models, particularly mice. Considerable information regarding developmental physiology has been gained from genomics and subsequent transgenesis.³⁷⁰ Studies in mammals phylogenetically closest to humans such as sheep, pig, dog and non-human primates represent a most appropriate experimental model for cardiorenal physiology in whole organisms.³⁷¹ There are, however, significant differences when other mammals are contrasted to humans and findings in other animals do not always correctly predict what would be found in humans. As a particular species is selected, inter- and intra- species variability should be taken

into account. The functioning of organs and systems as well as their modulating factors are different in different species and even in different strains of the same species such that they may react differently to environmental conditions.³⁷¹ Therefore, it is important to identify the experimental model that can provide the most reliable information from a human physiology standpoint.

Fetal and newborn sheep are an excellent *in vivo* model for studies of cardiovascular and renal development and function and provided much of our understanding of the RAS physiology during development.^{30,161,175,202,204,372}

The sheep is the species selected for the experiments of this doctoral dissertation for several reasons:

First, the development and growth of lambs' kidney follows a similar profile to that of the human kidney, nephrogenesis being completed during the latter part of the gestation. Like humans, the sheep has a long gestation with much of renal ontogeny occurring before birth.¹⁷³ Nephrogenesis is complete at birth in guinea pig, by 130 days of gestation in sheep (the term is at 145-150 days)¹⁷⁴ and in humans, where term is~ 40 weeks (38-42 weeks range) by 36 weeks of gestation.³⁷³ There is no postnatal spurt of kidney growth in lambs or human beings. Therefore, at term, the kidneys of fetal sheep, similar with humans, baboons and guinea pig, have a full complement of nephrons²¹⁴ as compared with other species, including rat, rabbit, dog and pig in which the kidney is not completely developed at birth, nephrogenesis continuing after birth.^{171,189,359,374}

Second, lambs have been used to investigate many different aspects of developmental physiology, including previous research conducted in our laboratory over the last two decades. There is also considerable information in the literature regarding the development and function of the fetal and newborn sheep kidney that could assist us in data interpretation. Similarities regarding some characteristics, such as the number of fetuses and size at term, as well as recorded variables of interest (RBF, GFR, urinary flow rate), make the sheep an ideal animal to study parameters of renal function during development.^{215,375-377} Furthermore, a large body of data regarding fetal and neonatal renal physiology has been carried out in chronically catheterized animals, conditions similar with those described here, to minimize the effects of anaesthesia and/or surgery.

Third, ovine exhibit vascular anatomy and renal gross anatomic structure similar to that of humans and have been the subject of multiple translational studies.³⁷⁸⁻³⁸⁰

Fourth, lambs are large enough to make the surgical and experimental manipulations necessary for the various physiological measurements. In addition, lambs are of sufficient size to allow chronic instrumentation compared to more altricial species such as rats, mice etc. which are only mm to cm in size at birth. Furthermore, lambs are placid animals that can be trained to stand calm and rest quietly in supportive slings during experiments, so that measurements reflect as close as possible to baseline physiology.

Fifth, there is a high level of structural homology between the RAS and NO system components and their renal and vascular distribution during ontogeny in humans and sheep as compared with dog, rabbit, rat and mouse.^{83,175,191,193,381}

Undoubtedly, large animals such as ovine, recapitulating human phenotype, play a crucial role in the elucidation of biological pathways involved in development.

3.2.2 Conscious vs. anaesthetized animals in physiology studies

The experiments described in this dissertation were carried out in conscious, chronically instrumented lambs. Physiological measurements obtained in conscious animals, in the absence of the effects of surgery and anaesthesia on the measured variables are reliable, repeatable and translatable. Surgery and volatile anaesthetics have been shown to depress ventricular function, evoke vasoconstriction, particularly of renal vasculature, and attenuate cardiovascular reflexes.³⁸²⁻³⁸³ All volatile anaesthetic agents produce a decrease in systolic and diastolic function and baroreflex depression to a varying degree, most probably through a central mechanism.³⁸³ A variety of anaesthetic agents are known to interfere with the neural and endocrine mechanisms that regulate cardiovascular and renal function.³⁸⁴ Three vasopressor systems have been shown to contribute to the regulation of arterial pressure during anaesthesia: the sympathetic nervous system, the RAS and AVP.³⁸⁵⁻³⁸⁶ Effects of RAS activation during anaesthesia is associated with an increase in plasma ANG II concentration from baseline values of 15-20 $\text{ pg}\cdot\text{mL}^{-1}$ up to >100 $\text{ pg}\cdot\text{mL}^{-1},^{387-388}$

impairment of the sympathetic nervous system activity through decreased epinephrine concentration, and increased AVP release.^{386,389} Arterial pressure during anaesthesia and surgery is also RAS-dependent resulting in robust hypotensive responses to RAS inhibition with ACE inhibitors or ATRs antagonists that further enhance the profound alterations of the anaesthesia in rat, cat, sheep, dog and humans.^{165,386,390-393} Deng *et al* (1996) have shown that renal effects of NO are altered during elevation of ANG II, such as occurs under surgery and anaesthesia.³⁹⁴ Studies conducted in barbiturate-anaesthetized rats reported that non-sterile surgery induces NOS expression which leads to local production of NO even in the presence of L-NAME.³⁹⁵ Therefore, reduced baseline blood flow and high endogenous levels of ANG II which occurs under the influence of anaesthesia may create a situation where further alteration of vascular tone and renal blood flow may be difficult to achieve. That is, treatment with NO synthesis inhibitor and/or the ANG II receptors antagonists (as used in the experiments described herein) may not be as effective, and would certainly not reflect their physiological effects.

Apart from the changes elicited by the anaesthetic drug itself, many secondary factors may affect renal and cardiovascular physiological responses, such as intravascular volume status and positive pressure ventilation. In addition, surgical stress leads to renal vasoconstriction and salt and water retention, which may last for days, while anaesthetic agents decrease GFR and urinary output.³⁸² Sidi *et al* (1982) showed that a minimum of 48h is necessary for cardiac output, arterial pressure and heart rate to restore to baseline levels in studies of cardiovascular function conducted in conscious newborn lambs undergone thoracotomy.³⁹⁶ Similar residual effects of surgery and anaesthesia, with alterations in baseline cardiovascular variables were observed in other species and required varied recovery periods to reach the haemodynamic steady state.³⁹⁷⁻³⁹⁸ Several studies have revealed that there is no elevation in plasma levels of catecholamines, PGs, PRA, ANG II or AVP by three days after surgery in sheep,^{174,202,375,399-401} confirming the findings from our laboratory.^{198,206,211,316,372,402-404} Although the use of conscious, chronically instrumented animals such as in the present experiments requires

considerable space and resources, are labor-intensive and time-consuming, the tremendous benefits of such physiological studies outweigh these negative consequences.

3.2.3 Age group selection

The choice of ages for the present studies (one and six weeks) was based on agedependent differences in: 1) baseline systemic and renal haemodynamics (as measured by us and others,^{198,206,316} 2) circulating levels of vasoactive factors,^{198,312,372,402-407} 3) the responsiveness of the renal vasculature to numerous vasoactive agents, 198,206,316,372,402-407 and 4) molecular expression of ATRs and NOS isoforms within the kidney and systemic vasculature.^{175-176,193,305,307,310-311} Importantly, the two age groups reflect age-dependent major physiological changes that concern vasoactive factors, ANG II and NO, during postnatal development, as detailed in previous sections of this dissertation: from the immediate newborn period (one week old lambs), characterized by high activation of RAS and NO systems to later in life (six weeks old lambs) when their activity returns towards the levels specific to the adult. The choice of ages is also based on our previous experiments, in which similar age groups were studied. For example, there are significant differences between one and six week old lambs in responses to blood volume depletion by furosemide⁴⁰⁸ as well as in the regulatory roles of endogenously produced vasoactive factors such as bradykinin,⁴⁰⁹ NO,^{307,316,319,410} endothelin-1,^{403,411} PGs,⁴⁰² and ANG II.^{193,198,211} Studies in lambs aged one, three and six weeks provided evidence for the maturational profile of the mechanisms underlying age-dependent effects of ANG II and NO in modulating renal haemodynamics as well as kidney function during ontogeny.^{307,316,319,410} The temporal and spatial expression of ATRs and NOS isoforms is age-dependent in sheep, with a switch in distribution pattern occurring around three to six weeks,^{193,310} as decribed in an earlier section of this dissertation. Therefore, it is reasonable to consider that the anatomical localization may be reflected in functional changes during postnatal maturation and that ANG II and NO may exert different physiological effects at one week as compared to older animals studied at six weeks.

3.3 Surgical procedures

This section describes the surgical protocol common to all studies carried out in each animal as follows:

3.3.1 Pre-surgery preparation

The vessel and bladder catheters were prepared in advance of the surgery and were used in surgical procedures outlined in the section 3.3.3. Details regarding this preparation are included in *Appendix A, Catheters preparation*.

3.3.2 Animal preoperative preparation

The details regarding preoperative preparation of the animals and the veterinary assessment prior surgery are provided in *Appendix B*, *section B.3 Preoperative preparation*.

3.3.3 General surgical procedures

Each animal underwent one surgical operation prior to experimentation. On the morning of surgery body weight was measured and the lamb was transported into the surgery suite after a thorough physical examination by the clinical veterinarian. Surgeries were performed using aseptic techniques as previously described.^{198,206,211,407,410} The animals were placed on a thermostatically controlled heating pad to maintain body temperature ~ 39.5°C, which was continuously monitored with a rectal thermometer (Becton Dickinson Canada Inc., Oakville, ON, Canada). The surgical procedures were carried out in animals in which anaesthesia was induced with a mask and 5% isoflurane (Abbott Laboratories Ltd., St-Laurent,QC, Canada) in oxygen and maintained, after intubation with a cuffed endotracheal tube, with isoflurane 1.5-2.5 % in a 3:2 mixture of nitrous oxide: oxygen via a ventilator (Harvard Apparatus Model 670, Holliston, MA, USA). To assess the level of anaesthesia and ventilation during surgery the arterial pressure, heart rate, peripheral pulse and oxygen saturation were measured continuously with multi-parameter monitoring equipment (SurgiVet Polymount[®], GCX[®] Corporation,

Petaluma, CA, USA) and the animal received crystalloid solutions (5% dextrose in 0.9% sodium chloride) to assist in maintaining plasma volume.

With the animal in a supine position, through the means of inguinal incision using an adjustable cautery (Valleylab Inc. Boulder, CO, USA), the femoral vessels were identified, dissected and the above mentioned vessel catheters were inserted into the right and left femoral vein and artery, and advanced to the level of inferior vena cava and aorta, respectively (Figure 3.1). The catheters were tunneled subcutaneously to exit the animal on the right and left flank, checked for competency by flushing with heparinized saline, and sealed with heparin to maintain patency. The skin incisions were sutured with surgical thread and sprayed with antibiotics. After surgery the catheters were secured inside the right and left pocket of the lamb's body jacket (described in Appendix B, section B.3). The vessel catheters allowed arterial and venous pressure monitoring and administration of drugs and solutions during experiments. To facilitate renal function measurements, a catheter (described in Appendix A) was placed in the bladder to assist with urine sampling and urinary flow rate measurements during experiments (Figure 3.1). A local analgesic block was performed prior to skin incision by infiltrating Marcaine[®] 0.50% (Bupivacaine Chlorhydrate, Hospira Healthcare Corporation, Montreal, QC, Canada) subcutaneously and intramuscularly over the incision line. This analgesia assisted relieving postoperative pain. Through means of an abdominal midline incision, the urinary bladder was exposed and the aforementioned prepared catheter was inserted directly across the bladder wall and secured in place. The positioning and the drainage capacity of the catheter were confirmed and the catheter was closed at the free end, secured in place and secured to the body jacket. Urine drainage through this catheter was allowed only during the experiments, leaving the urethra patent and functioning so that at any other time the bladder was emptied by normal micturition. After surgery, the bladder catheter was secured to the aforementioned body jacket.

To facilitate the ultrasound real-time measurements of RBF a flow transducer was placed around the right renal artery (Figure 3.1). The animal was placed on its left side. Before proceeding with the skin incision a Marcaine[®] 0.5% analgesic incision line block was performed as described above. Through a right flank incision and a retroperitoneal

approach the kidney was exposed and the renal artery was dissected. A pre-calibrated ultrasonic flow transducer (size 3-6S, Transonic Systems Inc., NY, USA) was placed around the renal artery for the measurements of RBF during experiments, as previously described.^{206,211} After closing the incision, the transducer cable was tunneled subcutaneously to exit the animal on the right flank. The skin incisions were sutured with surgical thread and sprayed with antibiotics. After surgery, the transducer cable was secured in the right pocket of the aforementioned body jacket.

At the termination of surgical procedures, animals were extubated and supported in sternal recumbency until standing. For ~ 30-45 min after surgery animals were allowed to recover from the effects of anaesthesia inside a Shor-Line intensive care unit for small animals (Schroer Manufacturing Company, Kansas, USA), in which the temperature and oxygen concentration were accurately controlled. Lambs were returned to the ewe after they were able to stand (approximately 45-60 min) and were closely monitored until suckling.

A schematic of the chronically instrumented animal is provided in Figure 3.1.



Figure 3.1 Schematic of chronically instrumented lamb

3.3.4 Animal postoperative care

The details regarding postoperative care of the animals are provided in Appendix A, section A.4. The animals were allowed to recover at least three days after the completion of the surgery. Previous reports from our laboratory^{372,412} and others³⁹⁶ have shown that this time interval is sufficient for lambs to recover from the effects of anaesthesia and surgery and to allow the cardiovascular and renal variables to return to the pre-surgery baseline values (as described in section 3.2.2 above). During the recovery period, animals were trained daily for at least one hour to rest quietly in a supportive sling in the laboratory environment to allow them to be accustomed to their surroundings during experiments.

3.4 Experimental protocols

To investigate the research questions, two major protocols were designed, each of which is subdivided into other two protocols as follows:

3.4.1 Protocol I

AT1R and/or AT2R mediate the effects of endogenous ANG II on systemic and renal haemodynamics and renal function during postnatal maturation

This protocol was designed to investigate whether the effects of endogenously produced ANG II on systemic and renal haemodynamics and renal function during the post natal period are mediated through either AT1R, or AT2R, or both receptors. According to the aims described in the section 2.2 *Hypotheses and objective*, this protocol was divided into two parts:

Protocol I.1. AT2Rs modulate the systemic and renal haemodynamics effects of endogenous ANG II mediated through AT1Rs during postnatal maturation.

Protocol I.2. AT1Rs and/or AT2Rs mediate the effects of endogenous ANG II on renal function during postnatal maturation.

Pharmacological inhibition of ATRs was achieved using I.V. administration of selective antagonist to the AT1R, ZD 7155, to the AT2R, PD 123319, and to both receptors, ZD 7155 and PD 123319 (the choice of drugs selection described in section

3.7). For each protocol three experiments were carried out in each animal, in random order, at intervals of 48h, and under physiological conditions:

Experiment one - administration of ZD 7155 and Vehicle,

Experiment two - administration of PD 123319 and Vehicle,

Experiment three - administration of ZD 7155 and PD 123319.

The Vehicle portion (detailes provided in section 3.4.3) in the experiment one and two has been included to control for the second ATR antagonist administration in the experiment three. A schematic of this protocol is provided in Figure 3.2.





The schematic shows the three experiments carried out in random order in each animal. Each experiment consisted of haemodynamic and renal measurements for 30 min before (Control) and 30 min after infusion of: ZD 7155 plus vehicle (experiment 1); PD 123319 plus vehicle (experiment 2); ZD 7155 plus PD 123319 (experiment 3).

3.4.2 Protocol II

NO effects on renal and systemic haemodynamics and renal function are modulated by ANG II through activation of AT1R or AT2R or both receptors during postnatal maturation

This protocol was designed to investigate whether the effects of endogenously produced NO effects on systemic and renal haemodynamics and/or renal function are modulated by ANG II through activation of AT1R, AT2R or both receptors, AT1R and AT2R. To facilitate the study, this protocol was divided in two additional parts:

Protocol II.1. Effects of NO on resting systemic and renal haemodynamics are modulated by ANG II through activation of AT1R or AT2R or both receptors during postnatal maturation.

Protocol II.2. Effects of NO on renal function are modulated by ANG II through activation of AT1R or AT2R or both receptors during postnatal maturation.

To assist in the investigation of endogenously produced NO, its production was inhibited pharmacologically by administering of the L-arginine analogue, N^{G} -nitro-L-arginine methyl ester (L-NAME) (the choice of drug detailed in section 3.7) in addition to ZD 7155, PD 123319 or both angiotensin receptor antagonists, ZD 7155 and PD 123319. For each protocol three experiments were carried out in each animal in random order, at intervals of 48h, under physiological conditions:

Experiment one - administration of ZD 7155 and L-NAME,

Experiment two - administration of PD 123319 and L-NAME,

Experiment three - administration of ZD 7155 plus PD 123319 and L-NAME.

A schematic of this protocol is provided in Figure 3.3. The order of drugs administration, respectively, ATRs antagonists followed by NO synthesis was chosen based upon the following rationale: 1) the haemodynamic effects of separate infusion of ATRs inhibitors ZD 7155 and PD 123319 are known from previous experiments in our laboratory; they occur within min of infusion and persist over 120 min¹⁹⁸ allowing, therefore, additional interventions; 2) the haemodynamic and renal effects of L-NAME alone have been determined in previous studies in conscious lambs in our laboratory in

similar experimental conditions and, therefore, could be used to assist with the interpretation of the findings from the present experiments; 3) the proposed sequence of drugs administration facilitates measurements of the renal effects of ATRs separately and together firstly followed by identification of ATRs contributions to modulation of NO effects on haemodynamics and renal function.

Drugs selection is described in a subsequent section of this chapter (Section 3.7 *Drugs selection*) and preparation of drugs is described in Appendix C.



Figure 3.3 Experimental Protocol II

The schematic shows the three experiments carried out in random order in each animal. Each experiment consisted of heamodynamics and renal measurements for 30 min before (Control) and 30 min after administration of ZD 7155 plus L-NAME (experiment 1); PD 123319 plus L-NAME (experiment 2); ZD 7155 plus PD 123319 plus L-NAME (experiment 3).

3.4.3 General experimental protocol

On the day of the experiment, the animal was transported to the laboratory and placed in the supportive sling. Experiments were scheduled at the same hour and commenced after 60 min of equilibration that allowed the animal to become accustomed to the experimental environment. A solution of 5% dextrose in 0.9% NaCl (B. Braun Medical Inc., Irvine, CA, USA) was infused IV at a rate of 4.17 mL·kg⁻¹·h⁻¹ throughout this period and the duration of the experiment, using a Microinfusion pump (MI 60-1B, World Precision Instruments, Sarasota, Fl, USA) to help maintain fluid balance. A rectal temperature probe (Thermalert TH-5, Physitemp, Clifton, NJ, USA) ensured continuous monitoring of the lamb's body temperature (~39°C, normal body temperature in sheep⁴¹³). The urine was allowed to drain and the urinary flow rate was measured. The left femoral venous and arterial catheters were connected to pressure transducers (Model P23XL, Statham, West Warwick RI, USA) for continuous monitoring of venous and arterial pressure, respectively (as detailed in Appendix D). The flow transducer placed around the renal artery was connected to a transonic flow meter (T101, Transonics Systems, New York, USA) for real time measurement of RBF (as detailed in Appendix D). The pressure and flow transducers were connected to a polygraph (Model 7, Grass Technologies, Astro-Med Inc., West Warwick, RI, USA) that continuously recorded the haemodynamic variables. The recordings were simultaneously digitized at 200 Hz using the data acquisition and analysis software package, PolyVIEWTM 16 (Grass Technologies, Astro-Med Inc., West Warwick, RI, USA) for later off-line analyses of haemodynamic variables (Figure 3.4).



Figure 3.4 Experimental setup

A: chronically instrumented conscious lamb, B: data acquisition on the polygraph, and C: data acquisition in PolyVIEWTM

The experiments consisted of measurements of systemic and renal haemodynamic and renal function variables for 30 min before, Control period, and 30 minutes after each drug administration as per specific protocol (Figures 3.2 and 3.3), at the following rates: AT1R antagonist, ZD 7155, was administered as an I.V. infusion of 70 μ g·kg⁻¹·h⁻¹ following an I.V. bolus of 100 μ g·kg⁻¹. AT2R antagonist, PD 123319, was administered as a bolus of 100 μ g·kg⁻¹, followed by an infusion of 70 μ g·kg⁻¹·h⁻¹. These doses were carefully selected from previous dose-response experiments,^{198,211} and administered using a microprocessor-controlled syringe pump (Mi 60-1B, World Precision Instruments Inc., Sarasota, Fl, USA) at a rate of 4.17 mL·kg⁻¹·h⁻¹. The doses of ZD 1755 were selected based on previous dose-response studies conducted in our laboratory as that which elicited a significant decrease of MAP for 2h and abolished by 100% the pressor response of to ANG II EC₅₀ (12.5 ng·kg⁻¹) administered I.V.¹⁹⁸ The efficacy of inhibition of AT1Rs was ensured by testing the pressor response to I.V. administration of ANG II.¹⁹⁸ The NO synthesis inhibitor, L-arginine analogue, L-NAME, was administered I.V. as a bolus at a concentration of 20 mg·kg⁻¹.^{312,316,410} This dose was determined previously as that which significantly attenuated intrarenal NOS, assessed by inhibition of intrarenal production of ACh for 2h.^{312,410} Vehicle was represented by 0.9% saline solution, infused at a rate that equals the rates of the drug infusion. An intravenous loading of lithium chloride (LiCl) was injected slowly (over 10 seconds) 30 min before starting the experiments as bolus injection of 200 μ m·kg⁻¹ for later determination of proximal tubular Na⁺ reabsorption, according to the method previously described.^{377,414-417} Preparation of drugs described in detail in Appendix C.

Urine was collected continuously by draining the bladder catheter and urine was sampled at every 30 min collection period. At the end of each urinary collection period urine flow rate (V) was recorded and samples were stored at -70°C for later determination of urinary electrolytes (Na⁺, K⁺, Li⁺, Cl⁻, NO₃⁻), creatinine and urinary osmolality. At the midpoint of each 30 min urinary collection, blood samples (~3 mL) were removed from the right femoral arterial catheter. Whole blood (~0.5 mL) was used for immediate measurement in duplicate of hematocrit using a microhematocrit centrifuge (Clay Adams, Parsippany, NJ, USA) and heparinized micro-hematocrit capillary tubes (VWR Scientific Inc., Drummond Scientific CO., USA). Whole blood (2.5 mL) was also withdrawn into a chilled heparinized tube and immediately centrifuged and the supernatant stored at -70°C for further analysis of plasma concentrations of electrolytes (Na⁺, K⁺, Li⁺, Cl⁻), creatinine and osmolality.

Urinary and plasma osmolalities were measured by freezing point depression, using a micro-osmometer (2430 Multi-OSMETTETM, Precision Systems Inc., Natic, MA, USA) (method described in Appendix E). Urine and plasma creatinine concentrations were measured using a commercially available kit (QuantiChrom Creatinine assay kit, BioAssays Systems, Hayward, CA) (method detailed in Appendix F). Urinary and plasma electrolytes were later measured on thawed samples by ion chromatography (IC 680, Methrom AG, Herisau, Switzerland) (as described in Appendix G).
3.5 Post-mortem

At the end of the three randomized experiments, lambs were administered a lethal dose of pentobarbital sodium (Euthanyl, Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada). After *postmortem* inspection for verifying catheter placement, the zero offset of the flow transducer was recorded (for correction of RBF). The kidneys were removed, and examined grossly, then weighed to normalize haemodynamics and renal function measurements between the two age groups.

3.6 Computations

3.6.1 Analytic procedures

The measured and calculated cardiovascular variables are summarized in Table 3.2. Arterial and venous pressures measured in real-time were continuously collected and recorded onto a polygraph (see also section 3.4.3 and Appendix D). Using ultrasound techniques, the blood flow to the kidney was continuously measured and recorded onto the polygraph (section 3.4.3 and Appendix D). All haemodynamic data were analyzed using PolyViewTM software (Appendix D). Continuous data were averaged every minute, retrieved using markers (Figure D.1, Appendix D), saved as text files, and imported into Microsoft Office Excel for further analysis. HR was determined from the systolic peaks of the arterial pressure waveform (Table 3.2). RVR was calculated as (MAP-MVP)/RBF, where MAP represents mean arterial pressure and MVP is mean venous pressure (Table 3.2). All variable were averaged for the 30 min Control period, then every 30 min for each treatment period.

All renal variables were measured (Table 3.3) or calculated (Table 3.4) for 30 min Control period, then for 30 min intervals over which the treatments were applied (Figures 3.2 - 3.3) and the data imported into Microsoft Office Excel sheets for further analysis. Renal plasma flow (RPF) was determined from the formula: RPF (mL/min) = [1-(RBF·Hct)], where Hct (%) represents the haematocrit. GFR was estimated as the clearance of creatinine (Table 3.4) (the rationale for this method and an in depth description of the technique is provided in Appendix F). Filtration fraction (FF) was determined as GFR/RPF. Fractional reabsorption (FR) of electrolytes X (FRX) (where X is Na⁺, K⁺, Li⁺, Cl⁻, NO3⁻) was determined as the ratio of electrolyte clearances to GFR from the formula: FRX (%) = $[1-(CX/GFR)]\cdot 100$, where Cx represents the clearance of electrolyte X. Fractional reabsorption of Li⁺ (FRLi) was calculated to provide an index of proximal tubular fractional reabsorption of Na⁺ (FRNa)^{312,377,414-415} (the principle and limitations of the method are provided in Appendix H) from the formula: FRLi= $[1-(CLi/GFR)]\cdot 100$, where CLi represents Li⁺ clearance.^{375,377} Details regarding the calculations for GFR, FF and other parameters of renal function as well as directly measured haemodynamic and renal variables are summarized in Tables 3.2 - 3.4. (the renal variables are reported in gram per kidney weight).

