THE ROLE OF ANGIOTENSIN II AND ENDOTHELIN-1 IN THE HYPERTENSION OF EXPERIMENTAL CHRONIC RENAL FAILURE

By

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ABSTRACT

THE ROLE OF ANGIOTENSIN II AND ENDOTHELIN-1 IN THE HYPERTENSION OF EXPERIMENTAL CHRONIC RENAL FAILURE

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The primary purpose of the experiments described here was to investigate the role of humoral factors specifically, angiotensin II (AngII) and endothelin-1 (ET-1), in the pathogenesis of hypertension in the reduced renal mass (RRM) model of chronic renal failure (CRF). I hypothesized that the relative contribution of these two hormones to both short-term and long-term BP regulation in CRF differs depending on the level of salt intake. The work is highly relevant to the treatment of human CRF, which currently entails both regulation of dietary salt and aggressive drug therapy aimed at controlling arterial pressure and thus slowing progressive deterioration of renal function.

My experimental approach was designed to study the mechanism(s) of hypertension associated with CRF using the RRM animal model. I examined the effects of acute and chronic treatment in RRM rats with specific pharmacological inhibitors of the renin angiotensin and endothelin systems.

The results of my work demonstrate that both AngII and ET-1 play important roles in the maintenance of RRM hypertension and their relative contribution depends on the level of salt intake. Under conditions of low salt intake, inhibition of AngII formation was shown to lower blood pressure to the greatest extent, while during high salt intake endothelin system blockade proved to be most beneficial.

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Attaining a Ph.D. in Pharmacology was the most challenging undertaking of my life thus far. The experience has profoundly changed my life (hopefully for the better). I must thank many of those who helped me along the way.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE ..... angiotensin converting enzyme
ACEI ..... angiotensin converting enzyme inhibitor
AngI..... angiotensin I
AngII ..... angiotensin II
ANOVA ..... analysis of variance
ANP ..... atrial natriuretic peptide
AT_1RA ..... angiotensin II type 1 receptor antagonist
AVP ..... arginine vasopressin
BP ..... blood pressure
BUN ..... blood urea nitrogen
Ccr..... creatinine clearance
CNS..... central nervous system
CO ..... cardiac output
CRF ..... chronic renal failure
CVD ..... cardiovascular disease
DOCA ..... deoxycorticosterone acetate
ESRD ..... end-stage renal disease
ET..... endothelin isoform 1
ET-1 ..... endothelin isoform 1
ETRA ..... endothelin receptor antagonist
ET_ARA ..... endothelin subtype A receptor antagonist
ET_A/ET_BRA ..... endothelin subtype A and B receptor antagonist
GFR ..... glomerular filtration rate
HR ..... heart rate
i.m..... intramuscular
i.p..... intraperitoneal
i.v..... intravenous
MAP ..... mean arterial pressure
Na^+/K^+-ATPase ..... sodium potassium adenosine triphosphate enzyme
NO..... nitric oxide
OLF ..... ouabain-like factor
1K1C..... one-kidney, one clip
PRA ..... plasma renin activity
RAS ..... renin angiotensin system
RRM ..... reduced renal mass
RVR..... renal vascular resistance

SBP ..... systolic blood pressure
s.c..... subcutaneous
Scr..... serum creatinine
SEM..... standard error of the mean
SHR ..... spontaneously hypertensive rat
SNA ..... sympathetic nervous system activity
SNGFR ..... single nephron glomerular filtration rate
SNS ..... sympathetic nervous system
SPE..... slow pressor effect of AngII
TPR ..... total peripheral resistance
2K1C..... two-kidney, one clip
$U_{Na}V$ ..... urinary sodium excretion
UO ..... urine output
Upro..... urinary protein excretion
VSMC ..... vascular smooth muscle cell
WB ..... water balance
WI ..... water intake

INTRODUCTION

I. Hypertension

A. Prevalence

In Westernized countries, cardiovascular disease (CVD) is the leading cause of death. In the United States, close to 1 