VARIABLE	DESCRIPTION	CALCULATION	UNITS
DIRECT MEASURED PARAMETERS			
МАР	Mean arterial pressure	Direct measurement	mmHg
SAP	Systolic arterial pressure	Direct measurement	mmHg
DAP	Diastolic arterial pressure	Direct measurement	mmHg
MVP	Mean venous pressure	Direct measurement	mmHg
RBF	Renal blood flow	Direct measurement	$mL \cdot g^{-1} \cdot min$
CALCULATED PARAMETERS			
RVR	Renal vascular resistance	(MAP-MVP)/RBF	mmHg·mL ⁻¹ ·g·min
RPF	Renal plasma flow	RBF • (1-Hct)	$mL \cdot g^{-1} \cdot min^{-1}$
HR	Heart rate	From R-R intervals	beats.min ⁻¹

Table 3.2 Measured and calculated cardiovascular variables

	Table 3.3	Measured	renal	variables
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VARIABLE	DESCRIPTION	CALCULATION	UNITS
V	Urinary flow rate	Direct measurement	$\mu L \cdot g^{-1} \cdot min^{-1}$
Hct	Haematocrit	Direct measurement	%
Pcreat	Plasma creatinine	Direct measurement	$\text{mmol} \cdot \text{L}^{-1}$
Ucreat	Urine creatinine	Direct measurement	$mmol \cdot L^{-1}$
Рх	Plasma concentration of electrolyte X	Direct measurement	$\text{mmol} \cdot \text{L}^{-1}$
UX	Urinary concentration of electrolyte X	Direct measurement	$\mu mol \cdot L^{-1}$
POsm	Plasma osmolality	Direct measurement	mOsm•kgH ₂ O ⁻¹
UOsm	Urine osmolality	Direct measurement	mOsm•kgH ₂ O ⁻¹

Table 3.4 Calculated renal variables

VARIABLE	DESCRIPTION	CALCULATION	UNITS
RPF	Renal plasma flow	RBF • (1-Hct)	mL•g ⁻¹ •min ⁻¹
GFR	Glomerular filtration rate	Ucreat•V/Pcreat	mL•g ⁻¹ •min ⁻¹
FF	Filtration fraction	GFR/RPF	%
UXV	Urinary excretion rate of electrolyte X	UX•V	µmol•L ⁻¹ •g ⁻¹
Сх	Clearance of electrolyte X	UX•V/PX	mL•g ⁻¹ •min ⁻¹
COsm	Clearance of osmoles	UOsm•V/POsm	mL•g ⁻¹ •min ⁻¹
CH ₂ O	Free water clearance	V-COsm	mL•g ⁻¹ •min ⁻¹
FEX	Fractional excretion of electrolyte X	[GFR/Cx]•100	%
FRX	Fractional reabsorption of electrolyte X	[1-(Cx/GFR)] •100	%
PFRNa	Proximal fractional reabsorption of Na	[1-(CLi/GFR)] •100	%
DFRNa	Distal fractional reabsorption of Na	FRNa - PFRNa	%
TTKG	Transtubular K gradient	(UK/PK)/(UOsm/POsm)	
UNa/UK	Urinary Na to K ratio	UNa/UK	

3.6.2 Statistical analysis

Data obtained from experiments were entered into MS Office Excel sheets and analyzed using IBM SPSS Statistics 19 for Windows software. Effects of drugs (ZD 7155, PD 123319, L-NAME) and age (one and six weeks) on the measured and calculated variables were evaluated using analysis of variance ANOVA procedures for repeated measures with factors being treatment and age. Where the F value was significant, Holm-Sidak multiple comparison procedures were applied to determine where the significant differences occurred. Baseline variables were analyzed using nonpaired Student's t-test. Significance was accepted at the 95% confidence interval. The normal distribution of the data was evaluated with Kolmogorov-Smirnov test.

A sample size of 9 animals per age group was calculated based upon previous observation in our laboratory of an expected 20 % difference in MAP between the group means. The sample size was adjusted according to the technical limitations (i.e. surgical instrumentation malfunctions that influenced renal haemodynamics measurements success rate); as a result a total of 22 lambs were included in the study. For the renal haemodynamic data presented in this dissertation N=8, one week group, and N=10, six weeks group; for the systemic haemodynamic and renal function data N=9, one week and N=13, six weeks.

The data were plotted and graphic represented using Sigma Plot 12 data analysis and graphing software (Systat Software Inc., San Jose, CA, USA). All data presented in the text, tables and figures are expressed as mean \pm standard deviation. The renal variables were normalized per gram of kidney weight to allow comparisons between the two age groups.

3.7 Drugs selection

3.7.1 Inhibitors of ANG II receptors

A variety of techniques have been employed to study the physiological and pathophysiological roles of ATRs. These include molecular techniques such as gene knockout and recombinant DNA models and a wide array of biochemistry and molecular biology methods. However, genetically engineered models have been employed in mice, but are not available in other species, including larger animals such as sheep. On the other hand, the conclusions of *in vitro* studies, although provided much of our insight into the functions of the vasoactive factors such as ANG II and the ATRs, should be viewed with caution due to specific, limited experimental conditions and absence of regulatory internal milieu present in living organisms. Pharmacological studies using agonists and antagonists provided much of our knowledge in deciphering the functions of the ATRs in *vivo*. Since both receptors, AT1R and AT2R, have similar affinity for the natural agonist ANG II, the present study employed the use of selective antagonists as a pharmacological approach to investigate their physiological roles *in vivo*. Therefore, in this study two non-peptide selective ANG II receptor antagonists, which show high affinities for the two distinct subtypes of ANG II binding sites were chosen to facilitate the study of ATRs physiological functions in sheep during postnatal maturation: ZD 7155 and PD 123319. While PD 123319 is well known and used on a large scale in molecular biology and physiological studies, much less is known about ZD 7155. These drugs have been successfully used in previous studies in our laboratory.^{198,211,360}

ZD 7155 hydrochloride is a potent and selective competitive antagonist for the AT1R with formula: $C_{26}H_{26}N_6O$.HCl and chemical name: 5,7-Diethyl-3,4-dihydro-1-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-1,6-naphthyridin-2(1H)-one hydrochloride (Tocris Bioscince, Tocris Cookson Inc, Ellisville, MO, USA). Between multiple AT1R antagonists, ZD 7155 is a less known product used exclusively in research since 1993,⁴¹⁸ however, its use has been shown to provide a number of advantages as compared with other AT1R antagonists. ZD 7155 potency is ~ten times higher than that of losartan in antagonizing ANG II-induced pressor effects in conscious normotensive and spontaneously hypertensive rats, yet unlike losartan, has no active metabolite which could impact on the physiological measurements.⁴¹⁹ EXP 3174, the active metabolite of losartan, is a highly effective ATR antagonist *in vivo* and *ex vivo*.^{420,421} ZD 7155 is a potent antagonist of ANG II-mediated contraction of the isolated guinea pig ileum,⁴¹⁸ inhibiting the pressor response to ANG II by 96% at one hour and 65% at 12h after dosing in conscious chronically catheterized rats.⁴¹⁸ In experiments in guinea pig adrenal gland membranes ZD 1755 displaces [¹²⁵I]-ANG II binding, *in vitro*, in a concentration-

dependent manner and is more potent and longer acting than the prototype AT1R antagonist, losartan.⁴¹⁸

The onset of arterial pressure responses to ZD 7155 is dose-dependent, rapid and the effects are long lasting.^{418,422} An interval of 48h was needed for ZD 7155 to be cleared from the blood and the haemodynamic effects to diminish.^{418,422} In newborn conscious sheep, previous studies in our laboratory have shown that, ZD 7155 abolishes the pressor response to ANG II within 5 min after I.V. bolus administration.¹⁹⁸ The hypotensive effects of ZD 7155 were accompanied by marked inhibition of AT1R binding predominant in medulla of adrenal gland, cortex and *vasa recta* of the kidney and media of thoracic aorta.⁴²²

PD 123319 ditrifluoroacetate (chemical name: 1-[[4-(Dimethylamino)-3-methylphenyl] methyl]-5-(diphenylacetyl)-4, 5, 6, 7-tetrahydro-1*H*-imidazo [4, 5-c] pyridine-6-carboxylic acid ditrifluoroacetate) (Tocris Bioscince, Tocris Cookson Inc, Ellisville, MO, USA) is a potent, selective, reversible non-peptide angiotensin AT2R antagonist.¹² It has been shown to have significant physiological, long lasting effects at low doses, such as the ones used in this study.^{96,141,423-424} PD 123319 has little or no activity at the AT1R, displacing bound ANG II with an IC₅₀>10,000nM, whereas it displaces ANG II with an IC₅₀ of ~ 20 nM⁹⁹ at AT2Rs. PD 123319 is 10,000 to 15,000 fold more selective for the AT2R than losartan.⁴²⁵

3.7.2 NO synthesis inhibition

Beside the molecular approach to investigate NO biosynthesis regulation, a variety of pharmacological perturbations of NOS enzymes and their cofactors were employed to study the large sphere of action of NO at the molecular and organ level. Since NO does not act on specific receptors, methods that assess specific NOS shyntase enzymes binding sites or intracellular targets that alter synthesis or function of NO have been developed to study the functions of this molecule in heath and disease. Pharmacological compounds that release or inhibit synthesis of NO have been useful tools for evaluating the broad role of NO in physiology and therapeutics.²⁴¹ The pharmacological approach of NO synthesis inhibition used in the experiments described

in this dissertation has the major advantage of permitting the investigation of endogenously produced NO. Furthermore, there is literature that use the same approach to study the roles of NO, including considerable previous experience from our laboratory. In addition, such a choice represents a consistent approach for this dissertation that uses as well pharmacological agents that inhibit ATRs to facilitate studying their functions.

L-NAME (chemical name: N^{G} -Nitro-L-arginine methyl ester hydrochloride) (Sigma Aldrich Canada Ltd., Oakville, ON, Canada) is a member of a large class of L-arginine analogues, which act by competitively inhibiting L-arginine binding with the NOS enzymes. It is the methyl ester of L-NNA (N^{G} -Nitro-L-Arginine) and competitively and non-selective inhibits basal NO production from constitutive NOS isoforms. This inhibition is stereospecific: only L-NAME (N^{G} -nitro-L-arginine methyl ester) and not D-NAME (N^{G} -nitro-D-arginine methyl ester), its inactive enantiomer, elicits inhibitory effects on NO synthesis, and therefore physiological effects.^{283,312} The metabolism of L-NAME has been demonstrated in human, rat and canine blood and plasma.⁴²⁶⁻⁴²⁸ The half-life of L-NAME in dog plasma was virtually identical (222 min) to the value found in human plasma (207 min), as compared with its hydrolysis in buffer at physiological pH of 7.4 (365 min) indicating that the metabolism of L-NAME is mainly catalyzed by blood and vascular endothelial cells esterases.⁴²⁸⁻⁴²⁹

Chapter Four: **RESULTS**

4.1 Baseline measurements in conscious lambs

Baseline haemodynamic variables measured in one and six weeks old lambs are shown in Table 4.1. There were significant differences in age (t=38.1, p<0.001), weight (t=12.7, p<0.001), and total kidney weight (t=3.5, p=0.002) between the two age groups. MAP (t=6.068, p<0.001), MVP (t=13.480, p<0.001) and RBF (t=11.744, p<0.001) were higher, while RVR (t=10.950, p<0.001) and HR (t=20.900, p<0.001) were lower in six as compared to one week old lambs. There were no significant differences between the control values of any of the haemodynamics variables measured during the three separate experimental days in either age group.

	One week	Six weeks
MAP (mmHg)	73 ± 6	78 ± 4 †
SAP (mmHg)	103 ± 8	108 ± 7
DAP (mmHg)	50 ± 5	53 ± 6
MVP (mmHg)	2 ± 2	$5 \pm 2 \ddagger$
HR (beats \cdot min ⁻¹)	212 ± 13	133 ± 2 1 †
$RBF (mL \cdot g^{-1} \cdot min^{-1})$	1.8 ± 0.4	4.1 ± 1.6 †
RVR (mmHg·mL- 1 ·g $^{-1}$ ·min $^{-1}$)	38.8 ± 7.2	18.3 ± 7.1 †

 Table 4.1 Baseline haemodynamic variables in conscious lambs

Values are mean \pm SD; MAP, mean arterial pressure; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; RBF, renal blood flow; RVR, renal vascular resistance; $\dagger p$ <0.05 compared to one week

Baseline plasma and whole blood measurements are shown in the Table 4.2. Control values of plasma Na⁺ and Cl⁻ and plasma osmolality were similar in one and six week old lambs. Control plasma hematocrit (t=4.462, p<0.001) values was higher at one than six weeks, whereas plasma K^+ (t= 4.894, P<0.001) was higher at six than one week old lambs (Table 4.2).

	One week	Six weeks
$PNa, mmol \cdot L^{-1}$	140 ± 10	143 ± 10
PK, mmol· L^{-1}	3.3 ± 0.2	$3.7 \pm 0.3 \ddagger$
PC1, mmol· L^{-1}	65 ± 4	62 ± 5
POsm,mOsm·kgH ₂ O ⁻¹	304 ± 8	302 ± 8
Hct, %	36 ± 5	29 ± 6 †

Table 4.2 Baseline plasma and whole blood variable in conscious lambs

Values are mean \pm SD; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; †p<0.05 compared to one week

Baseline renal variables in the two groups of conscious chronically instrumented lambs are presented in Table 4.3. There were no significant differences in baseline GFR between the two age groups when normalized per gram kidney weight. RPF was higher (t=7.352, p<0.001), whereas FF was significantly lower (t=6.277, p<0.001) in six as compared to one week old lambs. There were no significant differences between baseline urinary flow rate and excretion rates of Na⁺, K⁺ and Cl⁻ between the two age groups. Na⁺, K⁺ and Cl⁻ clearance were higher in six as compared with one week old lambs (t=10.461, p<0.001, t=8.611, p<0.001 and t=2.413, p=0.019, respectively). Baseline nitrate excretion rate was higher in six than in one week old lambs (t=7,314, p<0.001). Urinary osmolality and free water clearance were higher at six than one week (t=7.384, p<0.001, t=4.704, p<0.001, respectively). There were no significant differences between the control values of any of the renal variables measured or calculated during the three separate experiments in either age group.

	One week	Six weeks
GFR, mL·g ⁻¹ ·min ⁻¹	0.3 ± 0.1	0.5 ± 0.2
RPF, mL·g ⁻¹ ·min ⁻¹	1.1 ± 0.3	2.8 ± 1.0 †
FF, %	27 ± 7	18 ± 4 †
V, $\mu L \cdot g^1 \cdot min^{-1}$	4.6 ± 1.9	5.1 ± 3.3
UNaV, μ mol·g ⁻¹ ·min ⁻¹	0.04 ± 0.02	0.05 ± 0.03
UKV, μmol·g ⁻¹ ·min ⁻¹	0.52 ± 0.28	0.74 ± 0.47
UCIV, µmol·g ⁻¹ ·min ⁻¹	0.14 ± 0.09	0.18 ± 0.13
UNO ₃ V, mmol·g ⁻¹ ·min ⁻¹	5.9 ± 3.2	$32.9\pm20.0\ddagger$
CNa, $\mu L \cdot g^{-1} \cdot min^{-1}$	0.15 ± 0.08	0.85 ± 0.44 †
CK, mL·g ⁻¹ ·min ⁻¹	0.14 ± 0.08	$0.59\pm0.24 \ddagger$
CCl, mL·g ⁻¹ ·min ⁻¹	1.7 ± 1.0	2.4 ± 1.3 †
UNa/UK	0.09 ± 0.1	0.1 ± 0.1
TTKG	15.1 ± 8.0	17.8 ± 6.2
UOsm, mOsm·kgH ₂ O ⁻¹	582 ± 178	880 ± 216 †
$COsm, mL \cdot g^{-1} \cdot min^{-1}$	8.1 ± 3.4	12.9 ± 6.5 †
CH_2O , $mL \cdot g^{-1} \cdot min^{-1}$	-0.004 ± 0.003	-0.009 ± 0.005 †

 Table 4.3 Baseline renal variables in conscious lambs

Values are mean \pm SD; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; V, urinary flow rate; UXV, urinary excretion rate of electrolyte X; CX, clearance of electrolyte X; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UOsm, urinary osmolality; COsm, clearance of osmoles; CH₂O, free water clearance; †p<0.05 compared to one week. *Note:* In this dissertation, for all the graphic representation of results the color code of the graphs is as follows:

treatment with AT1R antagonist, ZD 7155

- treatment with AT2R antagonist, PD 123319
- treatment with both antagonists, ZD 7155 plus PD 123319

4.2 Effects of ANG II receptor antagonists on systemic and renal haemodynamics

There was an overall effect of age (F=11.584, p=0.003) and treatment (F=16.189, p<0.001) on MAP. As shown in Figure 4.1, MAP decreased in both groups of lambs within minutes of administration of ZD 7155 and reached a nadir at 20 min of infusion, after which there were no further changes. This response was not altered by the addition of the AT2R antagonist, PD 123319 and there were no effects of PD 123319 alone in either age group. MAP responses to ZD 7155 and combined treatment, ZD 7155 plus PD 123319 resulted from a decrease in both, SAP and DAP (Tables 4.4 - 4.6).

There was an overall effect of treatment (F=12.634, p<0.001) and age (F=80.787, p<0.001) on RVR. As shown in Figure 4.2, infusion of ZD 7155 elicited a greater decrease in RVR at one week than six weeks (F=9.643, p=0.007). This response was not altered by addition of the AT2R antagonist, PD 123319 in either age group (Figure 4.2). Infusion of PD 123319 alone did not alter RVR in either age group (Figure 4.2).

HR and MVP were not significantly influenced by any of the treatments, separately and combined, at one and six weeks. (Tables 4.4 - 4.6)



Figure 4.1 Effects of ATRs antagonists on MAP in conscious lambs Values are mean ± SD measured before (Control) and for 60 min after treatment with ZD 7155 (blue), PD 123319 (green), and both ZD 7155 plus PD 123319 (red); *p<0.05 compared to Control; †p<0.05 compared to one week.



Figure 4.2 Effects of ATRs antagonists on RVR in conscious lambs Values are mean ±SD measured before (Control) and for 60 min after treatment with ZD 7155 (blue), PD 123319 (green), and both, ZD 7155 plus PD 123319 (red); *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + Vehicle
SAP (mmHg)	One week	104 ± 7	91 ± 8*	91 ± 6*
	Six weeks	108 ± 8	99 ± 8*†	98 ± 6*†
DAP (mmHg)	One week	49 ± 6	43 ± 6*	43 ± 6*
	Six weeks	53 ± 5	46 ± 8*	46 ± 7*
MVP (mmHg)	One week	2 ± 2	2 ± 2	2 ± 2
	Six weeks	6 ± 2 †	5 ± 2 †	5 ± 2 †
HR (bpm•min ⁻¹)	One week	201 ± 31	213 ± 27	213 ± 31
	Six weeks	132 ± 24†	134 ± 26†	133 ± 30†
RBF (mL·g ⁻¹ ·min ⁻¹)	One week	1.7 ± 0.5	2.1 ± 0.7	2.0 ± 0.6
	Six weeks	4.4 ± 1.7†	5.4 ± 2.2*†	5.1 ± 2.1*†

Table 4.4 Haemodynamic effects of AT1R antagonist, ZD 7155

Values are mean \pm SD measured before (Control) and 30 min after treatment with the AT1R antagonist, ZD 7155 and ZD 7155 + Vehicle; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	PD 123319	PD 123319 + Vehicle
SAP (mmHg)	One week	103 ± 8	103 ± 8	102 ± 7
	Six weeks	107 ± 8	106 ± 7	106 ± 8
DAP (mmHg)	One week	50 ± 6	50 ± 5	48 ± 5
	Six weeks	52 ± 7	51 ± 7	51 ± 6
MVP (mmHg)	One week	2 ± 2	1 ± 2	1 ± 2
	Six weeks	6 ± 2†	6 ± 2†	6 ± 2†
HR (bpm•min ⁻¹)	One week	201 ± 30	201 ± 30	204 ± 27
	Six weeks	132 ± 23†	133 ± 21†	131 ± 20†
RBF (mL·g ⁻¹ ·min ⁻¹)	One week	1.7 ± 0.5	1.8 ± 0.6	1.8 ± 0.5
	Six weeks	3.9 ± 1.4†	4.0 ± 1.6†	$3.9 \pm 1.5 \ddagger$

Table 4.5 Haemodynamic effects of AT2R antagonist, PD 123319

Values are mean \pm SD measured before (Control) and at 30 min after treatment with the AT2R antagonist, PD 123319 and PD 123319 +Vehicle; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + PD 123319
SAP (mmHg)	One week	103 ± 7	93 ± 8*	91 ± 7*
	Six weeks	108 ± 8	100 ± 7*†	100 ± 8*†
DAP (mmHg)	One week	50 ± 4	43 ± 4*	42 ± 5*
	Six weeks	54 ± 6	47 ± 5*	$48 \pm 6^*$
MVP (mmHg)	One week	2 ± 1	1 ± 1	1 ± 1
	Six weeks	6 ± 2 †	6 ± 2 †	6 ± 2†
HR (bpm·min ⁻¹)	One week	218 ± 16	221 ± 26	$222~\pm~24$
	Six weeks	131 ± 22†	132 ± 25 †	$132 \pm 26\dagger$
RBF (mL·g ⁻¹ ·min ⁻¹)	One week	1.7 ± 0.5	2.0 ± 0.5	1.9 ± 0.5
	Six weeks	4.4 ± 1.6†	5.4 ± 2.2*†	5.2 ± 2.1*†

Table 4.6 Combined haemodynamic effects of ZD 7155 and PD 123319

Values are mean±SD measured before (Control) at 30 min after treatment with the AT1R antagonist, ZD 7155, and after both AT1R and AT2R antagonists, ZD 7155 and PD 123319; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; *p<0.05 compared to Control; †p<0.05 compared to one week.

4.3 Effects of ANG II receptor antagonists on renal function

There was no overall effect of treatment, age or an interaction between age and treatment of the ATRs antagonists on GFR. At 60 min of ZD 7155 infusion GFR decreased in six weeks. PD 123319 alone or combined with ZD 7155 did not alter GFR in either age group (Tables 4.7 - 4.9).

There was an effect of treatment (F=11.045, p<0.001) and age (F=14.675, p<0.001) of ZD 7155 on RPF, which increased in six but not in one week old lambs. This was not further altered by combined treatment with PD 123319. There was also no effect of PD 123319 infusion alone on RPF in either group of animals (Tables 4.7 - 4.9).

There was also an overall effect of treatment (F=4.799, p=0.016) and age (F=5.813, p=0.030) on FF. After ZD 7155 infusion, FF decreased in one but not in six weeks old lambs. Combined treatment with ZD 7155 plus PD 123319 did not significantly alter FF in either age group. PD 123319 did not alter FF when infused alone in one and six week old lambs (Tables 4.7 - 4.9).

Altough there were no overall effects of treatment, age or interaction between age and treatment on V, there was a decline in six but not in one week old lambs at 60 min of ZD 7155 alone infusion. Infusion of PD 123319 alone or combined with ZD 7155 did not influence urine production in either group (Figure 4.3).

There were also no overall effects of treatment, age or any interaction between age and treatment on total, proximal or distal fractional reabsorption, fractional excretion, urinary excretion rate or clearance of Na^+ of any of the ATRs antagonists treatments, alone or combined, in one and six week old lambs (Figure 4.4, Tables 4.10 - 4.12, and Tables I.4 - I.6, Appendix I).

There were no significant effects of treatment, age or an interaction between age and treatment on fractional reabsorption, fractional excretion, urinary excretion rate (Tables 4.10 - 4.12) or clearance of Cl⁻ (Tables I.4 - I.6, Appendix I) of any of the ATRs antagonists, alone or combined in one and six week old lambs.

There was, however, an overall age effect of the ATRs treatment on UKV (F=11.172, p=0.003). UKV decreased significantly in one week old lambs after 60 min of ZD 7155 infusion (Figure 4.5). Combined infusion ZD 7155 plus PD 123319 did not alter

UKV in either age group (Figure 4.5). There were also no effects of PD 123319 infused alone on UKV (Figure 4.5). Fractional reabsorption and fractional excretion of K^+ were not altered by any of the treatments with the ATR antagonists in both age groups studied (Tables 4.10 - 4.12). The responses of K^+ clearance to the ATRs antagonist treatments mirrored the changes in UKV (Tables I.4 - I.6, Appendix I). There was also an effect on TTKG (F=5.601, p=0.007), which resulted from a decrease in one but not in six week old lambs after ZD 7155 (Table 4.10). Treatment with PD 123319 alone or combined with ZD 7155 did not alter TTKG in either group of lambs (Tables 4.11 - 4.12). There were no significant effects of the ATRs antagonist alone or combined on the urinary Na⁺ to urinary K⁺ ratio in one and six weeks old lambs (Tables 4.10 - 4.12).

There was a significant overall age effect of the ATRs antagonists treatments on UNO_3V (F=9.505, p=0.006). UNO_3V fell in six weeks old lambs at 60 min of ZD 7155 infusion, whereas combined treatment with ZD 7155 plus PD 123319 and treatment with PD 123319 alone did not significantly alter UNO_3V in either age group (Tables 4.10 - 4.12).

Administration of ZD 7155 alone was associated with a significant increase in UOsm in six but not in one week old lambs that resulted from the effects of treatment (F=3.941, p=0.028), age (F=4.792, p=0.041) and an interaction between treatment and age (F=3.767, p=0.032). Addition of PD 123319 had no influence on these responses (Figure 4.6) whereas PD 123319 infused alone did not alter UOsm in either age group (Figure 4.6). As illustrated in Figure 4.6, after combined treatment with both antagonists, UOsm increased in one and six week old lambs.

There was also an overall age effect of the ATRs antagonists on CH_{2O} (F=21.144, p<0.001). After treatment with ZD 7155 alone, CH_{2O} significantly decreased in six but not in one week old lambs at 60 min (Figure 4.7). There were no significant effects of combined treatment ZD 7155 plus PD 123319 on CH_{2O} in one and six week old lambs nor of the PD 123319 treatment alone in either age group (Figure 4.7).

Administration of ATRs antagonists, alone or combined, had no significant effects on plasma Na^+ , K^+ and Cl^- concentration, nor did it influence plasma osmolality in either of the age groups (Table I.1 - I.3, Appendix I).



Figure 4.3 Effects of ATRs antagonists on urinary flow rate (V) in conscious lambs Values are mean ±SD measured before (Control) and after treatment with ZD 7155 (blue), PD 123319 (green), and both, ZD 7155 plus PD 123319 (red); *p<0.05 compared to Control; †p<0.05 compared to one week.</p>



Figure 4.4 Effects of ATRs antagonists

on Na⁺ urinary excretion rate (UNaV) in conscious lambs

Values are mean ±SD measured before (Control) and after treatment with ZD 7155 (blue), PD 123319 (green), and both, ZD 7155 plus PD 123319 (red); †p<0.05 compared to one week.



Figure 4.5 Effects of ATRs antagonists

on K⁺ urinary excretion rate (UKV) in conscious lambs Values are mean ±SD measured before (Control) and after treatment with ZD 7155 (blue), PD 123319 (green), and both, ZD 7155 plus PD 123319 (red); *p<0.05 compared to Control; †p<0.05 compared to one week.





Values are mean ±SD measured before (Control) and after treatment with ZD 7155 (blue), PD 123319 (green), and both, ZD 7155 plus PD 123319 (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.



Figure 4.7 Effects of ATRs antagonists on free water clearance (CH2O) in conscious lambs

Values are mean ±SD measured before (Control) and after treatment with ZD 7155 (blue), PD 123319 (green), and both, ZD 7155 plus PD 123319 (red); *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + Vehicle
GFR	One week	0.38 ± 0.11	0.26 ± 0.18	0.33 ± 0.19
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.47 ± 0.25	0.32 ± 0.20	$0.30 \pm 0.20*$
RPF	One week	1.07 ± 0.28	1.37 ± 0.54	1.32 ± 0.46
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.63 ± 1.02 †	$2.96 \pm 1.07*$ †	3.04 ± 1.13*†
FF	One week	33 ± 14	$20 \pm 5^*$	29 ± 8
(%)	Six weeks	18 ± 10 †	12 ± 6	11 ± 8

 Table 4.7 Glomerular effects of ZD 7155

Values are mean \pm SD measured before (Control) and 30 min after treatment with the AT1R antagonist, ZD 7155 and ZD 7155 + Vehicle; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; *p<0.05 compared to Control; †p<0.05 compared to one week.

Table 4.8 Glomerular effects of PD 123319

VARIABLE	AGE	Control	PD 123319	PD 123319 + Vehicle
GFR	One week	0.36 ± 0.15	0.38 ± 0.15	0.28 ± 0.13
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.45 ± 0.23	0.37 ± 0.16	0.41 ± 0.21
RPF	One week	1.10 ± 0.36	1.19 ± 0.37	1.13 ± 0.33
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	$2.50\pm0.66\dagger$	$2.47 \pm 0.69 \ddagger$	2.44 ± 0.65 †
FF	One week	31 ± 5	33 ± 13	28 ± 16
(%)	Six weeks	19 ± 12†	16 ± 8	18 ± 9

Values are mean \pm SD measured before (Control) and 30 min after treatment with the AT2R antagonist, PD 123319 and PD 123319 + Vehicle; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + PD 123319
GFR	One week	0.38 ± 0.24	0.22 ± 0.15	0.19 ± 0.11
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.43 ± 0.24	0.30 ± 0.12	0.38 ± 0.18
RPF	One week	1.07 ± 0.34	1.28 ± 0.41	1.35 ± 0.45
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.93 ± 1.23†	$3.42 \pm 1.42*$ †	3.35 ± 1.45*†
FF	One week	32 ± 15	17 ± 6*	24 ± 21
(%)	Six weeks	18 ± 10 †	9 ± 5	16 ± 15

Table 4.9 Glomerular effects of combined ZD 7155 plus PD 123319

Values are mean \pm SD measured before (Control) and 30 min after treatment with the AT1R antagonist, ZD 7155 and after both, AT1R and AT2R antagonists, ZD 7155 and PD 123319; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + Vehicle
FRNa	One week	99.96 ± 0.03	99.93 ± 0.04	99.94 ± 0.04
(%)	Six weeks	99.91 ± 0.11	99.86 ± 0.22	99.91 ± 0.11
PRNa	One week	78.5 ± 8.3	84.3 ± 6.3	86.1 ± 8.2
(%)	Six weeks	65.0 ± 16.8	66.4 ± 19.9	67.6 ± 13.0†
DRNa	One week	21.5 ± 8.3	15.5 ± 6.2	13.2 ± 8.5
(%)	Six weeks	35.0 ± 16.8	33.5 ± 19.9	32.3 ± 13.0†
FRK	One week	56.0 ± 23.9	64.6 ± 18.1	72.3 ± 19.1
(%)	Six weeks	55.3 ± 12.8	57.8 ± 15.5	57.3 ± 8.6
TTKG	One week	18.0 ± 7.0	16.3 ± 6.3	13.7 ± 4.9*
	Six weeks	17.0 ± 3.1	16.2 ± 2.8	16.5 ± 3.3
UNa/UK	One week	0.07 ± 0.03	0.10 ± 0.05	0.09 ± 0.06
	Six weeks	0.06 ± 0.04	0.07 ± 0.07	0.05 ± 0.04
UCIV	One week	0.12 ± 0.04	0.08 ± 0.06	0.06 ± 0.03
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.15 ± 0.09	0.21 ± 0.20	$0.15 \pm 0.10 \ddagger$
FRC1	One week	99.53 ± 0.21	99.43 ± 0.32	99.31 ± 0.92
(%)	Six weeks	99.43 ± 0.25	98.92 ± 0.97	99.22 ± 0.35
UNO ₃ V	One week	4.5 ± 3.0	3.0 ± 2.3	4.0 ± 2.2
$(\text{mmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1})$	Six weeks	$28.8 \pm 18.7 \ddagger$	30.6 ± 25.1†	13.4 ± 9.6*†‡

Table 4.10 Tubular effects of ZD 7155

Values are mean ±SD measured before (Control) and 30 min after treatment with the AT1R antagonist, ZD 7155 and ZD 7155 + Vehicle; PRNa, proximal reabsorption of Na⁺; DRNa, distal reabsorption of Na⁺; FRNa, fractional reabsorption of Na; FRK, fractional reabsorption of K; FRCl, fractional reabsorption of Cl⁻; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UClV, urinary excretion rate of Cl⁻; UNO₃V, urinary excretion rate of NO₃⁻; *p<0.05 compared to Control; †p<0.05 compared to one week; p<0.05 compared to previous treatment.

VARIABLE	AGE	Control	PD 123319	PD 123319 + Vehicle
FRNa	One week	99.95 ± 0.06	99.94 ± 0.06	99.87 ± 0.14
(%)	Six weeks	99.89 ± 0.09	99.88 ± 0.12	99.88 ± 0.13
PRNa	One week	78.7 ± 10.5	80.9 ± 8.6	78.7 ± 8.0
(%)	Six weeks	64.8 ± 17.6	63.8 ± 17.0	67.5 ± 18.7
DRNa	One week	21.3 ± 10.4	19.0 ± 8.5	21.1 ± 7.8
(%)	Six weeks	35.1 ± 17.5	36.1 ± 17.0	30.3 ± 18.9
FRK	One week	56.2 ± 15.4	63.7 ± 12.5	61.7 ± 15.0
(%)	Six weeks	54.5 ± 16.6	59.3 ± 12.8	64.3 ± 17.9
TTKG	One week	17.1 ± 6.1	15.7 ± 4.1	14.7 ± 4.6
	Six weeks	17.0 ± 3.5	14.0 ± 2.1	14.0 ± 4.1
UNa/UK	One week	0.07 ± 0.05	0.09 ± 0.06	0.15 ± 0.16
	Six weeks	0.07 ± 0.05	0.09 ± 0.06	0.15 ± 0.16
UCIV	One week	0.11 ± 0.09	0.10 ± 0.09	0.09 ± 0.09
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.17 ± 0.10	0.18 ± 0.13	0.20 ± 0.11
FRCI	One week	99.44 ± 0.51	99.46 ± 0.40	99.32 ± 0.61
(%)	Six weeks	99.37 ± 0.35	99.22 ± 0.47	99.15 ± 0.61
UNO ₃ V	One week	4.8 ± 2.7	5.7 ± 2.8	4.5 ± 2.3
$(\text{mmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1})$	Six weeks	26.7 ± 21.2 †	22.9 ± 13.7 †	21.0 ± 12.7 †

Table 4.11 Tubular effects of PD 123319

Values are mean ±SD measured before (Control) and 30 min after treatment with the AT2R antagonist, PD 123319 and after PD 123319 + Vehicle; PRNa, proximal reabsorption of Na⁺; DRNa, distal reabsorption of Na⁺; FRNa, fractional reabsorption of Na; FRK, fractional reabsorption of K, FRCl, fractional reabsorption of Cl⁻; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UNa/UK, urinary excretion rate of NO₃⁻; *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + PD 123319
FRNa	One week	99.95 ± 0.05	99.95 ± 0.04	99.90 ± 0.10
(%)	Six weeks	99.89 ± 0.07	99.83 ± 0.16	99.83 ± 0.14
PRNa	One week	78.4 ± 11.7	82.2 ± 9.8	83.5 ± 9.0
(%)	Six weeks	64.0 ± 13.2	67.5 ± 15.2	55.3 ± 12.7
DRNa	One week	21.5 ± 11.8	17.8 ± 9.7	16.3 ± 9.0
(%)	Six weeks	36.0 ± 13.2	32.5 ± 15.2	44.7 ± 12.7
FRK	One week	57.6 ± 16.6	62.4 ± 13.9	60.4 ± 10.8
(%)	Six weeks	54.6 ± 16.8	57.2 ± 12.4	50.2 ± 21.3
TTKG	One week	17.3 ± 5.0	15.9 ± 4.6	15.8 ± 3.1
	Six weeks	17.7 ± 8.3	16.1 ± 6.0	14.8 ± 5.1
UNa/UK	One week	0.07 ± 0.04	0.10 ± 0.07	0.09 ± 0.06
	Six weeks	0.07 ± 0.05	0.09 ± 0.07	0.10 ± 0.08
UCIV	One week	0.11 ± 0.06	0.07 ± 0.02	0.05 ± 0.03
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.15 ± 0.10	0.20 ± 0.24	0.23 ± 0.13†
FRCI	One week	99.53 ± 0.26	99.55 ± 0.30	99.59 ± 0.23
(%)	Six weeks	99.42 ± 0.28	99.15 ± 0.65	99.00 ± 0.56†
UNO ₃ V	One week	4.7 ± 2.8	3.0 ± 2.6	3.5 ± 2.5
$(\text{mmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1})$	Six weeks	28.1 ± 19.0†	31.9 ± 19.1†	32.6 ± 13.1†

Table 4.12 Tubular effects of ZD 7155 plus PD 123319

Values are mean ±SD measured before (Control) and 30 min after treatment with AT1R antagonist, ZD 7155 and after both AT1R and AT2R anatgonists, ZD 7155 and PD 123319; PRNa, proximal reabsorption of Na⁺; DRNa, distal reabsorption of Na⁺; FRNa, fractional reabsorption of Na; FRK, fractional reabsorption of K; FRCl, fractional reabsorption of Cl⁻; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; at of Cl⁻; UNa₃V, urinary excretion rate of NO₃⁻; †p<0.05 compared to one week.

4.4 Effects of ATRs antagonists and NO synthase inhibitor, L-NAME on systemic and renal haemodynamics

For MAP, there was an overall effect of treatment (F=22.273, p<0.001) and age (F=15.332, p=0.001) on MAP. As illustrated in Figure 4.8, MAP decreased after ZD 7155, then increased after L-NAME in one and six week old lambs (F=36.529, p<0.001). Addition of PD 123319 did not influence these responses in either age group (Figure 4.8). MAP did not change after PD 123319 pre-treatment, but increased after L-NAME addition in both groups of lambs (F=40.653, p<0.001) (Figure 4.8). The changes observed in MAP resulted from changes in both SAP and DAP that mirrored the responses of MAP in both groups of lambs. (Tables 4.13 - 4.15)

There was also an effect of treatment (F=9.239, p<0.001), age (F=60.739, p<0.001) and an interaction between age and treatment (F=4.847, p=0.004) of the combined co-infusion of L-NAME with ZD 7155 on HR, which increased in one but not in six weeks old lambs. This response was not altered by addition of PD 123319. L-NAME administration to PD 123319 alone increased HR only in one week old lambs (Tables 4.13 - 4.15).

MVP remained constant after all treatments in both groups of lambs (Tables 4.13 - 4.15).

For RVR responses to ATRs antagonist and L-NAME, there was an effect of treatment (F=10.019, p<0.001) and age (F=11.653, p=0.004). RVR increased in one and six weeks old lambs, after L-NAME with ZD 7155, (F=30.022, p<0.001); this response was not altered by the addition of PD 123319 (Figure 4.9) Addition of L-NAME after PD 123319 alone also elicited an increase in RVR in both groups of lambs (Figure 4.9)



Figure 4.8 Effects of combined treatment of ATRs antagonists and L-NAME on MAP in conscious lambs

Values are mean ± SD measured before (Control) and after treatment with ZD 7155 and L-NAME (blue), PD 123319 and L-NAME (green), and both antagonists, ZD 7155 plus PD 123319, and L-NAME (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.





Values are mean ± SD measured before (Control) and after treatment with ZD 7155 and L-NAME (blue), PD 123319 and L-NAME (green), and both antagonists, ZD 7155 plus PD 123319, and L-NAME (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 as compared with previous treatment.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + L-NAME
SAP (mmHg)	One week	104 ± 7	92 ± 6*	103±8‡
	Six weeks	108 ± 8	98 ± 6*†	109±8†‡
DAP (mmHg)	One week	49 ± 6	$43 \pm 6^*$	53 ±7‡
	Six weeks	53 ± 5	$45 \pm 8*$	59 ±13‡
MVP (mmHg)	One week	2 ± 3	1 ± 3	1 ± 3
	Six weeks	6 ± 2†	5 ± 2†	5 ± 2 †
HR (bpm⋅min ⁻¹)	One week	201 ± 31	213 ± 31	176±17*‡
	Six weeks	132 ± 24†	133 ± 30†	131±24†
RBF (mL·g ⁻¹ ·min ⁻¹)	One week	1.7 ± 0.5	2.0 ± 0.6	1.9 ±0.5
	Six weeks	4.4 ± 1.7 †	5.1 ± 2.1*†	4.3 ±1.6†

Table 4.13 Effects of combined treatment ZD 7155 and L-NAME on haemodynamics

Values are mean \pm SD measured before (Control) and after treatment with the AT1R antagonist, ZD 7155 and after ZD 7155 plus L-NAME. Values are averaged over 30 min of drug infusion. SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; *p<0.05 compared to Control; †p<0.05 compared to ZD 7155.

VARIABLE	AGE	Control	PD123319	PD123319 + L-NAME
SAP (mmHg)	One week	101 ± 9	100 ± 8	112 ± 7*‡
	Six weeks	107 ± 8	106 ± 8	123 ± 9*†‡
DAP (mmHg)	One week	50 ± 6	48 ± 5	57 ± 8‡
	Six weeks	52 ± 7	51 ± 6	67 ± 9*†‡
MVP (mmHg)	One week	2 ± 2	1 ± 2	1 ± 2
	Six weeks	6 ± 2†	6 ± 2†	5 ± 2†
HR (bpm⋅min ⁻¹)	One week	199 ± 27	204 ± 37	168 ± 14*‡
	Six weeks	132 ± 23†	131 ± 20†	132 ± 21†
RBF (mL·g ⁻¹ ·min ⁻¹)	One week	1.7 ± 0.5	1.8 ± 0.5	1.7 ± 0.5
	Six weeks	3.9 ± 1.4†	3.9 ± 1.5†	$3.6 \pm 1.1 \ddagger$

Table 4.14 Effects of combined treatment PD 123319 and L-NAME on haemodynamics

Values are mean \pm SD measured before (Control) and after treatment with the AT2R antagonist, PD 12319 and after PD 123319 plus L-NAME. Values are averaged over 30 min of drug infusion. SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; *p<0.05 compared to Control; †p<0.05 compared to PD 123319.

VARIABLE	AGE	Control	ZD7155 + PD123319	ZD7155 + PD123319 + L-NAME
SAP (mmHg)	One week	103 ± 7	91 ± 7*	99 ± 6‡
	Six weeks	108 ± 8	$102 \pm 10*;$	$115 \pm 8*$ †‡
DAP (mmHg)	One week	50 ± 4	42 ± 5*	52 ± 6‡
	Six weeks	53 ± 6	45 ± 13*†	58 ± 17*†‡
MVP (mmHg)	One week	2 ± 1	1 ± 1	1 ± 1
	Six weeks	6 ± 2†	6 ± 2 †	5 ± 1 †
HR (bpm·min ⁻¹)	One week	218 ± 16	222 ± 24	169 ± 26*‡
	Six weeks	131 ± 22†	132 ± 26 †	131 ± 20†
RBF (mL·g ⁻¹ ·min ⁻¹)	One week	1.7 ± 0.5	1.9 ± 0.5	1.9 ± 0.6
	Six weeks	4.4 ± 1.6 †	5.2 ± 2.1*†	4.7 ± 1.8†

Table 4.15 Effects of combined treatment ZD 7155 plus PD 123319 andL-NAME on haemodynamics

Values are mean \pm SD measured before (Control) and after treatment with the AT1R and AT2R antagonist, ZD 7155 and PD 12319 and after combined treatment ZD 7155 plus PD 123319 and L-NAME. Values are averaged over 30 min of drug infusion. SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MVP, mean venous pressure; HR, heart rate; *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to ZD 7155 and PD 123319.

4.5 Renal effects of ATRs antagonists and L-NAME

GFR remained constant after all three treatments in both age groups. After addition of L-NAME to the ATRs antagonists pre-treatments, however, GFR was higher at six than one week (F=9.896, p=0.005) (Tables 4.16 - 4.18). L-NAME addition did not alter the RPF or FF responses to ZD 7155, PD 123319 or ZD 7155 plus PD 123319 in both groups of lambs. After the combined treatments with ATRs antagonists and L-NAME, RPF was higher at six as compared with one week (Tables 4.16 - 4.18).

There was a significant increase in V at six but not one week old lambs after the combined treatment ZD 7155 plus L-NAME that resulted from an effect of treatment (F=7.174, p<0.001), age (F=4.604, p=0.044) and an interaction between age and treatment (F=3.791, p=0.015) (Figure 4.10). This robust diversis was not altered by addition of PD 123319 (Figure 4.10). However, after L-NAME addition to PD 123319, urine production increased in older lambs (Figure 4.10).

For UNaV, there was also an overall effect of age (F=5.103, p=0.035) and an interaction between age and treatment (F=4.617, p=0.016). Figure 4.11 shows that L-NAME administration after ZD 7155 was associated with a significant increase in UNaV in six but not in one week old lambs (F=7.687, p<0.001). Similarly, L-NAME addition after ZD 7155 plus PD 123319 increased UNaV only in six weeks old lambs (F=6.192, p=0.001), while L-NAME addition to PD 123319 did not alter PD 123319 responses in either age group (Figure 4.11). The changes observed in Na⁺ excretion were associated with age-dependent changes in fractional Na⁺ reabsorption, which fell after ZD 7155 and L-NAME and after ZD 7155 plus PD 12319 and L-NAME in six but not in one week old lambs (Tables 4.19 and 4.21). L-NAME addition after PD 123319 did not alter FRNa in either age group (Table 4.20). There were no significant changes after any of the treatments in proximal and distal fractional reabsorption of Na⁺ in either age group (Tables 4.19 - 4.21). CNa mirrored the changes in UNaV, from an effect of age (F=4.062, p=0.025) and an interaction between age and treatment (F=8.818, p=0.008) (Tables I.10 - I.12 (Appendix I).
The combined treatment with ATRs antagonists and L-NAME was followed by changes on Cl⁻ handling along the nephron that mirrored the changes in Na⁺. These effects are shown in Tables 4.19 - 4.21 and Tables I.10 - I.12 (Appendix I).

A kaliuresis occurred in six but not in one week old lambs associated with L-NAME addition to all three ATRs antagonists treatments. There was an effect of treatment (F=7.405, p<0.001), age (F=8.788, p=0.008) and an interaction between treatment and age (F=4.605, p=0.006) on UKV (Figure 4.12). Following L-NAME administration after ZD 7155, FRK decreased in six weeks, from an effect of age (F=6.214, p=0.027) and an interaction between age and treatment (F=5.705, p=0.002) (Tables 4.19 - 4.21). Addition of L-NAME to combined treatment ZD 7155 plus PD 123319 or PD 123319 alone did not alter FRK in either age group. CK mirrored the changes in UKV (Tables I.10 - I.12, Appendix I). In six but not in one week old lambs, only the combined treatment of L-NAME with ZD 7155 plus PD 123319 was associated with a significant increase in urinary Na to K ratio (F=4.127, p=0.010) (Table 4.21). There were no effects of the other two treatment combinations on urinary Na to K ratio in one and six weeks old lambs (Tables 4.19 - 4.20). There was an age-dependent decline in TTKG after combined treatment ZD 7155 and L-NAME in one but not in six weeks old lambs that resulted from an effect of treatment (F=3.716, p=0.016) and an interaction between age and treatment (F=6.001, p=0.001) (Table 4.19). There were no effects of the other two treatment combination on TTKG in one and six weeks old lambs (Tables 4.20 -4.21)

UN03V increased in six but not in one week old lambs after ZD 7155 plus L-NAME from an effect of treatment (F=5.547, p=0.002), age (F=8.962, p=0.007) and an interaction between age and treatment (F=5.547, p=0.002) (Table 4.19). L-NAME infusion after ZD 7155 plus PD 123319 did not alter nitrate excretion in either age group (Table 4.21).

Addition of L-NAME after pre-treatment with ATRs antagonists altered urine osmolality in an age-dependent manner. UOsm decreased in six week old lambs after L-NAME addition to ZD 7155 (F=5.614, p=0.029), and to PD 123319 (F=6.380, p=0.003) (Figure 4.13). The decrease in UOsm only in older lambs after combined treatment ZD

7155 plus PD 123319 and L-NAME and after combination of ZD 7155 plus PD 123319 resulted from an effect of age (F=6.304, p=0.021), treatment (F=4.834, p=0.004) and an interaction between age and treatment (5.818, p=0.001) (Figure 4.13).

There was an overall effect of treatment (F=3.240, p=0.050), age (F=20.821, p<0.001) and an interaction between age and treatment (F=3.673, p=0.034) of the combinations of ATRs antagonists and L-NAME on CH₂O. CH₂O increased in six but not in one week old lambs after L-NAME co-infusion with ZD 7155 alone and with combined treatment ZD 7155 plus PD 123319. L-NAME co-infusion with PD 123319 alone did not alter CH₂O in either age group (Figure 4.14). There were no changes after L-NAME addition to any of the ATRs antagonist treatments on either variable, UOsm and CH2O, in one week old lambs.

Co-administration of L-NAME with ATRs antagonists had no significant effect on plasma electrolytes or osmolalities, nor hematocrit in either age group (Tables I.7 – I.9, Appendix I).



Figure 4.10 Effects of combined treatment of ATRs antagonists and L-NAME on urinary flow rate (V) in conscious lambs

Values are mean ±SD measured before (Control) and after treatment with ZD 7155and L-NAME (blue), PD 123319 and L-NAME (green), and both antagonists, ZD 7155 plus PD 123319, and L-NAME (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.



Figure 4.11 Effects of combined treatment of ATRs antagonists and L-NAME on sodium excretion rate (UNaV) in conscious lambs Values are mean ±SD measured before (Control) and after treatment with ZD 7155and L-NAME (blue), PD 123319 and L-NAME (green), and both antagonists, ZD 7155 plus PD 123319, and L-NAME (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.





Values are mean ±SD measured before (Control) and after treatment with ZD 7155and L-NAME (blue), PD 123319 and L-NAME (green), and both antagonists, ZD 7155 plus PD 123319, and L-NAME (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.







Figure 4.14 Effects of combined treatment of ATRs antagonists and L-NAME on free water clearance (CH2O) in conscious lambs

Values are mean ±SD measured before (Control) and after treatment with ZD 7155and L-NAME (blue), PD 123319 and L-NAME (green), and both antagonists, ZD 7155 plus PD 123319, and L-NAME (red); *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + L-NAME
GFR	One week	0.38 ± 0.11	0.33 ± 0.19	0.27 ± 0.15
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.47 ± 0.25	0.30 ± 0.20	$0.55 \pm 0.36 \ddagger$
RPF	One week	1.07 ± 0.28	1.32 ± 0.46	1.35 ± 0.48
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	$2.63 \pm 1.02 \ddagger$	3.04 ± 1.13†	$2.67\pm0.93\dagger$
FF	One week	33 ± 14	29 ± 8	29 ± 8
(%)	Six weeks	18 ± 10 †	11 ± 8	23 ± 14

Table 4.16 Glomerular effects of ZD 7155 and L-NAME

Values are mean \pm SD measured before (Control) after treatment with the AT1R antagonist, ZD 7155 and after combined treatment, ZD 7155 and L-NAME; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; *p<0.05 compared to Control; †p<0.05 compared to one week.

 Table 4.17 Glomerular effects of PD 123319 and L-NAME

VARIABLE	AGE	Control	PD 123319	PD 123319 + L-NAME
GFR	One week	0.36 ± 0.15	0.28 ± 0.13	0.34 ± 0.17
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.45 ± 0.23	0.41 ± 0.21	0.55 ± 0.24 †
RPF	One week	1.10 ± 0.36	1.13 ± 0.33	1.10 ± 0.35
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	$2.50 \pm 0.66 \ddagger$	2.44 ± 0.65 †	$2.33 \pm 0.63 \ddagger$
FF	One week	31 ± 5	28 ± 16	36 ± 25
	Six weeks	19 ± 12†	18 ± 9	26 ± 9

Values are mean \pm SD measured before (Control) after treatment with the AT2R antagonist, PD123319 and after combined treatment, PD 123319 and L-NAME; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155 + PD 123319	ZD 7155 + PD 123319 + L-NAME
GFR	One week	0.38 ± 0.24	0.19 ± 0.11	0.31 ± 0.20
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.43 ± 0.24	0.38 ± 0.18	$0.63\pm0.42\dagger$
RPF	One week	1.07 ± 0.34	1.35 ± 0.45	1.25 ± 0.46
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.93 ± 1.23 †	3.35 ± 1.45 †	$2.98 \pm 1.45 \ddagger$
FF	One week	32 ± 15	24 ± 21	36 ± 23
(%)	Six weeks	18 ± 10 †	16 ± 15	22 ± 15

 Table 4.18 Glomerular effects of ZD 7155 plus PD 123319 and L-NAME

Values are mean \pm SD measured before (Control) after treatment with the ATRs antagonists, ZD 7155 plus PD123319 and after combined treatment, ZD 7155 plus PD 123319 and L-NAME; GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction; *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + L-NAME
FRNa	One week	99.96 ± 0.03	99.94 ± 0.04	99.80 ± 0.18
(%)	Six weeks	99.91 ± 0.11	99.91 ± 0.11	$99.29 \pm 0.67*$ ‡
PRNa	One week	78.5 ± 8.3	86.1 ± 8.2	80.3 ± 8.5
(%)	Six weeks	65.0 ± 16.8	67.6 ± 13.0†	66.4 ± 16.6
DRNa	One week	21.5 ± 8.3	13.2 ± 8.5	19.5 ± 8.5
(%)	Six weeks	35.0 ± 16.8	32.3 ± 13.0†	32.6 ± 16.3
FRK	One week	56.0 ± 23.9	72.3 ± 19.1	70.2 ± 22.3
(%)	Six weeks	55.3 ± 12.8	57.3 ± 8.6	31.0 ± 15.5†‡
TTKG	One week	18.0 ± 7.0	13.7 ± 4.9*	13.2 ± 7.9*
	Six weeks	17.0 ± 3.1	16.5 ± 3.3	18.4 ± 4.3
UNa/UK	One week	0.07 ± 0.03	0.09 ± 0.06	0.21 ± 0.18
	Six weeks	0.06 ± 0.04	0.05 ± 0.04	0.32 ± 0.22
UCIV	One week	0.12 ± 0.04	0.06 ± 0.03	0.17 ± 0.13
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.15 ± 0.09	0.15 ± 0.10 †	$0.87 \pm 0.48*$ †‡
FRCI	One week	99.53 ± 0.21	99.31 ± 0.92	99.08 ± 0.58
(%)	Six weeks	99.43 ± 0.25	99.22 ± 0.35	96.51 ± 2.05*†‡
UNO ₃ V	One week	4.5 ± 3.0	4.0 ± 2.2	3.0 ± 1.3
$(\text{mmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1})$	Six weeks	$28.8 \pm 18.7 \ddagger$	13.4 ± 9.6*†‡	21.6±14.6*†‡

Table 4.19 Tubular effects of ZD 7155 and L-NAME

Values are mean \pm SD measured before (Control) and after treatment with AT1R antagonist ZD 7155 and after combined treatment ZD 7155 and L-NAME; FRNa, fractional reabsorption of Na; FRK, fractional reabsorption of K; FRCl, fractional reabsorption of Cl⁻; PRNa, proximal reabsorption of Na⁺; DRNa, distal reabsorption of Na⁺; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UClV, urinary excretion rate of Cl⁻; UNO₃V, urinary excretion rate of NO₃⁻; *p<0.05 compared to Control; p<0.05 compared to one week; p<0.05 compared to previous treatment.

VARIABLE	AGE	Control	PD 123319	PD 123319 + L-NAME
FRNa	One week	99.95 ± 0.06	99.87 ± 0.14	99.43 ± 0.84
(%)	Six weeks	99.89 ± 0.09	99.88 ± 0.13	99.68 ± 0.35
PRNa	One week	78.7 ± 10.5	78.7 ± 8.0	79.4 ± 11.3
(%)	Six weeks	64.8 ± 17.6	67.5 ± 18.7	70.7 ± 13.6
DRNa	One week	21.3 ± 10.4	21.1 ± 7.8	21.5 ± 10.2
(%)	Six weeks	35.1 ± 17.5	30.3 ± 18.9	28.7 ± 13.5
FRK	One week	56.2 ± 15.4	61.7 ± 15.0	67.9 ± 22.2
(%)	Six weeks	54.5 ± 16.6	64.3 ± 17.9	59.3 ± 15.9
TTKG	One week	17.1 ± 6.1	14.7 ± 4.6	12.1 ± 6.8
	Six weeks	17.0 ± 3.5	14.0 ± 4.1	16.3 ± 4.9 †
UNa/UK	One week	0.07 ± 0.05	0.15 ± 0.16	0.20 ± 0.26
	Six weeks	0.07 ± 0.05	0.15 ± 0.16	0.25 ± 0.27
UCIV	One week	0.11 ± 0.09	0.09 ± 0.09	0.19 ± 0.17
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.17 ± 0.10	0.20 ± 0.11	$0.59 \pm 0.34*$ ‡
FRCI	One week	99.44 ± 0.51	99.32 ± 0.61	98.41 ± 1.58
(%)	Six weeks	99.37 ± 0.35	99.15 ± 0.61	98.21 ± 1.14*†‡
UNO ₃ V	One week	4.8 ± 2.7	4.5 ± 2.3	3.6 ± 2.9
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	26.7 ± 21.2	21.0 ± 12.7	$15.4 \pm 10.8*$

Table 4.20 Tubular effecst of PD 123319 and L-NAME

Values are mean ±SD measured before (Control) and after treatment with the AT2R antagonist PD 123319 and after combined treatment PD 123319 and L-NAME; FRNa, fractional reabsorption of Na; FRK, fractional reabsorption of K; FRCl, fractional reabsorption of Cl⁻; PRNa, proximal reabsorption of Na⁺; DRNa, distal reabsorption of Na⁺; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UClV, urinary excretion rate of Cl⁻; UNO₃V, urinary excretion rate of NO₃⁻; *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.

VARIABLE	AGE	Control	ZD 7155+ PD 123319	ZD 7155 + PD 123319 + L-NAME
FRNa	One week	99.95 ± 0.05	99.90 ± 0.10	99.68 ± 0.54
(%)	Six weeks	99.89 ± 0.07	99.83 ± 0.14	98.25 ± 1.75*†‡
PRNa	One week	78.4 ± 11.7	83.5 ± 9.0	86.6 ± 8.4
(%)	Six weeks	64.0 ± 13.2	55.3 ± 12.7†	$59.8 \pm 23.7 \ddagger$
DRNa	One week	21.5 ± 11.8	16.3 ± 9.0	13.1 ± 8.4
(%)	Six weeks	36.0 ± 13.2	44.7 ± 12.7†	40.2 ± 23.7 †
FRK	One week	57.6 ± 16.6	60.4 ± 10.8	64.7 ± 18.0
(%)	Six weeks	54.6 ± 16.8	50.2 ± 21.3	56.8 ± 22.1
TTKG	One week	17.3 ± 5.0	15.8 ± 3.1	16.8 ± 6.5
	Six weeks	17.7 ± 8.3	14.8 ± 5.1	13.5 ± 7.7
UNa/UK	One week	0.07 ± 0.04	0.09 ± 0.06	0.17 ± 0.19
	Six weeks	0.07 ± 0.05	0.10 ± 0.08	$0.96 \pm 0.92*\ddagger$
UCIV	One week	0.11 ± 0.06	0.05 ± 0.03	0.12 ± 0.07
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.15 ± 0.10	$0.23 \pm 0.13 \dagger$	1.38± 0.80*†‡
FRCI	One week	99.53 ± 0.26	99.59 ± 0.23	99.36 ± 0.43
(%)	Six weeks	99.42 ± 0.28	99.00 ± 0.56†	$96.52 \pm 2.05*\ddagger$
UNO ₃ V	One week	4.7 ± 2.8	3.5 ± 2.5	4.6 ± 3.1
$(\mu mol \cdot g^{-1} \cdot min^{-1})$	Six weeks	28.1 ± 19.0 †	32.6 ± 13.1†	$34.7 \pm 31.8 \ddagger$

Table 4.21 Tubular effects of ZD 7155 plus PD 123319 and L-NAME

Values are mean ±SD measured before (Control) and after treatment with the ATRs antagonists, ZD 7155 plus PD 123319 and after combined treatment ZD 7155 plus PD 123319 and L-NAME; FRNa, fractional reabsorption of Na; FRK, fractional reabsorption of K; FRCl, fractional reabsorption of Cl⁻; PRNa, proximal reabsorption of Na⁺; DRNa, distal reabsorption of Na⁺; UNa/UK, urine Na⁺ to K⁺ ratio; TTKG, transtubular K⁺ gradient; UClV, urinary excretion rate of Cl⁻; UNO₃V, urinary excretion rate of NO₃⁻; *p<0.05 compared to Control; †p<0.05 compared to one week; ‡p<0.05 compared to previous treatment.

Chapter Five: **DISCUSSIONS**

5.1 Cardiovascular effects of ATRs during postnatal development

The purpose of this study was to investigate the potential roles of AT2Rs in modulating the haemodynamic effects of AT1Rs during postnatal development in conscious, chronically instrumented lambs. This study shows for the first time that AT2Rs do not appear to influence the AT1R-induced systemic and renal haemodynamic effects of endogenous ANG II in conscious animals during postnatal development (Figure 5.1). Novel findings of this study are:

1) The effects on baseline systemic haemodynamics of endogenously produced ANG II mediated through AT1Rs are not modulated by AT2R in conscious animals during postnatal ontogeny. The effects of the AT1R antagonist, ZD 7155 on arterial pressure are not altered by addition of the AT2R antagonist, PD 123319 in one and six weeks old lambs.

2) The effects on baseline renal haemodynamics of endogenously produced ANG II mediated through AT1Rs are not modulated by AT2Rs in conscious developing animals under physiological conditions. The changes in RVR and RBF following ZD 7155 were not altered by the addition of PD 123319 in one and six weeks old lambs.



Figure 5.1 Effects of AT1R and AT2R on haemodynamics in conscious lambs

In addition, the observations from the present study confirm previous findings from our laboratory:

a) As demonstrated by Chapellaz and Smith (2007),²¹¹ AT1Rs appear to play a predominant role in mediating the effects of endogenous ANG II on systemic and renal haemodynamics and contribute to the maintenance of arterial pressure and blood flow to the kidney during postnatal maturation in sheep (Figure 5.1). ZD 7155 decreased MAP and RVR and increased RBF during the first six weeks of life in conscious lambs. The same findings were also reported more recently by Wehlage and Smith (2012),³⁶⁰ confirming the role of the AT1Rs in influencing cardiovascular homeostasis during the postnatal period.

b) AT2Rs do not appear to play a role in the ANG II-mediated control of systemic and renal haemodynamics (Figure 5.1). PD 123319 infusion did not alter baseline arterial pressure or renal vascular tone in both groups of animals studied, findings that confirm previous observations from our laboratory that PD 123319 had no influence on baseline haemodynamics nor on the pressor response to ANG II.²¹¹

Therefore, it is clear that the role of ANG II in regulating systemic and renal haemodynamics in conscious developing animals under physiologic conditions appears to result exclusively through activation of AT1Rs.

There is a general agreement that ANG II modulates blood pressure during the postnatal period.^{195,430-431} In newborn sheep, systemic ANG II infusion increases MAP in a dose-dependent manner, effects being comparable in 7-14, 15-21 and 22-35 day old lambs.¹⁹⁶ In addition, we have previously shown that the dose-dependent haemodynamic responses to ANG II are developmentally regulated. Administration of ANG II was associated with age- and dose-dependent increase in MAP and decrease in RBF in conscious lambs, responses that appeared to be mediated through activation of AT1Rs, while no effect was elicited by AT2Rs.¹⁹⁸ There is little known, however, regarding the functional roles of AT1Rs and AT2Rs in mediating the haemodynamic effects of ANG II in the newborn period, a time when ANG II levels are high, and when there is a preponderance of AT2Rs, as detailed in the introductory chapter (section 1.1.6.2).^{208,432}

Considerable evidence from studies in adult animals has emerged over the past decades revealing physiological roles for both AT1Rs and AT2Rs in regulating cardiovascular homeostasis.^{210,220,433} It has been shown that, under certain conditions, ANG II counterbalances its own vasoconstrictor effect mediated by activation of AT1Rs through the vasorelaxation mediated by activation of AT2Rs. Nonetheless, these vasodilator and hypotensive actions of AT2Rs are revealed only against a background of RAS up-regulation, such as in conditions of RAS-dependent increase in arterial pressure, and pharmacological inhibition of AT1Rs.⁴³⁴⁻⁴³⁶ For instance, experiments carried out under activation of the RAS in the renovascular hypertension rat model or chronic activation of RAS through depletion of Na⁺ intake, have provided evidence that AT2Rs are involved in arterial pressure regulation through a cross-talk with AT1Rs, mediating a vasodilation that counterbalances the vasoconstrictor effects of AT1R through activation of the bradykinin-NO-cGMP pathway.¹⁴¹ This has been the subject of previous reviews by Carey (2005),^{115,437} Carey and Padia (2008),¹²⁵ Jones *et al* (2008).⁶⁵

To date, there are no studies of any such cross-talk between ATRs in mediating ANG II-induced cardiovascular responses early in life. Since in the newborn period, the RAS is highly activated and ATR expression, particularly AT2Rs, is increased within the newborn vasculature,¹⁷⁶ it raises the possibility of a more active functional role for the AT2Rs in mediating the cardiovascular effects of ANG II postnatally. Based upon this premise, we anticipated that, during the newborn period, AT2Rs may influence the sensitivity of the systemic vasculature to the high levels of ANG II characteristic of this period, and serve to balance AT1R-induced pressor effects, such as occurs in adult mammals. In contrast, the findings from the present study are consistent with an absence of any modulatory effects of AT2Rs on the haemodynamic responses induced by activation of AT1Rs in the newborn period.

This is the first measurement of the combined effects of ATRs on haemodynamics in newborn animals studied in the awake state and in the absence of any confounding effects of surgery and anaesthesia, and therefore provides a new contribution to the literature of newborn physiology. In fact, to our knowledge, there are no measurements of the combined effects of AT1Rs and AT2Rs in any newborn animals. Therefore, this study is the first investigation of the ATRs interaction in mediating the cardiovascular effects of endogenously produced ANG II during postnatal development and will provide an important contribution to our understanding of the RAS during ontogeny.

The results are intriguing considering that AT2Rs appear to predominate in the systemic vasculature of the developing lambs.¹⁷⁵⁻¹⁷⁶ Except for the umbilical vessels, the AT2R is the primary receptor subtype expressed in the systemic vasculature near-term and during the first two weeks of postnatal life in sheep, after which there is a near complete transition from AT2Rs to AT1Rs by three months postnatally.¹⁷⁵⁻¹⁷⁶ Velaphi *et al* (2002)¹⁹⁶ examined the mechanisms through which ANG II modulates vasoconstriction and arterial pressure in conscious newborn sheep showing that ANG II does not influence peripheral vascular resistance (measured at the level of femoral artery) until two weeks after birth, although it does modulate systemic vascular resistance. Taken together, the difference between ATRs activity postnatally may be due to either incomplete vascular expression, differences in affinity to ANG II or to differences in the level of activation during ontogeny. Such a premise warrants further investigation. The experimental findings from the present study would also confirm such a theory, since AT2Rs do not appear to be regulating haemodynamics, nor are they regulating the effects of AT1Rs early in life.

With regard to the developing renal vasculature, several lines of evidence have shown that ANG II is an important vasoconstrictor. Previous experiments in our laboratory have shown an effect of exogenous ANG II on renal haemodynamics in conscious lambs at doses that did not affect MAP, suggesting that the renal vasculature is sensitive to ANG II.¹⁹⁸ In addition, selective inhibition of the AT1Rs in the developing sheep and rabbit was followed by alterations of renal haemodynamics,²¹⁰⁻²¹¹ suggesting, therefore, a role for the AT1Rs in mediating the renal haemodynamic effects of the endogenously produced ANG II during postnatal development.²¹¹ For example, selective AT1R inhibition elicited a dose-dependent decrease in RBF in anaesthetized six days old rabbits,²¹⁰ and a higher decrease in RVR in newborn anaesthetized piglet as compared with adult pig.³⁰⁹ In conscious lambs in our laboratory, administration of the AT1R antagonist, ZD 7155 but not the AT2R antagonist, PD 123319 decreased RVR and

increased RBF.²¹¹ This observation is in keeping with the present findings on the effects of ATRs antagonists, ZD 7155 and PD 123319 on the renal haemodynamics of conscious lambs as well as our previous studies.³⁶⁰ In addition, the present study complements and strengthens our previous observations by showing, for the first time that combined administration of ZD 7155 and PD 123319 did not alter the changes in renal haemodynamics elicited by ZD 7155 alone. This finding was surprising and contradicts our premise that AT2R modulate AT1R-mediated effects on renal haemodynamics early in life.

There is consistent evidence from studies in adult conscious and anaesthetized animals showing that, in conditions of increased RAS activity (that is a condition similar with up-regulation of RAS activity in newborn lambs from the present study), AT2R activation induces a renal vasodilation that counterbalances the AT1R-mediated renal vasoconstriction.^{95,140} This protective vasodilator response appears to be mediated by the renal production of bradykinin, NO and cGMP,^{96,140,438} as well as PGF2a.¹¹⁴ Taken together, these studies advocate for an active modulatory role of AT2Rs predominately under conditions of RAS activation, a situation that may relate to either up-regulation of AT2Rs expression and/or increased cross talk between the receptors. Our hypothesis regarding the role of ATRs in the newborn period was based upon such an assumption that there would also be a developmentally regulated cross-talk between the AT1Rs and AT2Rs in mediating renal haemodynamic effects of ANG II. Such an assumption was sustained by the observation of a developmentally regulated intrarenal expression and distribution of the ATRs in various species (detailed in section 1.1.7.2).^{83,188,439} For example, within the pre-glomerular vasculature of the newborn piglet AT1R gene expression, lowest at birth, progressively increases during maturation, whilst AT2R mRNA and protein expression is highest at birth and decreases thereafter.¹⁸⁸ At present, an explanation of the absence of any effects of AT2Rs and of an interaction between ATRs in modulating renal haemodynamics in conscious lambs cannot be provided. It is conceivable, however, that the stage of renal maturation and/or activity of the ATRs within the developing kidney of various species may influence the renal haemodynamics responses. Also, differences in the renal expression and distribution of the ATRs between

species may reflect the differences between the responses to ATRs antagonists. Studies by Sorensen *et al* (2000)⁴⁴⁰ provide an additional explanation of the age-dependent renal haemodynamic changes. In halothane-anaesthetized adult rats, a reduction in the renal perfusion pressure (that may have also occurred in our experiments) induces a gradual decrease in RBF due to pre- and post-glomerular vasoconstriction that is blunted by ANG II,⁴⁴⁰ and therefore, may explain the alterations in RBF in older animals from our experiments. Such an assumption warrants further investigation regarding the distribution and activity of ATRs within the glomerular vasculature of the developing sheep.

In addition, the arteriolar effects mediated by ANG II through the ATRs may be selectively potentiated/modulated, in an age-dependent manner, by other vasoactive factors,⁴⁴⁰ such as ANG 1-7 and other angiotensin fragment peptides, PGs, endothelin or NO. For example, previous studies in our laboratory in conscious lambs have shown a role for endogenously produced PGs in modulating the haemodynamic responses to ANG II,³⁶³ although the receptor through which this occurs was not studied. The possible involvement of ATRs in modulating the effects of the ANG II fragment peptides as well as the interaction of ANG II with other vasoactive factors in modulating haemodynamics early in life remains to be investigated.

The differences between published literature from studies in adult animals and our data from newborn animals may as well reflect differences in baseline arterial pressure. That is an interaction between AT1Rs and AT2Rs is present only when both, the arterial pressure and the circulating levels of ANG II are increased. The fact that, in the present study, ANG II levels are increased whereas arterial pressure is not, may explain the absence of a cross-talk between the two receptors subtypes. On the other hand, the increased level of expression of the AT2Rs within the developing kidney may be related rather to an involvement of these receptors in regulating kidney function rather than haemodynamics. This will be presented in the following section (5.2).

In conclusion, this study explored the roles of the AT1Rs and AT2Rs, separately and together, in mediating the cardiovascular effects of the endogenously produced ANG II during postnatal maturation in conscious animals. It demonstrates for the first time that AT2Rs do not play any modulatory role on the AT1R-induced pressor or renal haemodynamic effects of ANG II, substantiating a predominant role for AT1Rs in regulating cardiovascular homeostasis early in life.

5.2 Effects of ATRs on renal function during postnatal development

This protocol was designed to test the hypothesis that renal effects of ANG II are mediated through AT1R and AT2R during postnatal maturation and that AT2Rs modulate renal responses to activation of AT1Rs. To test this hypothesis, experiments were carried out in conscious, chronically instrumented lambs, aged one and six weeks of life, in which various parameters of renal function were measured before and after inhibition of AT1Rs using ZD 7155, AT2Rs using PD 123319 or both antagonists, ZD7155 and PD 123319 to inhibit both ATRs.

This study provides new information on the roles of ATRs during postnatal maturation (summarized in Figure 5.2) as follows:

1) ANG II modulates renal function in conscious animals through predominant activation of AT1Rs in an age-dependent manner;

2) AT2Rs alone do not appear to mediate ANG II renal effects during postnatal maturation;

3) AT2Rs modulate the renal effects mediated by AT1R in an age-dependent manner during postnatal development, a period of time when the RAS is activated.



Figure 5.2 Effects of AT1R and AT2R on renal function in conscious lambs

Therefore, this study is the first to explore the functional roles of the AT1Rs and AT2Rs in regulating fluid and electrolyte homeostasis during postnatal development in conscious animals. It brings an original contribution to the newborn physiology literature through a complex approach of studying each receptor individually followed by investigation of the ATRs interaction in mediating the renal effects of ANG II *in vivo* and under physiological conditions, during the challenging period of adaptation to life after birth. In addition, it is first study of the renal functional roles of AT1Rs in developing sheep and of AT2Rs in any newborn animals.

The above mentioned novel findings are sustained by the following observations from the present experiments (Figure 5.2):

a) Glomerular function is regulated predominantly by ANG II through activation of AT1Rs, but not AT2Rs in an age-dependent manner. However, AT2Rs appear to moderate the effects of AT1R on GFR in older lambs, suggesting a possible interaction between these receptors may ensue as maturation proceeds.

b) ANG II modulates age-dependently K⁺ handling, but not Na⁺ and Cl⁻ handling along the nephron during postnatal development through predominant activation of AT1Rs in one week old lambs. AT2Rs appears to buffer these effects in younger lambs.

c) Endogenous ANG II modulates water handling along the nephron in an agedependent manner through activation of both receptors, AT1Rs and AT2Rs. The effects on urine production, urinary osmolality and free water clearance are regulated predominantly through activation of AT1Rs whereas AT2Rs appear to diminish the AT1R effects on urine production later in life.

The renal effects of angiotensin antagonists, ACE inhibitors and ATRs antagonists have been described previously in adult anaesthetized and conscious animals.^{423,441-442} In addition, several studies have provided evidence on an active role of RAS in regulation of the kidney activity during development.^{208,210-211,407,443-444} However, to date, there have been no functional roles ascribed to AT2Rs on renal function, nor have the possible modulatory role of AT2R on the renal effects AT1R-induced been investigated during postnatal development. The data presented in this section are the first to describe participation of both ATRs in mediating glomerular and tubular responses of

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ANG II during postnatal development in conscious animals, providing new evidence for the active involvement of AT1Rs in mediating ANG II effects and for AT2Rs in buffering these renal effects early in life.

As described in a previous section of this dissertation (Chapter 5, Section 5.1.), intravenous administration of the AT1R antagonist ZD 7155 but not the AT2R antagonist PD 123319 was followed by a fall in RVR in both age-groups studied. The changes in renal vascular tone were associated with age-dependent alterations in blood flow to the kidney. Therefore, it was not surprising that the changes in haemodynamics may have contributed to the age-dependent alterations in GFR found in the present experiments after administration of ZD 7155 alone. Interestingly, PD 123319 prevented the fall in GFR when administered after pre-treatment with ZD 7155 in older lambs. These findings may be explained through age-dependent effects that ATRs may mediate during development on the two governing parameters of the glomerular filtration: the net filtration pressure across the glomerulus and the ultrafiltration coefficient Kf. It is known that the expression and localization of ATRs shifts within kidney structures after birth to reach the distribution seen in the adult kidney (as detailed in section 1.1.5. of this dissertation). Therefore, it is possible for ATRs to mediate divergent vasomotor regulatory mechanisms in the afferent and efferent arteriole VSMC, which are known to regulate the hydrostatic pressure within the glomerular capillaries, and therefore, GFR. Taking into consideration the abundance of AT2Rs during the transition to newborn life, we predicted that, at least partially, the physiological effects of ANG II on glomerular filtration immediately after birth would be mediated by AT2Rs. However, this was not the case. On the contrary, we observed an age-dependent decrease in GFR, with effects that were elicited rather later in life and mediated by AT1Rs.

It has been shown that, in glomerular arterioles of adult animals, in which AT1Rs are the predominant receptor subtype, ANG II regulates the vascular tone of the efferent arterioles, predominant through the AT1Rs, favoring glomerular filtration.⁴⁴⁵ This is in accord with the present findings in six weeks old lambs, which may imply that AT1Rs mediate a vasoconstrictor effect preferentially at the post-glomerular efferent arteriole. Similar effects on GFR were found in a previous investigation by our laboratory in three

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week old conscious lambs infused with the ACE inhibitor, captopril.^{204,206} Taken together, these findings point out to a more important role for AT1Rs in regulating GFR as maturation proceeds.

On the other hand, the present data show a significant fall in FF after ZD 7155 in the absence of significant effects on GFR in one week old lambs. Since RPF consistently tended to rise more than the extent to which GFR decreased in younger animals, there was an age-dependent fall in FF in one but not in six weeks old lambs, in which RPF and GFR changed equivalently in opposite directions. These findings may suggest, intriguingly, modulatory effects on glomerular arteriolar tone mediated by AT1Rs in the immediate newborn period. Therefore, an age-dependent functional maturation in addition to the spatial and temporal distribution profile of the ATRs at the glomerular level, may contribute to explain the present findings. In three week old anaesthetized piglets, in which AT1R gene expression is down-regulated in the afferent arteriole and up-regulated in the interlobular and arcuate arteries,¹⁸⁸ administration of the AT1R antagonist, A-81988, markedly increased RBF, while it did not alter GFR.³⁰⁹ In newborn anaesthetized rabbits, administration of the AT1R antagonist, losartan, did not alter RBF, GFR and FF,²¹⁰ while the ACE inhibitor, perindoprilat, increased RBF and did not influence GFR and FF.⁴⁴⁴ These findings are, however, difficult to interpret due to the known detrimental effects of surgery and anaesthesia on the measured variables as well as variability in the ages studied. Nonetheless, the lack of consensus of the above mentioned findings may reflect as well species-dependent maturational differences of ATRs on the glomerular capillaries, including differential effects on the afferent and/or efferent arterioles. To date, no studies have been carried out to investigate the temporal and spatial distribution of the ATRs in the pre- and post- glomerular vasculature in sheep. In addition, ATRs roles in mediating the vasomotor ANG II effects on the pre- and/or post-glomerular vessels during development are not known.

Furthermore, since GFR is governed by two parameters, (1) net filtration pressure at the glomerulus and (2) the filtration coefficient Kf, it is conceivable that the age- and species- dependent changes in GFR may also result from an age- and species-dependent maturational regulation of Kf, ^{221,364} although the contribution of the ATRs to Kf during

development has not been investigated to date. In addition, to our knowledge, there are no other studies regarding the regulation of GFR by AT2Rs or a possible interaction between these receptors in mediating ANG II effects on glomerular filtration in developing animals. Ours study, therefore, addresses for the first time this research question and shows that AT2Rs influence glomerular function during postnatal maturation by possibly counterbalancing the effects of the AT1Rs, therefore providing new information regarding the way in which ANG II regulates GFR early in life.

Studies investigating the roles of AT2Rs in regulating glomerular function have been performed in adult animals.^{423,425,442,446} For example, similar with the findings from the present experiments, in anaesthetized adult dogs, PD 123319 had no significant effects on renal haemodynamics, GFR or FF.⁴²³ In anaesthetized, sodium-depleted adult rats, in which the RAS is activated (similar with high RAS activity in one week old lambs from the present experiments) PD 123319 infusion over a wide range of doses, did not significantly affect renal haemodynamics and glomerular function.⁴²⁵ Interestingly, however, in our experiments addition of the AT2R antagonist PD 123319 prevented the effects mediated by AT1Rs on glomerular function in older animals. This observation may suggest that AT1R-induced arteriolar vasomotor activity may be offset by the activation of AT2Rs, and that AT2Rs modulate the responses AT1R-mediated in an agedependent manner. Such an effect was shown in other species. For example, AT2Rmediated renal vasodilation that potentiates the effects induced by the AT1R inhibition was elicited in conscious adult rats, albeit in conditions of RAS activation.^{95,141} It is also likely that ATR inhibition in our experiments may have caused activation or suppression of various rescue mechanisms such as NO, BK, endothelin, adenosine and sympathetic reflexes. The effects of these vasoactive factors on the renal vasculature have, in fact, shown to be developmentally regulated.^{403,407,411} Taken together, further investigations are warranted to address the underlying mechanisms of ATRs regulation of the glomerular function during postnatal development.

The salt-retaining effects of ANG II through stimulation of Na^+ proximal and distal tubular reabsorption are well documented in adult mammals. As described elsewhere in this dissertation (section 1.1.5), compelling evidence demonstrates the

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multifaceted contribution of ANG II to the regulation of Na⁺ transport within the kidney, effects that range from natriuresis to antinatriuresis, effects mediated through both receptors, not only via direct effects on renal tubular transport but as well through multiple effects on renal haemodynamics, GFR, and aldosterone secretion.

Much less is known regarding the contribution of ANG II to Na⁺ handling in newborn mammals. It has been shown, however, that Na⁺ handling along the nephron is different in the newborn as compared with the adult in several species,⁴⁴⁷⁻⁴⁴⁸ including sheep.³⁷⁷ In adult animals, in which the RAS is activated, inhibition of ANG II formation with ACE inhibitors elicits a robust increase in water and electrolyte excretion.⁴⁴⁹⁻⁴⁵⁰ Therefore, this study predicted that, in newborn lambs, in which RAS is also highly activated^{451,165}(detailed in section 1.1.7.1), there would also be a significant increase in water and electrolyte excretion during ATRs inhibition. We also predicted that AT2Rs, which are the predominant type of ATRs expressed within the kidney vasculature and distal tubule immediately after birth, may play a predominant role in water and electrolyte handling in the newborn. On the contrary, such effects were not observed. Surprisingly, in the present study, in one and six week old lambs, Na⁺ and Cl⁻ excretion were not affected by either the AT1R antagonist, ZD 7155 nor the AT2R antagonist, PD 123319 nor by the concomitant treatment with both antagonists. The absence of any effects of ANG II on renal handling of Na⁺ may suggest immaturity of the ANG IIdependent reabsorptive mechanisms from the proximal tubule and /or distal tubule. Such an assumption is sustained by the findings in neonatal rat and volume-expanded puppies, in which Na⁺ reabsorption in the distal tubule is enhanced and reabsorption in the proximal tubule is depressed relative to the adult,⁴⁴⁷⁻⁴⁴⁸ a situation similar with fetal sheep⁴⁵². In addition, the RAS may play a species- and age-dependent role in regulating Na⁺ handling along the nephron during postnatal development and may explain the variability of the AT1Rs effects on renal function regulation in observed in newborn. For example, in newborn anaesthetized rabbits, the AT1R inhibitor, losartan had no significant effects on urinary flow or Na⁺ excretion²¹⁰, whereas in pre-weaned anaesthetized rat pups, Chevalier *et al* (1996)²⁰⁸ observed a robust diuresis, natriuresis and kaliuresis and a marked increase in fractional Na⁺ excretion after losartan infusion. In contrast to Na⁺ and Cl⁻, K⁺ excretion significantly fell in one week old lambs after continuous infusion of ZD 7155 but not PD 123319 in the present experiments. The fact that the effects on K⁺ excretion occurred later, after 60 min of ZD 7155 infusion, and in the absence of changes in tubular Na^+ handling, suggests that the effects on K^+ handling by ANG II through AT1Rs might predominate at the distal rather than at the proximal nephron. Furthermore, it appears that AT2Rs may play a modulatory role on the effects of AT1Rs in K⁺ handling regulation. In addition, our findings suggest that ATRs modulate K^+ and water handling differently in one as compared with six weeks. Such a theory is sustained by experimental evidence which suggests that, in contrast with the adult, fully differentiated kidney in which final regulation of K⁺ excretion occurs at the level of the cortical collecting duct, no significant net K⁺ transport has been identified within the first three weeks of postnatal development in rabbit.⁴⁵³⁻⁴⁵⁶ Clearance studies in several species, including the newborn rabbit as well as human infants, revealed low baseline rates of K^+ excretion that contribute to maintaining a state of positive K^+ balance necessary for somatic growth.⁴⁵⁷ Studies in the rabbit and rat newborn kidney have shown a developmentally regulated temporal and spatial expression of various $K^{\scriptscriptstyle +}$ channel proteins within the renal tubule.^{456,458-459} The presence of such developmental events within the sheep kidney has not been investigated to date and the mediating signal which may involve ATRs regulation of K^+ handling remains to be identified. On the other hand, the decrease in TTKG observed in our present experiments in the newborn lamb suggests a low aldosterone activity that may be related either to immaturity of the ATRs in the distal and collecting tubule or to a reduction in aldosterone secretion elicited by AT1R inhibition at the adrenal gland.⁴⁶⁰ Several studies have shown the presence of an aldosterone-mediated effect on distal tubular Na⁺ and K⁺ handling in the neonate,^{448,461} whereas Aperia et al (1975),⁴⁶³ (1981),⁴⁶² have reported that aldosterone exerts a permissive role on the activity of the proximal Na⁺-K⁺-ATPase in immature but not in adult rat proximal tubular cells. The present experiments did not measure the circulating levels of aldosterone and although do not allow precise determination of the tubular site of K⁺ reabsorption, suggest rather age-dependent modulatory effects of AT1Rs predominant in the distal nephron.

With respect to water handling, active participation of AVP in homeostatic mechanisms after birth has been suggested by studies in conscious lambs in which AVP may mediate the systemic pressor effects of ANG II on MAP through binding on its specific vascular receptors.⁴⁶⁴ The age-dependent effects on water handling may be due to the increased sensitivity of the collecting tubule to AVP with maturation as suggested from previous work in newborn rats⁴⁶⁵ and rabbits⁴⁶⁶ and fetal sheep.⁴⁶⁷ Although the presence of functional AVP receptors in the immature kidney has been supported by experimental evidence, other factors such as a short loop of Henle, high medullary blood flow that limit the formation of the osmotic gradient, or counter-regulatory effects of vasoactive factors such as PGs and NO may explain the differences in the concentrating abilities between younger and older lambs. Our hypothesis regarding a direct role of AT2Rs in water handling regulation during postnatal development is not sustained by the present findings; PD 123319 when infused alone had no effects on water handling in one and six week old lambs. Such effects were shown, however, in adult animals. In conscious adult rats, intrarenal PD 123319 infusion was associated with a marked diuresis.¹³³ In sodium-depleted anaesthetized adult dogs,⁴²³ PD 123319 significantly increased urine volume and free water excretion. These effects were thought to be result of direct effects of PD 123319 on distal tubule and collecting ducts or to possible central effects of the drug on AVP secretion.⁴²³ Although AT2Rs are present in the developing kidney,⁷⁷ a specific effect of PD 123319 on plasma levels of AVP have not been reported.⁴²³ In contrast, similar with our findings in newborn lambs, no effects on water handling were elicited by PD 123319 in sodium-depleted anaesthetized adult rats,⁴²⁵ sustaining the present findings in the newborn lambs.

In conclusion, the wide range of renal effects mediated by ATRs, related to differences in species, dose of the drug and administration route as well as level of maturation and experimental conditions, point out to the variability of the physiologic roles ATRs may play during the period of adaptation to life after birth. Taken together, the present study contributes to the newborn physiology literature through the findings that suggests a major, age-dependent role for AT1Rs in regulating kidney function during development, whereas AT2Rs appear to modulate the effects mediated by AT1Rs. The

underlying molecular mechanisms, however, cannot be explained through the light of the findings from this study and our observations warrant further investigation. However, an interaction between ANG II and other vasoactive factors in regulating kidney function is possible and may contribute to explain the wide variability of the observed ATRs effects from various studies. The changes in nitrate excretion observed in the present experiments suggest that ANG II modulates age-dependently NO production within the kidney, effects which appear to be mediated predominatly through activation of the AT1Rs. Such an interaction between ANG II and NO in developing sheep has been explored through the experiments described in this dissertation and the findings are presented in following sections.

5.3 Cardiovascular effects of ANG II - NO interaction during postnatal development: roles of AT1Rs and AT2Rs

This study was designed to test the hypothesis that there is a significant interaction between the vasoconstrictor ANG II and the vasodilator NO in regulating systemic and renal haemodynamics during postnatal maturation. Experiments were carried out in conscious, chronically instrumented lambs aged one and six weeks to investigate the roles of ANG II in modulating the effects of endogenously produced NO on systemic and renal haemodynamics through activation of AT1Rs and AT2Rs, separately and together, in the developing animal. The present research findings are interpreted based upon previous studies in our laboratory, in which the physiological effects of L-NAME (as well as D-NAME) were investigated.³¹⁶ Novel findings of this study are that, in conscious lambs during postnatal maturation:

1) AT2Rs do not modulate the effects of endogenously produced NO on systemic haemodynamics. The AT2R antagonist, PD 123319, does not attenuate the effects elicited by L-NAME on arterial pressure and heart rate in one and six week old lambs.

2) There is no interaction between AT1Rs and AT2Rs in modulating the effects of endogenously produced NO on systemic haemodynamics. The effects of ZD 7155 on arterial pressure and HR responses to L-NAME are not altered by addition of PD 123319 in either group of animals.

3) AT2Rs modulate renal haemodynamic responses to L-NAME in age-dependent manner. PD 123319 pre-treatment attenuates the effects elicited by L-NAME on renal haemodynamics predominantly at one week.

4) There is no interaction between AT1Rs and AT2Rs in modulating the effects of the endogenous NO on renal haemodynamics. Addition of PD 123319 does not buffer the modulatory effects of ZD 7155 on the increase in RVR after L-NAME, at one and six weeks. A synthesis of the aforementioned findings is provided in the Figure 5.3 below:



Figure 5.3 Roles of ATRs in mediating the effects of ANG II - NO interaction on haemodynamics in conscious lambs

In addition, the observations from the present study confirm previous findings in our laboratory:

a) AT1Rs do not appear to modulate L-NAME effects on systemic haemodynamics, confirming recent findings by Wehlage and Smith (2012).³⁶⁰ Taken

together, then, it appears that AT1Rs do not mediate an interaction between ANG II and NO in regulating cardiovascular homeostasis in the newborn period.

b) AT1Rs modulate age-dependently L-NAME responses on renal haemodynamics. Previous reports from our laboratory have shown that pre-treatment with ZD 7155 buffers the L-NAME effects on RBF in an age-dependent manner.³⁶⁰

Taken together these findings provide new evidence for the presence of an interaction between ANG II and NO in regulating cardiovascular homeostasis in the newborn period which appears to be mediated through activation of both, AT1Rs and AT2Rs.

As mentioned elsewhere in this dissertation (see details in Chapter 1, sections 1.1.7.1. and 1.2.8.) both vasoactive systems, RAS and NO, are highly activated at birth and may contribute to the cardiovascular maturational changes that characterize the early newborn period. Therefore, we hypothesized that, during the transition to life after birth, ANG II may modulate NO effects on haemodynamics through activation of ATRs. Since there is a predominance of AT2Rs in the systemic vasculature of sheep until two weeks postnatally, when transitioning to AT1Rs¹⁷⁵⁻¹⁷⁶ we also hypothesized that the vasodilator NO counterbalances the ANG II effects through predominant activation of the AT2Rs in the immediate newborn period and through AT1Rs later in life. In addition, we presumed that there may be an important, developmentally-regulated, interaction between AT1Rs and AT2Rs in mediating the modulatory effects of ANG II on NO. Our observations from the present experiments suggest that is not the case. The increase in MAP observed after addition of L-NAME was similar after the ATRs antagonists separately and together and comparable to the systemic haemodynamic responses to L-NAME alone infusion as shown by previous studies in our laboratory.^{312,316,319} Therefore, the present findings show, for the first time, that AT1Rs and/or AT2Rs do not modulate an interaction between ANG II and NO in regulating systemic haemodynamics during postnatal maturation. Our observations are, however, supported by the findings of Solhaug et al (1996)³⁰⁹ from experiments in anaesthetized newborn piglet, in which they showed that pre-treatment with the AT1R antagonist, A-81988, did not alter the MAP responses to L-NAME.^{309,313} To our knowledge, there have been no previous studies to investigate the

potential role of AT2Rs in mediating ANG II-NO interaction on cardiovascular homeostasis early in life.

In adult animals, several studies have demonstrated an absence of any such interaction between ANG II and NO in regulating systemic haemodynamics, sustaining, therefore, our current findings in newborns. For instance, in conscious and anaesthetized adult normotensive rats neither ACE inhibition nor AT1R inhibition alters the pressure response to NO synthesis inhibition.⁴⁶⁸⁻⁴⁷⁰ The absence of an interaction between NO and ANG II in regulating the systemic circulation immediately after birth in the newborn period raises the assumption that the increased activity of RAS at birth may be counterbalanced by other vasodilatory systems such as PGs. This supposition is sustained by previous experiments in our laboratory which have shown that immediately after birth, endogenously produced PGs, but not endogenously produced NO balance the vasoconstrictor actions of ANG II on the systemic vasculature.³⁶³ Furthermore, no interaction was apparent between NO and PGs in modulating the systemic ANG II responses postnatally in conscious lambs.³⁶³ As maturation proceeds, it is possible that a more consistent interaction between the vasodilator NO and vasoconstrictor ANG II may ensue in governing cardiovascular homeostasis.

The present findings suggest that the NO regulation of renal haemodynamic in conscious animals is modulated by ANG II to a greater extent immediately after birth than later in life. through contribution of both receptors, AT1Rs and AT2Rs. The possibility that ANG II may be an important regulator of NO synthesis and release in the kidney immediately after birth is supported by the following observations: (1) both systems are activated at birth,^{309,471-473} (2) both, ATRs and NOS isoforms undergo similar postnatal changes, (3) ATRs are localized and abundantly expressed in the near proximity of the NOS isoforms suggesting, potentially, a functional interaction.^{188,193,310} In fact, several studies have linked ANG II to regulating NOS isoforms expression and activity during development, revealing an age-dependent participation of both ATRs in structural and functional regulation of NOS isoforms.^{189,317} For example, Ratliff *et al* (2010)¹⁸⁹ showed that administration of the AT1R antagonist, candesartan and the AT2R antagonist, PD 123319 decreased nNOS mRNA and protein levels in the afferent

arterioles of the newborn piglet but not in mature arterioles of adult pig, whereas eNOS transcript and protein followed opposite patterns.¹⁸⁹ Studies in developing sheep in our laboratory have also shown a developmentally regulated pattern of ATRs and NOS expressions within the developing kidney.^{193,310} This dissertation adds an innovative contribution to the literature of newborn physiology by clarifying the roles of ATRs in mediating ANG II-NO interaction in the newborn kidney. Furthermore, this study provides for the first time insight into the ATRs cross-talk in mediating such an interaction during postnatal development. The fact that AT2Rs do not buffer the AT1R-mediated haemodynamic effects of L-NAME in the developing newborn may suggest either (a) there is a blunting effect on NOS activity that is already maximal after AT1R inhibition, or (b) absence of a cross-talk between AT1Rs and AT2Rs in modulating NOSs expression and/or enzymatic activity early in life.

An important interaction between NO and ANG II has been identified within the renal vasculature in conscious and anaesthetized adult animals. There is evidence that ANG II modulates renal haemodynamics responses to systemic³³² and intrarenal inhibition of NO synthesis.⁴⁷⁴ For example, ANG II potentiates the renal vasoconstrictor responses to L-NAME in conscious dogs and rats in which the RAS was activated.^{468-469,474} In anaesthetized adult rats, in which RAS is up-regulated, the AT1R antagonist, losartan attenuated the increase in blood flow to the kidney elicited by L-NAME.³³³ Conversely, the L-NAME-induced increase in arterial pressure appears to be mediated, at least partially, through activation of AT1Rs in sodium-depleted rats³³⁴. Several other studies provide evidence for an increased interaction between ANG II and NO in adult in regulating renal haemodynamics in conditions of RAS activation, mediated through AT2Rs.^{333,470}

The present findings support the premise of an interaction between the vasoactive factors ANG II and NO in regulating renal haemodynamics during postnatal development, at a time when the RAS is also activated. In addition, the present experiments demonstrate for the first time the important, age-dependent roles that ATRs play in modulating the effects on renal haemodynamics of endogenously produced NO during postnatal development.

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The differences between the systemic and renal haemodynamic responses as well as between the two age groups observed in the present experiments may reflect the fact that the mediators of these responses, the ATRs and the NOS isoforms, undergo developmentally regulated expression in the peripheral and renal resistance vasculature,^{160,176,304-305,475} (also detailed in sections 1.1.7.2 and 1.2.7). Another plausible explanation may be offered as well by the maturational effects of other vasoactive factors, such as endothelin, PGs or the sympathetic nervous system that also regulate cardiovascular homeostasis and may contribute as well in the immature kidney to the haemodynamic responses to NO synthesis inhibition.^{363,411} Further studies are warranted to elucidate the intricate mechanisms of regulatory factors in influencing kidney blood flow during development.

On the basis of the results presented here, it can be concluded that these two systems - ANG II and NO - interact in modulating cardiovascular homeostasis during the critical adaptation to the extra-uterine life, setting the base for questioning a possible interaction between ANG II and NO mediated through AT1R and AT2Rs in regulating the function of the developing kidney. This will be addressed in section 5.4 of this chapter.

5.4 Renal effects of ANG II - NO interaction during postnatal maturation: roles of AT1Rs and AT2Rs

The purpose of this study was to test the hypothesis that there is a significant interaction between endogenous ANG II and NO in regulating renal function during postnatal maturation and that this interaction is mediated through AT1Rs and/or AT2Rs. In these experiments in conscious, chronically instrumented lambs, numerous renal variables were measured before and after inhibition of NO synthesis with the L-arginine analogue, L-NAME, in the presence of the AT1R antagonist, ZD 7155, the AT2R antagonist, PD 123319 or both antagonists, ZD 7155 and PD 123319. Novel findings of this study are that ANG II modulates the effects of NO on renal function through AT1Rs and AT2Rs in an age-dependent manner as follows:

1) AT1Rs and AT2Rs mediate age-dependently the counter-regulatory effects of ANG II and NO on GFR.

2) ANG II modulates L-NAME responses on tubular Na⁺ and water handling in six weeks old lambs through both AT1Rs and AT2Rs, although a synergistic involvement of both receptors is also possible.

3) ANG II modulates L-NAME-induced kaliuresis, chloriuresis and diuresis in six weeks through both AT1Rs and AT2Rs and AT2Rs potentiate the effects induced by AT1Rs. A schematic of these findings is illustrated in Figure 5.4:





This study is the first to describe the presence of an AT1R- and AT2R-mediated interaction between ANG II and NO on renal function during maturation in conscious animals, providing a new and important contribution to the literature. The following paragraphs provide explanations of the above-mentioned findings of the ANG II interaction with NO in regulating renal function during development. These explanations

are in the light of previous studies in our laboratory, in which in similar experimental conditions, only the effects of L-NAME were investigated.³¹⁹ The previously published studies of NO inhibition effects on haemodynamics and renal function in conscious lambs during postnatal maturation from our laboratory provide a solid background that facilitates the interpretation of the results from the present experiments, preventing possible overestimations of the apparent physiological effects of ANG II-NO interaction during postnatal period. The rationale for this choice is furher detailed in Section 3.4 and Chapter 6 of this dissertation.

With regard to glomerular function, the aforementioned studies showed that inhibition of NO synthesis with the L-arginine analogue, L-NAME, was followed by a dramatic decrease in GFR and FF in one week old conscious lambs, whereas both GFR and FF remained constant in six week old lambs.³¹⁹ Interestingly, the fall in GFR and FF observed previously by Sener and Smith³¹⁹ in one week old conscious lambs was not elicited in the present experiments by L-NAME after co-infusion with both ATR antagonists, ZD 7155 and PD 123319. Since AT2Rs are predominately expressed within the kidney in early development and localized in the close vicinity of the nNOS isoforms,¹⁸⁸ we predicted a more important role for this receptor type in mediating NO effects on GFR at this age. This may suggest that, earlier in life, ANG II modulates the effects of NO on glomerular filtration through activation of both, AT1R and AT2R. This is the first description of such an important interaction between ANG II and NO in glomerular function regulation in the newborn. In contrast, in the present experiments, L-NAME restored the GFR towards baseline values, and consequently, altered the AT1Rinduced decrease in GFR in six weeks old lambs. An interesting observation was that there was no apparent role for AT2Rs or a synergistic role for both ATRs in mediating the interaction of ANG II and NO on glomerular filtration at six weeks. Since no additional effects were observed when both receptors were inhibited concomitantly, it can be inferred that there are no synergistic effects of AT1Rs and AT2Rs in modulating NO effects on glomerular haemodynamics in the newborn. A similar response was also observed by Solhaug *et al* $(1996)^{309}$ in experiments in anaesthetized developing swine, in which the decrease in GFR elicited by the intrarenal infusion of L-NAME was abolished by pre-treatment with AT1R antagonist, A-81988.

As mentioned elsewhere in this dissertation (section 1.1.7.2), RAS is highly activated at birth and remains so during maturation in several species including sheep, swine and human.^{162,358,471} In addition, the NO system is also activated at birth, several studies showing that NO is a more important intrarenal vasodilator in the developing kidney, probably counterbalancing highly activated vasoconstrictors such as ANG II³¹⁷ (detailed in section 1.2.7). Recent studies have shown that inhibition of either AT1Rs or AT2Rs results in significant blunting of both nNOS mRNA and protein, but not eNOS, and attenuates the enzymatic activity of NOS isoforms¹⁸⁹ in the developing afferent arteriole of the newborn piglet. This supports a role for nNOS in ANG II-mediated NO release in the afferent arteriole of the newborn. Modulatory effects of NO activity by the ATRs on efferent arteriolar tone have not been investigated to date during ontogeny. In addition, in accord with the developmental patterns of ATRs and NOS expression¹⁸⁸⁻ 189,305 in the developing porcine kidney, Ratliff *et al* (2010)¹⁸⁹ have also shown that the regulation of NOS activity by AT1Rs increases while AT2Rs-mediated regulation decreases during maturation. The aforementioned findings are in keeping with the observations from the present study. That is, our results provide the first direct evidence of a developmentally regulated modulation of NO responses on glomerular function by ANG II at birth, with a predominant role for AT1Rs later in life.

Several studies have demonstrated that ANG II stimulates NO release via AT1Rs with direct effects on intrarenal vascular tone, providing evidence for possible AT1R-modulated NO effects on glomerular filtration in adulthood. For instance, Patzak *et al* (2004)³⁴⁹ and Thorup *et al* (1998)^{350,351} found that ANG II administration increases NO production in isolated afferent arteriole of the adult mice and, rats respectively; this response is abolished upon inhibition of the AT1Rs. In addition, in isolated microperfused rabbit glomeruli, NO selectively modulates the ANG II-mediated vasoconstriction at the afferent but not efferent arteriole,⁴⁷⁶ whereas in the rat nephron, De Nicola *et al* (1992)⁴⁷⁷ have shown that administration of losartan prevents the effects of NOS inhibition on efferent arteriole.

The observed changes in GFR may also be attributable to alterations of the other major determinant of GFR, the coefficient of ultrafiltration, Kf. Such a possibility should be taken into consideration since ATRs are expressed on the membrane of glomerular mesangial cells which are capable of generating biologic responses to NO at least in the adult kidney.⁴⁷⁸ The effects of an interaction between ANG II and NO modulated through ATRs on Kf have not been studied in the newborn. Whether AT1Rs alone or by working synergistically with AT2Rs modulates NO-induced effects on the afferent and/or efferent arteriolar tone of the developing kidney and/or Kf it is not known and warrants further investigations.

It is also important to acknowledge that the age-dependent observations in glomerular function may be attributable, at least partially, to the extra-renal effects of ATRs antagonist and/or L-NAME. As discussed in the preceding sections (section 5.3) and confirmed by previous studies in our laboratory^{211,316} the present data provide evidence that the age-dependent effects of ATRs antagonists and L-NAME on renal function do not appear to be determined by secondary systemic effects and rather are supported by alterations in the expression and activity of ATRs and NOS isoforms. Even so, the possibility of an age-dependent contribution of other active peptides such as endothelin or PGs or activation of renal sympathetic nerves cannot be excluded at this time.

The following paragraphs will focus on the age-dependent roles of ATRs in mediating the ANG II-NO interaction responses on tubular function, explained from the perspective of L-NAME administration alone, investigated previously in conscious lambs in our laboratory and published by Sener and Smith (2002).³¹⁹

Previously, experiments from our laboratory showed that L-NAME administration alone was associated in six but not in one week old lambs with a robust natriuresis that resulted from a marked reduction in total fractional reabsorption of Na^{+ 319} that was thought to be mediated by a decrease in circulating levels of ANG II.³¹⁹ The present experiments provide evidence for an age-dependent contribution of ATRs in influencing the natriuresis associated with L-NAME administration. Neither ANG II nor NO appear to regulate Na⁺ handling in the immediate newborn period, while in older lambs both, ZD

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7155 and PD 123319 profoundly altered the L-NAME response, suggesting a role for both, AT1Rs and AT2Rs in modulating the interaction between ANG II and NO on Na^+ reabsorption.

Studies in adult animals show, for example, a robust AT2R-mediated natriuresis associated with increased expression and translocation of the AT2Rs on the apical membrane of the proximal tubule in anesthetized normotensive adult rats.¹²⁶ However, the AT2R-induced natriuresis was engendered in conditions of intrarenal AT1R inhibition with candesartan.¹³³ The aforementioned studies may offer an explanation for an ANG II-dependent decrease in Na⁺ tubular reabsorption during L-NAME treatment mediated through the synergistic action of both AT1Rs and AT2Rs. Another possible explanation for the age-dependent ATR modulation of L-NAME effects on tubular function resides in age-dependent distribution of NOS enzyme and ATRs. As previously mentioned in this dissertation, nNOS, the predominant isoform in early development, is elevated in the thick ascending limb of Henle's loop including macula densa cells in newborn rats³⁰⁴ and piglets.³⁰⁶ A developmentally regulated expression of AT1Rs and AT2Rs within the epithelial cells of the distal tubule and juxtaglomerular apparatus has also been observed in several species including rat⁸³ and sheep.^{173,191} In addition, an agedependent maturation of the protein transporters such as Na⁺-H⁺ exchanger and Na⁺-K⁺-ATPase has been suggested by several studies in newborn kidney.⁴⁷⁹⁻⁴⁸¹ Studies in adult animals provide strong evidence that NO inhibits Na⁺ reabsorption through direct action at the renal tubule by inhibiting the activity of these transporters,²⁷¹ which are also known to be regulated by ANG II through AT1Rs and AT2Rs.^{130,482}

To our knowledge, there are no studies to date regarding the effects of ATRs in modulating L-NAME effects on renal handling of Cl⁻. Since renal reabsorption and excretion of Cl⁻ is paralleled to that of Na⁺, it is conceivable that the changes in Cl⁻ excretion may have resulted from direct correlation to the Cl⁻ isoelectric transport with Na⁺ across the tubular epithelium. There will be no further discussion of Cl⁻ transport in this dissertation.

Most interestingly, the present study suggests a participation of both, AT1R and AT2R in modulating the kaliuresis induced by L-NAME infusion in an age-dependent

manner. The present findings are the first to propose a developmentally regulated role for AT2Rs in buffering L-NAME-mediated kaliuresis and counterbalancing the promoting effects of AT1Rs on tubular K^+ handling. Although to date there has been no investigation into any role for ATRs in modulating NO responses on renal handling of K^+ , it is possible that these effects may have resulted from the alteration of mineralocorticoid production in the adrenal gland. Both receptors, AT1R and AT2R are present in the glomerulosa cells which are responsible for aldosterone synthesis in the adrenal gland during development.^{179,483} The expression of NOS isoforms has also been demonstrated within the adrenal glands.³²⁶ In contrast with the adult, in which AT1Rs predominantly stimulates synthesis of aldosterone,484 a more important role for adrenal AT2Rs in mediating aldosterone release has been demonstrated in fetal sheep.^{179-180,483} Several other studies have found that ANG II-induced aldosterone synthesis/secretion in adrenal cortex in adult animals is decreased by NOS inhibition⁴⁸⁵⁻⁴⁸⁶ (reviewed by Millat et al (1999).³²⁶ Although the plasma levels of aldosterone were not measured in the present experiments, its relative activity could be assessed from the urinary Na⁺ to K⁺ excretion as well as TTKG. The increase in the urinary Na⁺ to K⁺ ratio after combined treatment of ATRs antagonists and L-NAME in six week old lambs point out to an agedependent modulatory effect of these vasoactive factors on either secretion from the adrenal gland or/and on the aldosterone actions at the distal renal tubule. The fall in TTKG observed at one week in the absence of alterations in plasma K^+ indicates low activity of aldosterone in the newborn period that may be related to immaturity of the aldosterone receptors at this age.⁴⁶⁰ It is also possible that AT1Rs mediate a permissive effect of NO at the distal tubule early in life that may relate as well to the maturational changes in K^+ channels activity and/or expression.⁴⁵⁴⁻⁴⁵⁵

Sener and Smith (2002)³¹⁹ have shown previously that when infused alone L-NAME elicited an age-dependent increase in urinary flow. The present experiments support a developmentally regulated, predominant role for AT2R in modulating this diuretic response to L-NAME. The robust increase in free water clearance following L-NAME suggests the effects are mediated through AVP release. Although the plasma levels of AVP were not measured in the present experiments, its relative activity was

estimated through the UOsm to POsm ratio. The decrease in this ratio after L-NAME alone³¹⁹ and after L-NAME addition to ATR antagonists support an increase activity in AVP. It is not known, however, if these effects are related to ANG II and NO regulation of AVP release from the paraventricular and supraoptic nuclei of the hypothalamus or to modulation of V₂ receptors (through which AVP regulates AQP2, and therefore, water reabsorption), and/or aquaporin expression and/or activity⁴⁸⁷ in the principal cells of the collecting tubule.

In conclusion, this study is the first to investigate the roles of ATRs in modulating NO responses on renal function during development. We show for the first time that there is an important interaction between ANG II and NO in regulating kidney function early in life, and that this interaction appears to be developmentally regulated. Our findings suggest a predominant role for AT1Rs and AT2Rs in mediating NO effects on glomerular function in the newborn, while having little influence on tubular function. As maturation proceeds, a more important role emerges for both vasoactive factors regulating tubular function. The mechanism(s) underlying these exciting developmentally regulated renal effects warrants further investigation and remains to be elucidated.

Chapter Six: CONCLUSIONS

Summary of findings

As detailed in the literature review section of this dissertation, the majority of the physiological functions of ANG II are mediated through AT1R in adult animals and humans, whereas functional roles for AT2R in cardiovascular regulation are less defined: it appears to play a modest role in homeostasis regulation under physiologic conditions and buffers AT1R effects when the RAS is activated (Figure 1.4). In contrast, the potential role of AT2Rs in mediating renal effects of ANG II and in modulating haemodynamics and renal effects of AT1Rs has not been investigated during postnatal development.

To our knowledge, the research presented in this dissertation is the first report regarding the individual roles of ATRs as well as their cross-talk in mediating renal effects of ANG II as well as of the interaction between ATRs in mediating the cardiovascular effects of ANG II in conscious developing animals. The present findings clearly demonstrate that AT1Rs play a predominant, age-dependent role in mediating the effects of ANG II in regulation of renal function during postnatal development. AT2Rs alone do not mediate the renal effects of ANG II but appear to buffer the renal effects induced by AT1Rs activation. Also, our findings demonstrate that AT2Rs do not modulate the haemodynamic effects elicited by AT1R activation during postnatal maturation. In addition, our study in conscious animals reliably describes the physiological outcomes of the ATRs and ATRs interaction in mediating the effects of endogenously produced ANG II and NO under physiological conditions (Figure 6.1).



Figure 6.1 Proposed view of the physiological effects of AT1Rs and AT2Rs (based on the findings described in the present dissertation)

As outlined elsewhere in this dissertation, there is compelling evidence that the vasoactive factors, ANG II and NO as well as the balance between them plays, among other vasoactive factors, a major role in regulating cardiorenal homeostasis in adulthood (Figure P.1). NO antagonizes the ANG II pathway via several mechanisms in the adult, including modulation of expression and interactions of ATRs, via second messenger signalling systems. Conversely, ANG II modulates NO release as well as the expression of NOS isoforms through activation of ATRs. In contrast with this extensive knowledge from the adult, this interaction has not been established during postnatal development. While the roles of ATRs in mediating the interaction of ANG II and NO have been studied in adult animals, their functioning during postnatal maturation, a period in life when both systems are elevated, is not fully understood (Figure P.2). This research question has been addressed in this dissertation. Our findings provide evidence that such an interaction between ANG II and NO does, in fact, exists and that the balance between the vasoconstrictor ANG II and the vasodilator NO plays an important, age-dependent

role in regulating renal haemodynamics and fluid and electrolyte homeostasis during postnatal development in conscious animals (Figure 6.1).

Another element of novelty of this study is the complex approach in investigating not only the presence of such an interaction but, as well, the means through which this interaction is facilitated, respectively the physiological roles played by the ATRs, separately and together, in modulating the haemodynamic effects of NO. This study demonstrates that renal haemodynamic responses to NO are modulated by AT1Rs and AT2Rs in an age-dependent manner. As well, the effects of NO on renal function are mediated, at least partially, through activation of AT1Rs and AT2Rs. These responses are also developmentally regulated. It is also apparent that there is an interaction between the ATRs in modulating age-dependently the effects of NO on renal function (Figure 6.1).

Limitations

Although the current findings have clearly advanced our knowledge of physiological development of the kidney, there are a few limitations that hinder our full understanding of how AT1Rs and AT2Rs and the ANG II – NO balance function in the newborn kidney. We interpreted our data regarding the roles of ATRs in modulating NO physiological effects in the newborn with caution for several reasons. Firstly, we explained our findings in rapport with previous experiments carried out in our laboratory that studied the haemodynamic and renal effects of L-NAME alone. Albeit the experimental design was similar, those experiments were performed in a different set of animals as well as from another source provider. Therefore we could not accurately appreciate the magnitude in haemodynamic and renal function changes after administration of combined ATRs antagonists and L-NAME. Secondly, there was a time limitation in designing the time frame of the *in vivo* experiments that was determined by the postnatal interval in which the physiological changes in the ATRs expression occurs in sheep (as detailed in section 1.1.7.2). In addition, the experiments were scheduled at minimum intervals of 48h to allow for fully elimination of the experimental drugs. As a result, a forth experiment to study the effects of L-NAME alone could not be included in the experimental design. Thirdly, the limited information regarding the distribution and expression of the ATRs and NOS within the kidney of the developing sheep did not allow speculating more into the possible vasodynamic changes within the renal microvasculature that may have occurred under the treatments applied in our experiments. In addition, we could not clearly delineate the central (brain) from the peripheral (kidney and peripheral vasculature) effects observed in our experiments. Furthermore, we could not interpret the results regarding nitrate excretion as a measure of NO biologic activity for several reasons: (1) we did not control for dietary nitrate intake (the major differences in diet of the two groups of lambs (detailed in section B.2., Appendix B) may contribute as well to explain the higher baseline nitrate excretion in six as compared to one week old lambs (Table 4.3)); (2) we did not control for the level of physical activity of the animals that may also influence NO generation and impact the true estimate of the nitrite/nitrate levels in biological samples; (3) there were no reference values for nitrite/nitrate excretion in newborn lambs available from previous studies to facilitate the interpretations of the findings from the present study; (4) even with the accurate measurement of the nitrate excretion in these experiments, the results should be interpreted with caution as they may not be indicative solely of the biologic activity of NO since the nitrate concentration in urine decreased after ZD 7155 and increased increased after NO production was inhibited with L-NAME.

Future directions

In spite of these limitations, through the research questions addressed in this dissertation, our findings can be placed at the front line of the research inquiries of the contribution of vasoactive factors to perinatal physiology. There are still various avenues to explore in order to fully understand the physiological mechanisms that facilitate the adaptation of the newborn kidney after birth.

More research is warranted to elucidate, for example, the molecular pathways through which (a) AT2Rs modulate the effects of AT1Rs, and (b) both ATRs mediate the effects on ANG II and the interaction between ANG II and NO early in life as well as in adulthood. Although we demonstrated physiological roles for ANG II and NO in regulating newborn haemodynamics, we cannot explain the reason why both vasoactive agents are activated at the time of birth. Therefore, this fascinating observation dating back to the 1970's still remains an open question that requires further investigation. Furthermore, as mentioned elsewhere in this dissertation, a complex, reciprocal interaction between ANG II and NO exist in adult. The present dissertation investigated the role of ANG II in modulating the NO physiological effects in the newborn, whereas the possible modulatory effects of NO on the effects of ANG II on cardiovascular and renal function during postnatal development remain to be addressed in future studies.

There also still remain several questions with regard to possible contributions of other vasoactive factors such as PGs, endothelin or sympathetic nerves in mediating the physiological changes at birth which may or may not interact with ANG II and NO or alter the balance between them. More research is warranted to elucidate if the structural and physical changes within the nephron and vasculature contribute as well in modulating the adaptation of newborn kidney to postnatal life and if ANG II and NO play any role in these morphological alterations.

In conclusion, more work is required to characterize the physiological roles of the RAS and NO within the kidney and other tissues in order to broaden our understanding of the functional aspects of these important vasoactive systems in cardiovascular and renal regulation and development of cardio-renal systems during ontogeny. It is our hope that the new insights provided by this dissertation on the cardiovascular and renal roles of RAS and NO during postnatal maturation may serve as a catalyst to stimulate further studies and debates in the field to better understand how the vasoactive factors, act either independently or by interaction with each other, to modulate the adaptation to life after birth.

Significance of the study

By providing the first measurements of the physiological effects of ANG II on the function of the newborn kidney, as well as the combined effect with NO in newborn sheep this dissertation contribute to clarifying the mechanisms of newborn mammalian kidney physiology, which may impact the development of new therapeutic strategies in controlling fluid and electrolyte homeostasis in the pediatric setting. In clinic, the extensive resuscitation of the newborns born at different stages of gestation employs the use of a variety of drugs some of which may disturb the balance of the vasoactive factors and, therefore, affect the cardiovascular and renal systems. This study provides evidence that influences on one vasoactive factor may trigger chain reactions changes altering the functioning of other factors, which may result in repercursions on cardiovascular and renal function in short, and possible in long term. Therefore, cautions should be taken with such interventions to the benefit of the newborn and to prevent causing harm. Elucidating the physiological underpinnings of the role of NO and RAS in modulating physiological functions of the kidney during mammalian ontogeny may also have an impact on the development of new diagnostic strategies and therapeutic approaches for management of kidney congenital and acquired diseases in the term infant and child.

APPENDIX A: CATHETERS PREPARATION

This section describes the preparation of vessels and bladder catheters used in surgical procedures outlined in the section 3.3.3.; this preparation time is approximately one week.

Vessel catheters were constructed in the laboratory from different sizes silastic Tygon® tubing (Tygon® Microbore Tubing, Norton Performance Plastics, Akron, Ohio, USA), respectively 0.040"ID x 0.070" OD for the arterial and 0.050" ID X 0.090" OD for the venous catheter to accommodate the vessels diameter. This biocompatible, clear, and flexible surgical tubing is made by a material that is non-toxic, non-hemolytic, nonpyrogenic and chemical resistant. The vessels catheters were cut to a predetermined length of 110 cm. One end of each catheter was chosen as the catheter tip (proximal end) to be introduced into blood vessels. Five centimeters intervals marks were drawn with a permanent ink marker on this catheter end for a length up to 30 cm to assist the surgeon during insertion into femoral vessels. At the 30 cm mark from the catheter tip a 0.5 cm cuff of silastic laboratory tubing (0.192" OD, Dow Corning Corporation, Midland, MI, USA) was added with the help of a blunt hub aluminum needle (Tyco Healthcare Ltd, Wollerau, Switzerland) and glued in place. During surgeries, the cuff was tied up to the vessels walls to keep the catheter in place of insertion into the vessels and prevent it from sliding along the vessel walls. The opposite end of the catheters was inserted through the plastic protective cap of a Monoject aluminum needle. Another 5 mm cuff was slid and glued to this end to keep in place the plastic cap. At the same end, an 18 G blunt hub aluminum needle (Tyco Healthcare Ltd, Switzerland) was inserted into the catheter up to the needle neck. The plastic needle cap was slid over this construct and served during the surgery and experiments for attaching a Standard Bore three-way stopcock with rotating Luer lock (Baxter Healthcare Corporation, Deerfield, IL, USA).

The bladder catheters were adapted from ArgyleTM infant feeding tubes (Tyco Healthcare Group LP, Mansfield, MA, USA). The catheters (15" long) were marked from the end at 2.5 cm and 3.5 cm and two 0.5 cm silastic cuffs were slid and glued to these two levels. The cuffs were used during the surgery to secure in place the catheter to the

bladder walls. Several additional holes were cut on the catheter end to allow free flow drainage of urine during experiments.

The catheters prepared as described above were packed into surgery kits in individual sterilization instant sealing pouches (Propper Manufacturing Co. Inc., Long Island City, NY, USA) that contained: four vessels catheters (two arterials and two venous, labeled red and respectively blue on the plastic caps), five three-way stopcocks, eight caps, four 5mL and one 20 mL syringes with Luer-LokTM tip (Becton Dickinson, Franklin Lakes, NJ, USA), one 18G needle (Becton Dickinson, Franklin Lakes, NJ, USA) and one vial of 10 mL Heparin Sodium 1000 USP units/mL (Organon Canada Ltd, Toronto, ON). The ultrasonic RBF transducers, size 3S-6S (Transonics Sytems Inc., NY, USA) were individually placed in sterilization pouches that contained also a small Philips screw driver corresponding in size to the transducer size, 2-3 extra screws and a 5 mL syringe containing 3 to 4 mL acoustic coupling highly conductive multi-purpose electrode gel (Signa Gel, Parker Laboratories Inc, Fairfield, NJ, USA) attached to a 18G Monoject blunt hub aluminum needle. The bladder catheters and the low volume pressure monitoring infusion lines (Medex Inc, Hilliard, Ohio) (used for IV infusions during experiments) were sterilized in separate pouches, together with the stopcocks and caps necessary to adapt them to the catheters during experiments. All packages were sterilized prior to surgery with ethylene oxide (Amprolene gas ampoules, Andersen Sterilizers, Haw River, NC, USA).

APPENDIX B: ANIMAL CARE AND HANDLING

B.1. Clinical evaluation

Upon arrival at the animal facility center, the animals (lambs accompanied by their mothers) were allowed a period of adjustment of minimum 2 h from the transportation before completion of a thorough checking their health status done by the Clinical Veterinarian from the Health Sciences Animal Resource Facility and morphometric measurements. The overall health check of the lambs included, beside the veterinarian assessment, recording of the movements and suckling abilities, body weight and temperature. Each animal received an ear tag with a unique identification number. All these parameters together with possible distinctive marks and a given unique name were recorded on the Animal ID sheet. The animals were allowed to adapt to their new enclosures for a minimum of 48 h before surgery.

B.2. Daily care

Housing. All lambs were housed with their mothers in individual pens in the *vivarium* of the Health Science Centre, University of Calgary, AB, Canada except during surgery, training and the experiments. The rooms were maintained with a 12:12 hour light-dark cycle, temperature at ~ 21° C and ~40% humidity, with lights on at 7:00 am.

Health and growth. During the period spent in the facility the animals were checked daily regarding their health conditions and development. The general health state and behaviour, temperature and body weight, any manoeuvre performed such as treatment, surgery, pre- and postoperative care, as well as time of beginning and end experiments and training were recorded.

Nutrition. The newborn lambs were allowed to suckle *ad libitum* from their mother. The level of feeding was monitored through the progressive increase in body weight. In addition, the older lambs were fed ½ scoop (2 lb) per day with Prostock Feeds 16% Lamb Grower Weaning Ration that contains proteins, fat, fibre, vitamins A, D, E

and minerals: calcium, phosphorus, selenium and sodium 0.2%. Tap water was provided *at libitum*.

The lactating ewes were provided with three cups twice daily of the following: 1 part Feed Unifeed C/M 18% Lactating Ewe Ration (Unifeed Limited, Alberta) and 2 parts 14% Lamb Grow- Finisher Ration pellets (Masterfeeds, Alberta) in addition to one large flake of hay twice daily and tap water *ad libitum* to provide the ewes with an equilibrated diet of proteins, fat, crude fibres, minerals and vitamins.

B.3. Preoperative preparation

On the day prior to surgery the lamb's wool was shaved and body jackets were placed on the animals (Lomir Inc., Montreal, Canada). [The body jackets contain pockets to house the catheters and the flow transducers and protect for safe keeping between experiments.] Excenel[®] (Ceftriofur) (Pfizer, Kirkland, QC, Canada) 2.2 mg/kg I.M. antibiotic treatment was started 24 h prior surgery in all lambs. At least 12h prior to surgery, the six week old lambs prepared as mentioned above were removed from the ewe and placed into separate pens from their mothers for overnight fasting. The animals were allowed to drink water *ad libitum* until the morning of the surgery. The procedure is not required for the newborn lambs which were removed from the ewe within minutes before surgery. For the animals that feed solid foods. The preoperative fasting precaution is necessary to prevent paralytic ileus and subsequent bloating that may occur in relation with surgery and anaesthesia medication, jeopardizing the procedure and the health of the animal. These methods are well established in our laboratory.

B.4. Postoperative care

For three to four days after surgery animals were assessed daily for general health, healing of surgical incisions, body weight and temperature. Antibiotics, Excenel® (Ceftriofur) 2.2 mg/kg I.M. (Pfizer, Kirkland, QC, Canada), prior to surgery was continued at 24 h intervals for the following 48 h post surgical instrumentation. The surgical incisions were cleaned and sprayed with Betadine 10% twice per day.

B.5. Animal Training

Animals were trained daily in the experimental rooms in conditions that mimic the experiments: the lambs were transported to the experimental rooms and placed in the sling for \sim 1-2h daily to allow them to rest quietly and become accustomed to the laboratory environment. Furthermore, this period permitted the animals to become familiar with the separation periods during experiments and, as well, the immediate presence of the researcher. Particular care was taken regarding the room temperature and ventilation to ensure the comfort for these awake and conscious animals.

Table B.1 shows the animals included in the study from both age groups and their ages and body weights at the three experiments.

GROUP	NAME	GENDER	AGE (days)			BODY WEIGHT		
				(uays)			(Kg)	
			1 st	2 nd	3 rd	1 st	2 nd	3 rd
			Exp	Exp	Exp	Exp	Exp	Exp
One								
week				1			r	
	Linda	F	5	7	9	6.6	7.0	7.5
	Kevin	М	6	8	10	6.5	6.9	7.5
	Ryan	М	5	7	9	5.9	6.1	6.5
	Theodore	М	6	8	10	4.9	5.6	6.2
	Victor	М	7	9	11	5.7	6.5	7.3
	Zara	F	6	8	10	7.5	8.1	8.3
	Q	F	7	9	11	7.9	8.5	8.9
	Malik	М	6	8	10	9.1	10.0	11.2
	Nathan	М	7	9	11	9.2	10.1	11.0
Six weeks								
	Yama	М	39	43	45	18.6	18.7	18.9
	Francesca	F	36	38	40	12.5	13.2	14.1
	Henrietta	F	37	39	41	13.8	14.3	14.8
	Giselle	F	43	45	54	15.5	16.4	17.3
	Janice	F	33	36	38	13.9	14.8	15.4
	Irene	F	37	40	42	16.0	16.4	17.4
	Mara	F	40	42	51	15.2	15.9	17.6
	Wanda	F	40	42	44	10.3	10.4	10.8
	Xerxes	М	41	43	49	14.4	14.9	16.5
	Gala	F	34	36	41	12.5	12.4	13.6
	Hagar	М	37	42	44	12.5	13.9	14.7
	Isac	М	35	39	41	19.9	21.9	22.7
	Kathy	F	37	39	41	13.3	13.8	14.4

 Table B.1 Demographics of conscious lambs at the time of experiments

 1^{st} Exp, first experiment; 2^{nd} Exp, second experiment, 3^{rd} Exp, third experiment (chronological order of experiments)

APPENDIX C: DRUGS PREPARATION

C.1. Preparation of drugs for I.V. bolus administration

Angiotensin receptor selective antagonists, ZD 7155 and PD 123319 were prepared as stock solutions of 1 mg·mL⁻¹ by adding in a 50 mg drug powder to 50 mL distilled sterile water. Upon dissolving the drug, the solution was aliquoted in Eppendorf tubes that were stored at -20°C until use. The amount of solution to be used at a dose of 100 μ g·kg⁻¹ administered as bolus I.V. was calculated at the beginning of each experiment.

L-arginine analogue, L-NAME I.V. bolus solution was prepared as a stock solution of 200 mg \cdot mL⁻¹ by adding 2 g of drug to 10 mL normal saline. The solution was aliquoted in Eppendorf tubes and stored at -20°C until use. The amount of solution to be used at a dose of 20 mg/kg administered as bolus IV was calculated at the beginning of each experiment.

Lithium chloride (LiCl) I.V. bolus solution was prepared as a stock solution of 2M by adding 2.12g powder LiCl in 25 mL distilled sterile water and aliquoted in Eppendorf tubes stored at 4°C. The amount of solution to be used at a dose of 200μ mol·kg⁻¹ was calculated at the beginning of each experiment. Fresh aliquots were used for each set of experiments.

C.2. Preparation of drugs for I.V. infusion

Angiotensin receptor selective antagonists ZD 7155 and, respectively PD1233919 I.V. infusion solutions were prepared by adding 8.4 mg ZD 7155 and, respectively PD123319 into 500 mL 5% dextrose in 0.9% NaCl and administered at a rate of 4.17 mL·kg⁻¹·h⁻¹.

The combined angiotensin receptor antagonists ZD7155 plus PD123319 I.V. infusion solution was prepared by adding 8.4 mg of each, ZD 7155 and PD123319, into 500 mL of 5% dextrose in 0.9% NaCl and administered at a rate of 4.17 mL·kg⁻¹·h⁻¹. Freshly made solutions of ATR antagonists were used for each set of experiments.

APPENDIX D: HAEMODYNAMIC MEASUREMENTS

D.1. Haemodynamic measurements overview

Real time haemodynamic measurements performed in all experiments were recorded onto a model 7 Grass Technologies polygraph (Astro-Med Inc. subsidiary, West Warwick, Rhode Island, USA) and simultaneously digitized at 200 Hz using the data acquisition and analysis software package, PolyVIEWTM (Astro-Med Inc., Grass Technologies subsidiary, West Warwick, Rhode Island, USA). Figure C-1 shows an example of haemodynamic measurments as displayed by the PolyVIEWTM software.





The vertical black line represents the marker of L-NAME infusion. Black arrows show the increase in MAP and the decrease in RBF in responses to L-NAME I.V. infusion. The

figure shows the cardiovascular events occurring over a 200 sec time span.

D.2. Arterial and venous pressure measurements

Arterial and venous pressures were measured by the means of P23XL pressure transducers (Viggo-Spectramed, CA, USA), connected through a cable to a pressure monitor of the polygraph and through low volume pressure monitoring lines (Medex Inc, Hillard, Ohio) to the animals' femoral arterial and venous catheters. (The pressure lines are described in Appendix A and Section *3.3.3 General surgical procedures*). The silicon chip from the pressure transducer is mechanically connected to a metal diaphragm and

functions based on the Wheatstone bridge electrical principle. When the diaphragm is deflected by positive or negative pressure changes, the silicon chip is stressed, causing a proportional imbalance in the resistance of electrical circuit of the bridge. This electrical output which is proportional to the pressure change is amplified, measured and recorded. The pressure transducers were securely placed in the pole mounting holders and positioned at the level of the heart. A direct calibration with a mercury manometer was carried out before the start of each experiment. Arterial and venous pressure measurements data acquired by PoyVIEWTM during the experiments were retrieved at a later date, averaged over 1 min using the PolyVIEWTM software and over 10 and 30 min using the Microsoft Office Excel 2007 and statistically analysed using IBM SPSS Statistics 19.





Figure D.2 Baseline MAP values recorded over 30 min control period in the three experiments performed at 48 h intervals (experimental day 1, day 2, day 3) in one conscious chronically instrumented lamb; each dot symbol represents the MAP values averaged over 1 min of measurements

D.3. Renal blood flow measurement

Renal blood flow was measured using a Transonic T 101 Flowmeter (Transonic Systems Inc., Ithaca, NY, USA) based upon an ultrasonic transit-time principle whereby a wide beam of ultrasound is applied to a blood vessel and surrounding medium within the ultrasonic sensing window. The blood flow measurement represents an average of the velocity encountered by any ray of the beam that intersects the blood vessel and the length of the path over which that velocity is averaged. The transonic flow transducer consists of an epoxy probe body enclosing two ultrasonic transducers (on upstream and one downstream), an acoustic deflector and a flexible percutaneous cable as illustrated in Figure D.3. The transducers were positioned on one side of the blood vessel and the reflector was positioned midway between the two transducers on the opposite side of the vessel. An electrical excitation causes the transducers to alternately emit a plane wave of ultrasound that is detected by the second. Therefore, emission and detection occur at each transducer. The ultrasonic signal is then converted into an electrical signal, the intensity of which is proportional to the flow through the vessel. The downstream transit time is subtracted from the upstream transit time and this measurement is displayed, representing the volume flow through that vessel (Figure D.3). The transit time ultrasound technology represents the "gold standard" method for measurement of real-time blood flow. This accurate, direct and continuous method for volume flow measurements has previously been validated in several species, including swine, dog, sheep and cat.^{372,452,488-491}

RBF was recorded onto a polygraph digitized at 200 Hz and analyzed using PolyVIEWTM software in a manner similar to the aforementioned pressures measurements. Calibration of the measurements was carried out before the start of each experiment as per manufacturer instructions. The repeatability of the baseline RBF measurements is illustrated in figure D.4.

Flow transducer body



Reflector

Figure D.3 Schematic view of the perivascular ultrasonic flow transducer



Figure D.4 Baseline RBF measurements recorded over 30 min control period in the three experiments performed at 48 h intervals (experimental day 1, day 2 and day 3) in one conscious chronically instrumented lamb; each dot symbol represents the RBF values averages over 1 min of measurement

APPENDIX E: MEASUREMENTS OF PLASMA AND URINE OSMOLALITY

E.1. Introduction

The osmolality of plasma and urine specimens was determined by freezing point depression, a technique widely used for determination of the osmotic concentration of biological fluids. By this method, the specimen to be analysed is pipette into a sample tube, which is placed in the cooling chamber of an osmometer. The analysis cycle consists of several steps that include supercooling and crystallization of the specimen, process that results in temperature variations that are measured by use of thermistors. Further, the thermistors readings are converted to osmotic concentrations. All the osmolality measurements were performed automatically by the Multi-OsmetteTM instrument that displayed the osmolality readings at the end of the abovementioned processes.

E.2. Materials and method

Instruments

- 2430 Multi-OsmetteTM Auto-Sampling Turntable Osmometer (24 positions turntable, 0-2000mOsm/kg range), Precision Systems Inc., Natick, MA, USA
- 30 µL disposable polypropylene sample microtubes, Cat. No. 2020, Precision Systems Inc., Natick, MA, USA

Reagents

• CON-TROLTM reference standards, Precision Systems Inc., Natick, MA, USA, in sealed 5 mL glass ampoules (12/pk) of :

100 mOsm/kg H₂O, Cat. No. 2201 290 mOsm/kg H₂O, Cat. No. 2205 500 mOsm/kg H₂O, Cat. No. 2205

Standard solutions, Precision Systems Inc., Natick, MA, USA, bottles of 125 mL of:

100 mOsm/kg H2O, Cat. No. 2101 500 mOsm/kg H2O, Cat. No. 2105 1500 mOsm/kg H2O, Cat. No. 2115 2000 mOsm/kg H2O, Cat. No. 2120

Assay calibration

To ensure accuracy of the measurements, the 2430 Multi-OsmetteTM Osmometer was carefully calibrated during each assay using CON-TROLTM reference standards provided by the manufacturer. The calibration was verified daily at the beginning of the assay using the above-mentioned readily prepared Standard Solutions. In addition, the reference standards were used to ensure weekly quality of the Standard Solutions, as per manufacturer instructions. The instrument calibration followed the manufacturer's instructions using the "Recommended Calibration procedure for Labs running serum and urine" pamphlet provided with the instrument. The precision of the method was evaluated using standard solutions. The reproducibility of the osmolality assay calibration is presented in the Tables E.1 and E.2.

Calibration Standard Solutions	Standard Solutions osmolalities (grand mean) (mOsm/kgH ₂ O)	Standard deviation	Coefficient of variation (%)
100mOsm/kg H ₂ O	100.2	0.463	0.461
500mOsm/kg H ₂ O	500.7	1.337	0.267
1500mOsm/kg H ₂ O	1501.8	3.853	0.256
2000mOsm/kg H ₂ O	1999.2	4.104	0.205
290mOsm/kg H ₂ O	292.3	2.751	0.941

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Lanc Lai	Osmolality	assay	canor auon	mua	abbay	precision

Coeficient of variation (%) calculated for osmolality of ten replicates from each Standard Solution measured in one assay

Calibration Standard Solutions	Standard Solutions osmolalities (grand mean) (mOsm/kgH ₂ O)	Standard deviation	Coefficient of variation (%)
100mOsm/kg H ₂ O	100.4	1.381	1.375
500mOsm/kg H ₂ O	500.5	1.699	0.339
1500mOsm/kg H ₂ O	1501.5	4.989	0.332
2000mOsm/kg H ₂ O	2001.3	6.874	0.343
290mOsm/kg H ₂ O	292.2	2.045	0.699

Table E.2 Osmolality assay calibration - inter assay precision

Coefficient of variation calculated for osmolality of duplicates from each Standard Solution measured in ten days consecutive assays.

E.3. Sample preparation and handling

Heparinized plasma sample and urine samples collected during experiments in clean, dry containers (Eppendorf polypropylene tubes, Eppendorf Canada, Mississauga, ON) and stored at -20°C were used for analysis. Before analysis, the samples were thawed at room temperature and gently mixed to allow dissolution of precipitated solutes. To remove all undissolved particulate matter all samples were subject to centrifugation at 4000 rpm, for 10 min (Eppendorf Centrifuge 5417C, Eppendorf Canada, Mississauga, ON). 30 μ L of each sample was then directly pipetted in a polypropylene sample cup and placed in the osmometer carousel for osmolality measurement. All samples were run at least in duplicates. A double distilled water control and a standard of 1500 and 290 mOsm/kg H₂O for urine and plasma, respectively, were included at the beginning of each carousel of urine and plasma samples.

E.4. Evaluation of the assay performance

Intra assay reproducibility

Two different urine samples were run in replicates of ten in one assay. The coefficient of variation (%) was calculated from the grand means and standard deviations of measured samples osmolality (Table E.3).

Table E.3 Coefficient of variation of the osmolality intra assay precision testing

Sample	Urine osmolality (grand mean) (mOsm/kgH ₂ O)	Standard deviation	Coefficient of variation (%)	
Sample 1	714.4	2.567	0.3593	
Sample 2	423.0	3.033	0.7171	

Inter assay reproducibility

The osmolality of one urine sample was measured in duplicates in four assays that were run in four consecutive days. The coefficient of variation (%) was calculated from the grand means and standard deviations of measured samples osmolality (Table E.4).

Table E.4 Coefficient of variation of the osmolality inter assay precision testing

Sample	Urine osmolality (mean) (mOsm/kgH2O)	Standard deviation	Coefficient of variation (%)
Day1	779	1.414	0.182
Day2	777.5	2.121	0.273
Day3	778.5	0.707	0.090
Day4	779.5	0.707	0.091

APPENDIX F: PLASMA AND URINE CREATININE CONCENTRATION MEASUREMENTS

F.1. Validity of endogenous creatinine clearance in measuring GFR

Creatinine clearance was developed as a method to determine GFR by measurement of concentrations of this metabolite in blood plasma and spontaneous urine ⁴⁹²⁴⁹³. Considerable evidence has consistently demonstrated that creatinine clearance is a reliable method to estimate GFR in human patients and animal species 493 494 495 496. Creatinine (2-amino-1-methyl-5H-imadazol-4-one) is a metabolite of creatine, a naturally nitrogenous organic acid that is predominantly localized in skeletal muscle. In vivo, creatine and p-creatine are converted non-enzymatically and irreversible to creatinine, a process that is favoured by higher temperatures and lower pH in vitro, and is eliminated by the kidneys through glomerular filtration and then excreted. Under normal conditions, in healthy individuals, creatinine formation occurs at a rate that is relatively constant, proportional to the muscular mass, and its excretion is independent of the diet, with an intra-individual variation less than 15% from day to day. Therefore, the endogenous creatinine clearance as a method of measuring filtration rate offers the advantage of a substance endogenously produced, at a constant rate, which can be used without employing an exogenous substance infusion. The measurements of creatinine concentration in blood and urine are simple, reproducible, reliable and readily available^{494,496} and the use of creatinine clearance as an estimate of GFR is widely used in paediatric and adult clinical practice.497

Creatinine is not the perfect substance to measure the GFR, as approximately 10% is secreted by the organic cation secretory system in the proximal tubule, a pitfall that is cancelled by the ~10% overestimating error employed by the evaluation method. Several lines of evidence have shown the validity of this method, creatinine clearance correlating well with inulin clearance in humans and various animal species.⁴⁹³⁻⁴⁹⁶ Inulin clearance as a measure of glomerular filtration (proposed in the thirties by Smith and Shannon⁴⁹⁸) although considered the gold standard of measuring true filtration rate in most species, including human,⁴⁹⁹ has its own limitations and, unless carried out rigorously, can also be

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inaccurate.⁴⁹⁹⁻⁵⁰⁰ Furthermore, the method involves infusion of this substance in the body. Therefore, the suitability of other substances to measure GFR, such as creatinine, has been tested by comparison with inulin clearance. This has been demonstrated by experimental studies that measured and compared renal function measurements determined simultaneously through both methods⁵⁰¹⁻⁵⁰² and showed that in healthy adult sheep, under physiological conditions, creatinine clearance represents an accurate and easily conducted measure of GFR.⁵⁰³ Bickhardt and Dungelhoef (1994)⁵⁰⁴ measured simultaneously renal clearance of endogenous creatinine and inulin clearance in healthy sheep of different genders and body weights and reported that creatinine clearance was well correlated with inulin clearance (13.8 +/- 1.3 and respectively, 13.2 +/- 2.0 ml/min/10 kg body weight). Other studies showed similar findings in sheep^{495,505} as well as in other species such as dogs, cats (reviewed by Von Hendy-Willson *et al* (2011)⁵⁰⁶), swine,⁵⁰⁷ and humans (reviewed by Fesler *et al* (2011)⁵⁰⁸) suggesting that the renal clearance of endogenous creatinine is a satisfactory measure of GFR in these species.⁵⁰⁵.

Comparative examinations of renal function using inulin and endogenous creatinine clearances have also been conducted in developing animals. In chronically catheterized fetal sheep a good correlation between clearance of inulin and creatinine,^{375,510-511} with a ratio of creatinine to inulin clearance slightly over unity (1.18±0.02) was shown.³⁷⁵ The creatinine to inulin ratio was reported to be almost similar in catheterized lambs, with no significant differences in unanaesthetized vs. anaesthetized animals.³⁷⁵ Several other studies showed that the endogenous creatinine clearance is a reliable indicator of GFR validity of creatinine clearance in newborn lambs,^{174,375,512,513} and newborn piglets.^{504,507,514}

Taken together, aforementioned studies in newborn animals from various species, including sheep, provide evidence for creatinine clearance method as a means for determining GFR with high reliability and accuracy. Estimation of creatinine clearance using simple formulas such as Cockcroft-Gault has been proven acceptably accurate and precise for estimating GFR.^{508,515} Thus, this method was used in the experiments described in this dissertation to estimate GFR. The method described herein was tested

for the first time in our laboratory during the experiments from this dissertation and validated for GFR measurements in conscious lambs.

F.2. Materials and method

QuantiChromeTM Creatinine Assay Kit (DICT -500) (BioAssay Systems, Hayward, CA) was used to measure the creatinine concentration in plasma and urine according to the manufacturer's instructions. This assay is a quantitative colorimetric creatinine method designed to determine creatinine concentration directly in biological samples without any pre-treatment of the samples. The technique consists in an automation-ready procedure based on the Jaffé method facilitated by the use of an optimized reagent formulation that substantially reduces interference by substances such as bilirubin in the raw sample. The reagent utilizes picrate which forms a red colored complex with creatinine. The intensity of the color, measured spectrophotometrically at wave length \sim 510 nm, is directly proportional to creatinine concentration in the sample. The detection limit is 0.10 mg/dL (8µM) creatinine for the 96-well plate assay. The optimized formulation of the reagents offer enhanced signal stability and versatility to the method. The assay used in the present experiment has been validated with human, rabbit, sheep and mouse serum, EDTA and heparinized plasma samples.⁵¹⁶⁻⁵²⁰ The assav is designed to provide the creatinine concentration in mg/dL units. For the purpose of GFR calculation, the concentration of creatinine was expressed in mmol/L by the following transformation:

<u>Materials</u>

Instruments:

- Plate reader Wallac Victor V3 1420 multilabel counter (Perkin Elmer Life Sciences, Boston, MA) with filter for wave length of 531nm was used to measure the optic density of the samples.
- Eppendorf centrifuge 5417C (Eppendorf AG, Hamburg, Germany)
- 96 wells clear bottom plates (VWR International, Radnor, PA)

Reagents:

• Reagent A - sodium hydroxide

- Reagent B- picric acid and Creatinine standard 50 mg/dl
- Creatinine standards stock solution of 50mg/dL

All chemicals were provided by the manufacturer (BioAssay Systems, Hayward, CA).

<u>Reagent preparation</u>

The Working Reagent was freshly prepared before use by mixing per well reaction of 100 μ L Reagent A and 100 μ L Reagent B for plasma sample and 50 μ L of each reagent for urine samples. The Reagent A and B provided with the manufacturer kit, were stored at 4°C and allowed to equilibrate to room temperature prior to use.

Sample handling and preparation

Urinary and heparinized plasma samples were run in separate assays in duplicates after thawing samples to room temperature. One cycle of freeze/thaw was performed on each sample to avoid loss of bioactive analyte. The samples were allowed to thaw and equilibrate at room temperature for ~3h. Urine samples containing visible particulates were filtered. All urine samples were centrifuged at 4000rpm for 10 min and plasma samples at 14000 rpm for 15min (Eppendorf Centrifuge 5417C) and used within 2 h from the time of preparation. To ensure a linear relationship between the optical density and creatinine concentration the assay was designed to use different concentrations of sample/standard and reagent strength for plasma and urinary assay. Since the anticipated concentration of plasma and urine creatinine was within the limits provided by manufacturer, no further dilution of the samples was necessary.

Creatinine standard preparation

The creatinine standard was prepared separately for plasma and urine assays. For plasma, 5 μ L of the 50 mg/dL standard stock was diluted by mixing with 120 μ L distilled water to a final concentration of 2mg/dL. 30 μ L of the diluted standard was transferred in duplicates into the wells of each of the 96-well plates that were used to assay plasma creatinine concentration (Figure F.1). For urine, 5 μ L of the 50 mg/dL standard stock was used undiluted and added in duplicate to the wells of each of the 96-well plates that were used to assay used to assay urinary creatinine concentration (Figure F.2).



Figure F.1 Standard curve of creatinine concentration in plasma



Figure F.2 Standard curve of creatinine concentration in urine

Assay procedure

Duplicates of creatinine standards and distilled water were run simultaneously as positive and negative controls, respectively. The assays were performed in clear-bottom 96-well plates (Corning Costar, VWR International) in accord with the manufacturer recommendations as follows (Figure F.3):

1. Duplicates of urine and plasma freshly prepared samples as mentioned above were transferred into the wells of a clear bottom 96-well plates according with the plate design (an example of the plate layout sheet is provided in Figure F.3), 5 μ L of urine and respectively, 30 μ L of plasma.

2. Duplicates of standards and water were pipetted into the bottoms of the designated wells.

3. 200 μ L of Working Reagent was quickly added to all wells using a multichannel pipette (Eppendorf 8 channel Explorer electronic pipettor 10 to 300 μ L). The plates were briefly tapped to mix and incubated at room temperature.

4. The optic density at 1 min (OD1) and 5 min (OD5) at 531nm was measured spectrophotometrically with Wallac Victor V3 1420 microplate multilabel counter and displayed with Wallac 1420 Explorer Program software for optical density readings conversion. The data were retrieved for later computation and calculation of creatinine concentration in samples using formula:

creatinine concentration in sample = $\frac{\text{OD sample 5 - OD sample 1}}{\text{OD STD 5 - OD STD 1}} \cdot [\text{STD}] \text{ (mg/dL)},$

where ODsample5, ODsample1, OD STD5 and OD STD1 are OD 531 nm values of sample and standard at 5 min and 1 min, respectively. [STD] represent the creatinine standard concentration used, that was 2 mg/dL for plasma assay and 50 mg/dL for urine assay. The optic density of sample at 1 min was subtracted from the optic density at 5 min and divided by the difference between the optic densities of the creatinine standard at 5 and respectively, 1 min. The creatinine concentration values (transformed in mmol/L) obtained for the same sample were averaged as a single value that was used in further calculations of the GFR.

	1	2	3	4	5	6	7	8	9	10	11	12
Α	STD mg/dL	L1 s1	L1 s3	L2 s1	L2 s3	L3 s1	L3 s3	L4 s1	L4 s3	L5 s1	L5 s3	
В	STD mg/dL	L1 s1	L1 s3	L2 s1	L2 s3	L3 s1	L3 s3	L4 s1	L4 s3	L5 s1	L5 s3	
С	dd H20	L1 s2	L1 s4	L2 s2	L2 s4	L3 s2	L3 s4	L4 s2	L4 s4	L5 s2	L5 s4	
D	dd H20	L1 s2	L1 s4	L2 s2	L2 s4	L3 s2	L3 s4	L4 s2	L4 s4	L5 s2	L5 s4	
E	blank	L6 s1	L6 s3	L7 s1	L8 s3	L9 s1	L9 s3	L10 s1	L10 s3			
F	blank	L6 s1	L6 s3	L7 s1	L8 s3	L9 s1	L9 s3	L10 s1	L10 s3			
G	blank	L6 s2	L6 s4	L7 s2	L8 s4	L9 s2	L9 s4	L10 s2	L10 s4			
Н	blank	L6 s2	L6 s4	L7 s2	L8 s4	L9 s2	L9 s4	L10 s2	L10 s4			

Figure F.3 96 wells plate design for creatinine concentration measurements in biological samples (plasma and urine)

 L_x = experiment number; S_y = urine or plasma sample (four samples of urine or plasma were run in duplicate (i.e.s1,s1) for each experiment (color code)); STD = creatinine standard (2mg/dL in plasma assays and 50mg/dL in urine assays) (positive control and used for calculation on creatinine concentrations); ddH_2O = distilled water (negative control)

F.3. Evaluation of the assay performance

Intra assay reproducibility

Two different urine samples were run in replicates of ten in one assay. The coefficient of variation (%) was calculated from the grand means and standard deviations of calculated creatinine concentrations (Table F.1).

Table F.1 Coefficient of variation of the creatinine intra assay precision testing

Sample	OD 1min	OD 5min	Creatinine concentration (grand mean) (mmol/L)	Standard deviation	Coeficient of variation (%)
Sample 1	0.090	0.104	3.385	3.331	8.6%
Sample 2	0.085	0.094	2.142	1.011	4.1%

OD 1 min - optic density measured at 1 min; OD 5 min - optic density measured at 5 min

Inter assay reproducibility

Ten urine samples were run in duplicates in three assays over three days. The coefficient of variation (%) was calculated from the grand means and standard deviations of calculated creatinine concentrations of each sample over three consecutive assays (Table F.2).

Sample	Creatinine concentration (grand mean) (mmol/L)	Standard deviation	Coeficient of variation (%)
Sample 1	4.468	6.695	13.247
Sample 2	4.635	7.726	14.736
Sample 3	3.970	8.517	18.996
Sample 4	3.280	2.279	6.144
Sample 5	4.881	4.670	8.457
Sample 6	5.334	8.485	14.062
Sample 7	2.440	8.613	9.601
Sample 8	1.222	1.341	9.699
Sample 9	3.224	7.698	15.244
Sample 10	3.607	6.473	7.948

Table F.2 Coefficient of variation of the creatinine inter assay precision testing

Figures F.4 and F.5 show the measurements of baseline creatinine concentration (Control period) in plasma and urine in conscious lambs (the creatinine concentration expressed in mg/dL provided. In Figures F.4 and F.5 open symbols represent ~one week old lambs and closed symbols represent ~six weeks old lambs. The formula used to calculate clearance of creatinine that was used to estimate the GFR is:

$$GFR = Clearance\ creatinine = rac{Urine\ creatinine\ ullet\ Urinary\ flow\ rate}{Plasma\ creatinine}$$



Figure F.4 Baseline creatinine concentration in plasma of conscious lambs



Figure F.5 Baseline creatinine concentration in urine of conscious lambs

APPENDIX G: PLASMA AND URINE ELECTROLYTES CONCENTRATION MEASUREMENTS

G.1. Measurements of electrolytes (cations/anions) concentration in plasma and urine

The concentration of electrolytes (inorganic cations Li^+ , Na^+ , K^+ , and anions Cl^- , NO_3^-) in biological samples (plasma and urine) was measured by ion chromatography. The ion chromatography (IC) method, described in detail below, was developed and tested for the first time in our laboratory during the experiments described in this dissertation.

The IC method has been proven a highly accurate reference method for the determination of electrolytes in biological fluids.⁵²¹⁻⁵²³ The results are comparable with other methods such as flame atomic emission spectrometry; chemiluminescence or more automated clinical systems.⁵²¹ The use of this analytical method in the present experiments offered the advantage of a unique method of detection and measurement of both types of electrolytes, anions and cations. Furthermore, the method allowed measurement of NO participation in the physiological processes *in vivo* through the analysis of NO metabolites in biological fluids. Due to complete oxidation of the endogenously produced NO₂⁻ to NO₃⁻, only NO₃⁻ concentration was assayed.⁵²³⁻⁵²⁵ The nitrate anions were detected only in urine samples. Other advantages such high precision and accuracy and great flexibility made the IC a versatile method to determine the wide range of electrolytes concentrations from biological samples.⁵²⁴ Disadvantages are a relatively long analysis time and the frequent injections of standards for compensations of drifts.⁵²¹

The identity of the ions in the biological samples was determined by their retention times. The detection of the anions and cations was achieved by measuring the conductivity of the separate anions and respectively, cations as they elute from the separation column. Detection limits for the two anions and three cations were determined. The concentration of the ions was determined from a calibration curve of standard solutions of known concentrations of all the anions and cations of interest.

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G.2. Materials and method

Instruments

- 861 Advanced Compact Ion Chromatograph (Metrohm AG, Herisau, Switzerland)
- Triathlon 900 auto sampler (Spark Holland, Emmen, Netherlands)
- 750 µL limited volume polypropylene vials (Cat. No. 98050, Grace Davison Discovery Science, IL, USA)
- Snap red silicone septa caps (Cat. No. 5182-0564, Agilent Technologies Canada Inc, Mississauga, ON)

Reagents for Cation Application:

Cation eluent:

- 4.0 mM Tartaric acid (99+%, Acros Organics, NY, USA, Cat. No. 137855000)
- 0.75mM Dipicolinic acid (2,6 Pyridindicarboxylic acid PDCA) (Cat. No. 02321-5G-F, Fluka Analytical, Sigma-Aldrich Canada, Ltd)

Eluent preparation: For 1L cation eluent, 0.6 g of Tartaric acid and 0.125 g of PDCA were added to a volumetric flask and dissolved in 1 L deionized, degassed water following manufacturer's instructions. The amount of the daily fresh prepared eluent was calculated for a flow of 1.4 mL/min, temperature of 25.0°C, for a run of 77 sample injections of 20 μ L each.

Cations calibration standards:

- Lithium (source Li₂CO₃), 1000mg/L, Cat. No. CS-Li2-2Y, Spex CertiPrep, NJ, USA
- Sodium (source Na₂CO₃), 1000mg/L, Cat. No. CS-NA2-2Y, Spex CertiPrep, NJ, USA
- Potassium (source KNO₃), 1000mg/L, Cat. No. CS-K2-2Y, Spex CertiPrep, NJ, USA

Reagents for Anion application:

Anion eluent:

- 3.2 mM Sodium carbonate anhydrous (Na₂CO₃), 500g, Cat. No. S 495-500, Fisher Scientific
- mM Sodium bicarbonate (NaHCO₃), Cat. No.S 233-500, Fisher Scientific

Eluent preparation: For 1L anion eluent, 0.339 g sodium carbonate and 0.084 g of sodium bicarbonate were added to a volumetric flask and dissolved in 1 L deionized, degassed water following manufacturer's instructions. The amount of the daily fresh prepared eluent was calculated for a flow of 0.7 mL/min, temperature of 25.0°C, for a run of 77 sample injection of 40 μ L each.

Anions calibration standards:

- Chloride (source NaCl), 100 mg/L, Cat.No. AS-Cl9-1Y, Spex CertiPrep, NJ, USA
- Nitrate (source Na₂CO₃), 1000mg/L±4mg/L, Cat.No.74276, Fluka Analytical, Sigma-Aldrich, Steinheim, Switzerland)
- Nitrite (source NaNO₂) 1000mg/L±4mg/L, Cat.No.67276, Fluka Analytical, Sigma-Aldrich, Steinheim, Switzerland)

Suppressor regenerant solution: 100 mM sulphuric acid (H₂SO₄), Cat. No. A 468-500, Fisher Chemical, Fisher Scientific, USA

Suppressor preparation: 5.6 mL of concentrated sulphuric acid was added to 1L of ultrapure water.

<u>Method</u>

The anions and cations detection in plasma and urine was carried out with the 861 Advanced Compact Ion Chromatograph that was connected to the automated sample injector Triathlon 900 autosampler and through a RS 232 interface to a PC to allow the instrument operation as well as recording and integration of chromatographic signals. Ion concentration in the biological samples was calculated with the help of IC Net integration software.
The technique allowed separation, identification and quantitative evaluation of the concentration of the anions and cations of interest. The method implied separate runs for cations and anions, using different separating columns: 6.1050.420 Metrosep A Supp 5-150 (4.0x100mm) for cations and 6.1006.520 Metrosep (4.0x150 mm) for anions that were operated according to the manufacturer's instructions. From as many as seven anions identified (fluoride, chloride, nitrite, bromide, nitrate, phosphate and sulphate) only Cl⁻, NO₃⁻ were analyzed. From the six cations identified in the samples analyzed (lithium, sodium, ammonium, potassium, calcium, magnesium) only Li⁺, Na⁺ and K⁺ concentrations were measured. As mentioned above, the detection of the anions and cations was achieved by measuring the conductivity of the separated anions and cations as they eluted from the separation column at different, specific retention times. The concentration of the electrolytes was further calculated from a calibration curve of ions standards that was run with each batch of samples. Linear relationships between the peak areas and the analyte concentrations were found for all the anions and the cations identified. Blanks (deionized water) were injected at the beginning and at the end of each batch to evaluate any carryover in the system. No carry over was reported during the analysis. To exclude external contamination during the IC analysis, all containers were tripled rinsed with deionised water before use.

Ion standard preparation:

A five point ion standards calibration curve was used for calculation of anions and cations concentration using the commercial standards solutions containing the target analytes. The above-mentioned ion standard solutions of a certified purity (ISO 9001 Quality Assurance System) and predetermined concentration were used to prepare standard stock solutions containing all the anions and cations of interest that were used for determination of calibration curves. The ions standards were included with each batch of samples for determination of calibration curve from which the electrolytes concentration in the samples was calculated. The accuracy of the prepared standard solutions was cross-checked against the references provided by the manufacturer. The linearity of the curve allowed calculation of a wide range of electrolytes concentrations in

samples. All the calibration curves of the anions and cations showed good linear correlations (correlation coefficients $r^2>0.99$). The standard curves for anions and cations are shown in the figures G.1 and G.2 (as retrieved from the IC Net integration software).

Sample preparation and handling:

Ions concentrations in plasma and urine were analyzed in biological samples prepared in advance as previously described.^{521,524,526} Eletrolytes were measured in urine samples collected over 10 min, therefore for each 30 min of the experiments three samples were analyzed. For electrolytes concentrations in plasma, one sample per 30 min of the experiments was analyzed. The urine and heparinized plasma sample were stored in -80°C freezer from the collection time and were thawed on the day of the measurement. The samples were allowed to thaw and equilibrate at room temperature for few hours. To protect the column against foreign particles and adverse influences on the separation efficiency each sample was subject to centrifugation at 14000 rpm for 15min (Eppendorf Centrifuge 5417C, Eppendorf Canada, Mississauga, ON) and microfiltration (0.45 µm filter, Nalgene) and used within 1h from the time of preparation.⁵²¹ After centrifugation, the samples were transferred to the polypropylene capped vial for injection and loaded into the Triathlon auto sampler. One individual injection of a sample was introduced into the system via an automatic injection device. Thirteen urine samples and four plasma sample were run for each experiment. The anticipated electrolyte concentrations in collected sample (tested in both, urine and plasma in advance) required 20-fold dilution of the original biological samples with deionized water. 20 µL and respectively 40 µL of each prepared sample were injected into the IC system for the detection of cations and anions, respectively. Separate assays were designed for cations and anions and run separately as they required switching between the detection columns. Original recordings of analytical runs showing the separation of anions and cations are illustrated in Figure G.3.

А.

CALIBRATION OF COMPONENT C1





CALIBRATION OF COMPONENT NO2

Method:		AnionsAngela.mtw
Equation:		Q = 0.0535259-A
RSD:		11.736 %
Correlation	coefficient:	0.996659



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C.
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CALIBRATION OF COMPONENT NO3

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Method:AnionsAngela.mtwEquation:Q = 0.0512311-ARSD:7.575 %Correlation coefficient:0.995871
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Figure G.1 Anion calibration curves (A, Chloride; B, Nitrite; C, Nitrate)

A.

CALIBRATION OF COMPONENT lithium

Method:	CationsAngela.mtw
Equation:	$Q = 0.229114 \cdot \lambda$
RSD :	25,848 %
Correlation coefficient:	0.960466



B.

CALIBRATION OF COMPONENT sodium

Method:	CationsAngela.mtw
Equation:	Q = 0.210632-A
RSD:	2,494 %
Correlation coefficient:	0,999881



C.



Figure G.2 Cation calibration curve (A, Lithium; B, Sodium; C, Potassium)



Figure G.3 Separation of electrolytes by ion chromatography

(A, anion chromatogram; B, cation chromatogram)

G.3. Evaluation of the assay performance

Inter assay reproducibility

One standard solution of anions and cations was run in duplicates in ten assays over ten days. The relative standard deviation (RSD %) of electrolytes concentration was calculated from the grand means and standard deviations of each solution samples over the ten consecutive assays (Table G.1).

Eletrolyte	Creatinine concentration (grand mean) (mmol/L)	Standard deviation	Relative standard deviation RSD (%)
Anions			
Cl	0.2829	0.0060	3.379
NO ₂ ⁻	0.0991	0.0089	8.949
NO ₃ -	0.1544	0.0125	8.127
Cations			
Li ⁺	6.797	0.064	0.947
\mathbf{K}^+	1.271	0.002	0.126
Na^+	10.741	0.046	0.431

Table G.1 Reproducibility of the IC inter assay precision testing

Intra assay reproducibility

Table G.2 presents the summarized data for the mean, standard deviation, relative standard deviation (RSD %) and limit of quantitation (LOQ) of anions and cations determined in seven replicate of one standard solution of anions and cations run in one assay.

Electrolyte	Electrolyte concentration (grand mean) (mM/L) Standard deviation		Relative standard deviation (RSD) (%)	Limit of quantitation (LOQ)
Anions				
Cl	0.1378	0.0022	1.6348	0.0225
NO ₂ ⁻	0.1028	0.0085	8.3518	0.0858
NO ₃ -	0.0795	0.0033	4.1988	0.0333
Cations				
Li ⁺	219.69	0.5292	0.2000	0.0588
Na ⁺	205.43	0.5366	0.3000	0.0597
K^+	29.386	0.5269	1.8000	0.0666

Table G.2 Reproducibility of the IC intra assay precision testing

APPENDIX H: PROXIMAL SODIUM REABSORPTION MEASURMENTS

H.1. Lithium clearance as an indicator of proximal sodium reabsorption

Measurement of the fractional Li⁺ reabsorption in the renal tubule offers a simple and useful tool to determine Na⁺ transport along the nephron that has been used in clinical⁵²⁷⁻⁵²⁸ and experimental studies.^{452,529-530} In the experiments described in this dissertation, Li⁺ clearance was used as a method to determine proximal Na⁺ reabsorption.

In the late sixties, Li⁺ clearance was proposed as a method to quantitatively estimate the delivery of Na⁺ and water from the proximal tubule of the mammalian kidney.^{529,414} Thomsen and colleagues showed that the clearance of Li⁺ is a reliable method to measure proximal Na⁺ reabsorption.⁴¹⁴ Evidence has suggested that, as for Na⁺, Li⁺ is freely filtered through the glomerulus and is reabsorbed through the proximal tubules, pars convolute and pars recta, while no Li⁺ reabsorption takes place in the distal tubule.^{414,416,529,531-532} While fractional reabsorption of Na⁺ in the renal tubules is 99%, fractional reabsorption of Li^+ is ~ 80%, the same as the sodium filtered that is reabsorbed in the proximal tubule. Partial or complete substitution of Na⁺ by Li⁺ in perfusate and peritubular fluid partially or completely prevented fluid reabsorption in proximal tubules.⁵³³ The fraction of filtered Li⁺ that is excreted in urine (CLi/GFR), about 20-30%, equals the Na⁺ and water fraction delivered to the distal tubule (CNaprox/GFR).^{534,452} Thus this indicates that the reabsorption of Li^+ takes place mainly at the proximal tubule through mechanisms similar to Na⁺ reabsorption.⁴¹⁴⁻⁴¹⁵ In contrast to Na⁺, Li⁺ reabsorption is considered to occur through passive mechanisms via a paracellular route along the concentration gradient.^{533,535}

The technique has been further validated in several studies in a variety of species, including rats, sheep, dogs, primates and humans.^{414,452,527-530}

Previous work from our laboratory in conscious, chronically catheterized newborn lambs has shown that the Li⁺ clearance method is a reliable method to measure the proximal Na⁺ reabsorption.³¹⁹

H.2. Method

Lithium chloride (Cat No, Fischer Scientific) was prepared as a 2 M stock solution and administered as a single dose of 200 μ mol/kg IV bolus over 10 sec, flushed with 3 mL isotonic saline, 30 min before the beginning of experiments. Plasma and urinary concentrations of Li⁺ were determined by ion chromatography (method described in Appendix F).

H.3. Calculations

Proximal fractional reabsorption of sodium (PRNa) was assumed equal with that of lithium (PRLi) and calculated using formula:

 $PRNa(\%) = PRLi(\%) = [1-(CLi/GFR)] \cdot 100,$

where, $CLi^+ = ((ULi) \cdot (V))/PLi$

In this formula:

CLi - lithium clearance

PLi - plasma concentration of lithium

ULi - urinary lithium excretion

V - urinary flow rate

The changes in lithium clearance with treatment in the two age groups are shown in Table I.13, Appendix I.

APPENDIX I: EXPERIMENTAL DATA

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + Vehicle
PNa	One week	142 ± 9	143 ± 6	139 ± 9
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	144 ± 8	141 ± 9	141 ± 9
РК	One week	3.3 ± 0.2	3.4 ± 0.2	3.2 ± 0.4
$(\text{mmol} \cdot \text{L}^{-1})$	Six weeks	$3.8 \pm 0.3 \ddagger$	3.7 ± 0.4 †	$3.8 \pm 0.3 \ddagger$
PCI	One week	65 ± 5	66 ± 4	66 ± 7
$(\text{mmol} \cdot \text{L}^{-1})$	Six weeks	64 ± 3	62 ± 7	62 ± 4
POsm	One week	304 ± 11	298 ± 8	305 ± 10
$(mOsm\cdot kgH_2O^{-1})$	Six weeks	303 ± 4	297 ± 6	299 ± 7
Hct	One week	37 ± 5	36 ± 6	36 ± 5
(%)	Six weeks	$28 \pm 6 \ddagger$	33 ± 8*	30 ± 5

Table I.1 Effects of ZD 7155 on plasma variables

Values are mean \pm SD measured before (Control) and 30 min after treatment with the AT1R antagonist, ZD 7155 and ZD 7155 + Vehicle; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; †p<0.05 compared to one week.

VARIABLE	AGE	Control	PD 123319	PD 123319 + Vehicle
PNa	One week	140 ± 11	141 ± 8	140 ± 12
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	141 ± 7	140 ± 9	138 ± 4
Рк	One week	3.3 ± 0.3	3.2 ± 0.2	3.3 ± 0.3
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	$3.7 \pm 0.3 \ddagger$	3.7 ± 0.3 †	3.7 ± 0.3 †
PCI	One week	65 ± 7	65 ± 5	66 ± 6
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	63 ± 5	65 ± 4	63 ± 5
POsm	One week	302 ± 7	305 ± 11	305 ± 11
$(mOsm \cdot kgH_2O^{-1})$	Six weeks	302 ± 9	299 ± 7	297 ± 9
Hct	One week	36 ± 4	33 ± 4	34 ± 6
(%)	Six weeks	30 ± 4†	29 ± 5	30 ± 5

Table I.2 Effects of PD 123319 on plasma variables

Values are mean \pm SD; measured before (Control) and 30 min after treatment with the AT2R antagonist, PD 123319 and PD 123319 + Vehicle; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + PD 123319
PNa	One week	142 ± 11	141 ± 9	140 ± 9
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	142 ± 8	140 ± 12	144 ± 8
Рк	One week	3.4 ± 0.1	3.3 ± 0.2	3.3 ± 0.2
$(\text{mmol} \cdot L^{-1})$	Six weeks	3.7 ± 0.3 †	3.7 ± 0.4 †	3.8 ± 0.4 †
PCI	One week	64 ± 2	65 ± 3	65 ± 4
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	64 ± 5	62 ± 7	66 ± 3
POsm	One week	304 ± 7	297 ± 4	300 ± 4
$(mOsm \cdot kgH_2O^{-1})$	Six weeks	305 ± 6	300 ± 5	303 ± 8
Hct	One week	37 ± 5	37 ± 3	35 ± 4
(%)	Six weeks	29 ± 6 †	33 ± 6*	31 ± 6

Table I.3 Effects of ZD 7155 plus PD 123319 on plasma variables

Values are mean \pm SD measured before (Control) and 30 min after treatment with the AT1R antagonist ZD 7155 and both AT1R and AT2R antagonists, ZD 7155 + PD 123319, respectively; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + Vehicle
CNa	One week	0.19 ± 0.08	0.18 ± 0.06	0.16 ± 0.14
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.85\pm0.53\dagger$	$0.86\pm0.44\dagger$	$0.55\pm0.32\dagger$
Ск	One week	0.20 ± 0.09	0.11 ± 0.10	0.07 ± 0.06
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.68 \pm 0.33 \dagger$	$0.49\pm0.17\dagger$	$0.43 \pm 0.13*$ †
CCI	One week	1.5 ± 0.8	1.2 ± 0.9	0.9 ± 0.5
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.1 ± 0.9	2.5 ± 1.6	$2.3 \pm 1.5 \ddagger$
COsm	One week	8.4 ± 2.8	6.6 ± 4.6	5.4 ± 2.8
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	11.1 ± 4.2	8.9 ± 4.2	8.0 ± 4.3 †

Table I.4 Effects of ZD 7155 on electrolytes and osmolality clearances

Values are mean \pm SD before (Control) and at 30 min after treatment with the AT1R antagonist ZD 7155, and after ZD 7155 + Vehicle; CX, clearance of Na⁺, K⁺, Cl⁻; COsm, clearance of osmoles; *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	PD 123319	PD 123319 + Vehicle
CNa	One week	0.12 ± 0.06	0.19 ± 0.17	0.16 ± 0.15
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.96\pm0.50\dagger$	1.11 ± 0.88 †	1.25 ± 1.18 †
Ск	One week	0.19 ± 0.09	0.13 ± 0.06	0.09 ± 0.04
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.72 ± 0.33 †	$0.52 \pm 0.17*$ †	$0.53 \pm 0.25 * \ddagger$
CCI	One week	1.5 ± 1.2	1.4 ± 0.9	1.2 ± 0.8
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.7 ± 1.6	2.8 ± 1.9	3.2 ± 1.6
COsm	One week	7.0 ± 3.7	8.0 ± 4.1	7.2 ± 2.9
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	13.0 ± 5.7	11.6 ± 3.9	13.7 ± 7.7 †

Table I.5 Effects of PD 123319 on electrolytes and osmolality clearances

Values are mean \pm SD before (Control) and at 30 min after treatment with the AT2R antagonist PD 123319 and after PD 123319 + Vehicle; CX, clearance of Na⁺, K⁺, Cl⁻; COsm, clearance of osmoles; *p<0.05 compared to Control; \dagger p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + PD 123319
CNa	One week	0.14 ± 0.11	0.16 ± 0.10	0.13 ± 0.10
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.94 ± 0.37 †	$0.92\pm0.78\dagger$	1.79 ± 1.49 †
Ск	One week	0.20 ± 0.06	0.07 ± 0.04	0.07 ± 0.04
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.70\pm0.47\dagger$	$0.58 \pm 0.27*$ †	0.64 ± 0.34 †
CC1	One week	1.6 ± 0.9	1.0 ± 0.3	0.8 ± 0.5
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.3 ± 1.7	2.5 ± 1.9	3.7 ± 2.2 †
COsm	One week	8.7 ± 3.8	5.3 ± 4.0	4.5 ± 2.3
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	12.5 ± 7.1 †	9.9 ± 4.2	$13.9\pm5.5\dagger$

Table I.6 Effects of ZD 7155 plus PD 123319 on electrolytes and osmolality clearances

Values are mean \pm SD before (Control) and 30 min after treatment with the AT1R antagonist ZD 7155 and after both, AT1R and AT2R antagonists, ZD 7155 + PD 123319; CX, clearance of Na⁺, K⁺, Cl⁻; COsm, clearance of osmoles; *p<0.05 compared to Control; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + L-NAME
PNa	One week	142 ± 9	139 ± 9	138 ± 12
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	144 ± 8	141 ± 9	141 ± 9
Рк	One week	3.3 ± 0.2	3.2 ± 0.4	3.2 ± 0.4
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	$3.8\pm0.3\dagger$	3.8 ± 0.3 †	3.7 ± 0.4 †
PCI	One week	65 ± 5	66 ± 7	64 ± 7
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	64 ± 3	62 ± 4	65 ± 5
POsm	One week	304 ± 11	305 ± 10	302 ± 14
$(mOsm \cdot kgH_2O^{-1})$	Six weeks	303 ± 4	299 ± 7	299 ± 7
Hct	One week	37 ± 5	36 ± 5	35 ± 6
(%)	Six weeks	28 ± 6 †	30 ± 5	30 ± 7

Table I.7 Effects of ZD 7155 and L-NAME on plasma variables

Values are mean \pm SD measured before (Control) and after treatment with the AT1R antagonist, ZD 7155 and ZD 7155 plus L-NAME; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; †p<0.05 compared to one week.

VARIABLE	AGE	Control	PD 123319	PD 123319 + L-NAME
PNa	One week	140 ± 11	140 ± 12	140 ± 12
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	141 ± 7	138 ± 4	142 ± 8
Рк	One week	3.3 ± 0.3	3.3 ± 0.3	3.4 ± 0.3
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	3.7 ± 0.3 †	3.7 ± 0.3 †	$3.8\pm0.3\dagger$
PCI	One week	65 ± 7	66 ± 6	67 ± 4
$(\text{mmol}\cdot\text{L}^{-1})$	$(\text{mmol}\cdot\text{L}^{-1})$ Six weeks 6		63 ± 5	63 ± 6
POsm	One week	302 ± 7	305 ± 11	300 ± 11
$(mOsm \cdot kgH_2O^{-1})$	Six weeks 302 ± 9		297 ± 9	300 ± 8
Hct	One week	36 ± 4	34 ± 6	35 ± 5
(%)	Six weeks	30 ± 4 †	30 ± 5	29 ± 5

Table I.8 Effects of PD 123319 and L-NAME on plasma variables

Values are mean \pm SD measured before (Control) and after treatment with the AT2R antagonist, PD 1233319 and after PD 123319 plus L-NAME; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; $\dagger p$ <0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155 + PD 123319	ZD 7155 + PD 123319 + L-NAME
PNa	One week	142 ± 11	140 ± 9	140 ± 9
$(\text{mmol} \cdot \text{L}^{-1})$	Six weeks	142 ± 8	144 ± 8	141 ± 8
Рк	One week	3.4 ± 0.1	3.3 ± 0.2	3.3 ± 0.3
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	3.7 ± 0.3 †	3.8 ± 0.4 †	3.7 ± 0.3 †
PCI	One week	64 ± 2	65 ± 4	67 ± 2
$(\text{mmol}\cdot\text{L}^{-1})$	Six weeks	64 ± 5	66 ± 3	65 ± 5
POsm	One week	305 ± 8	300 ± 4	306 ± 9
$(mOsm \cdot kgH_2O^{-1})$	$n \cdot kgH_2O^{-1}$) Six weeks 305 ± 6		303 ± 8	300 ± 7
Hct	One week	37 ± 5	35 ± 4	36 ± 3
(%)	Six weeks	$29 \pm 6^{\dagger}$	31 ± 6	31 ± 7

Table I.9 Effects of ZD 7155 plus PD 123319 and L-NAME on plasma variables

Values are mean \pm SD measured before (Control) and after treatment with the ATRs antagonists, ZD 7155 plus PD 1233319, and after ZD 7155 plus PD 123319 and L-NAME; PX, plasma concentration of Na⁺, K⁺, Cl⁻; POsm, plasma osmolality; Hct, hematocrit; †p<0.05 compared to one week.

VARIABLE	AGE	Control	ZD 7155	ZD 7155 + L-NAME
CNa	One week	0.19 ± 0.08	0.16 ± 0.14	0.46 ± 0.45
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.85\pm0.53\dagger$	0.55 ± 0.32 †	11.39 ± 8.21*†‡
Ск	One week	0.20 ± 0.09	0.07 ± 0.06	0.14 ± 0.09
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.68 ± 0.33 †	0.43 ± 0.13 †	$1.08 \pm 0.56 \ddagger$
CCI	One week	1.9 ± 0.3	1.0 ± 0.4	3.1 ± 2.3
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.4 ± 1.3	2.4 ± 1.5 †	$15.0 \pm 10.1*$ †‡
COsm	One week	8.4 ± 2.8	5.4 ± 2.8	6.9 ± 3.5
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	11.1 ± 4.2	8.0 ± 4.3	19.6 ± 8.5†‡

Table I.10 Effects of ZD 7155 and L-NAME on electrolytes and osmolality clearances

Values are mean \pm SD calculated for before (Control) and after treatment with the AT1R antagonist ZD 7155, and ZD 7155 plus L-NAME; CX, clearance of Na⁺, K⁺, Cl⁻; COsm, clearance of osmoles; *p<0.05 compared to Control; †p<0.05 compared to one week; \pm p<0.05 compared to previous treatment.

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VARIABLE	AGE	Control	Control PD 123319		
CNa	One week	0.12 ± 0.06	0.16 ± 0.15	0.57 ± 0.62	
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.96\pm0.50\dagger$	1.25 ± 1.18 †	7.37 ± 9.30	
Ск	One week	0.19 ± 0.09	0.09 ± 0.04	0.11 ± 0.07	
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.72 ± 0.33 †	$0.53 \pm 0.25 \ddagger$	0.74 ± 0.29 †‡	
CCI	One week	1.5 ± 1.2	1.2 ± 0.8	2.9 ± 2.6	
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.7 ± 1.6	3.2 ± 1.6	$9.9 \pm 6.2*$ †‡	
COsm	One week	7.0 ± 3.7	7.2 ± 2.9	8.6 ± 4.9	
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	13.0 ± 5.7	13.7 ± 7.7†	15.7 ± 5.8	

Table I.11 Effects of PD 123319 and L-NAME on electrolytes and osmolality clearances

Values are mean \pm SD calculated for before (Control) and after treatment with the AT2R antagonist PD 123319, and PD 123319 plus L-NAME; CX, clearance of Na⁺, K⁺, Cl⁻; COsm, clearance of osmoles; *p<0.05 compared to Control; $\dagger p$ <0.05 compared to one week; $\ddagger p$ <0.05 compared to previous treatment.

VARIABLE	AGE	Control	ZD 7155+ PD 123319	ZD 7155 + PD 123319+ L-NAME
CNa	One week	0.14 ± 0.11	0.13 ± 0.10	0.32 ± 0.29
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	0.94 ± 0.37 †	1.79 ± 1.49†	33.06 ± 29.2 †
Ск	One week	0.20 ± 0.06	0.07 ± 0.04	0.13 ± 0.07
$(mL \cdot g^{-1} \cdot min^{-1})$	Six weeks	$0.70\pm0.47\dagger$	0.64 ± 0.34 †	1.01 ± 0.45 †‡
CCI	One week	1.6 ± 0.9	0.7 ± 0.5	1.8 ± 1.1
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	2.3 ± 1.7	3.7 ± 2.2†	22.0 ± 12.3*†‡
COsm	One week	8.7 ± 3.8	4.5 ± 2.3	6.7 ± 3.3
$(\mu L \cdot g^{-1} \cdot min^{-1})$	Six weeks	12.5 ± 7.1	13.9 ± 5.5 †	$27.5 \pm 11.5^{*}$ †‡

 Table I.12 Effects of ZD 7155 plus PD 123319 and L-NAME on electrolytes

 and osmolality clearances

Values are mean \pm SD calculated for before (Control) and after treatment with the ATRs antagonists ZD 7155 plus PD 123319, and after ZD 7155 plus PD 123319 and L-NAME; CX, clearance of Na⁺, K⁺, Cl⁻; COsm, clearance of osmoles; *p<0.05 compared to Control; p<0.05 compared to one week; p<0.05 compared to previous treatment.

EXPERIMENT I	AGE (weeks)	Control	ZD 7155	ZD 7155+ Vehicle	ZD 7155+ Vehicle+ L-NAME
	One	5.1 ± 2.3	3.1 ± 2.5	2.5 ± 1.6	3.6 ± 2.7
	Six	11.9 ± 5.7	10.2 ± 6.0	7.5 ± 4.1	9.7 ± 5.6
EXPERIMENT II		Control	PD 123319	PD123319+ Vehicle	PD 123319+ Vehicle+ L-NAME
	One	4.3 ± 3.0	3.9 ± 1.9	2.9 ± 1.5	3.9 ± 2.0
	Six	12.9 ± 7.2	11.6 ± 7.0	10.4 ± 7.2	9.5 ± 6.1
EXPERIMENT III		Control	ZD 7155	ZD 7155+ PD 123319	ZD 7155+ PD 123319+ L-NAME
	One	4.4 ± 2.9	2.2 ± 1.5	1.6 ± 1.3	2.0 ± 1.7
	Six	13.6 ± 7.0	12.2 ± 6.5	14.6 ± 6.9	15.6 ± 9.5

Table I.13 Effects of ATR antagonist and L-NAME on clearance of lithium

Values are mean±SD. Lithium clearance (CLi) measured before (Control) and at 30 min after administration of ZD 7155 (experiment I), PD123319 (experiment II) and ZD7155 plus PD123319 (experiment III) before and after L-NAME.

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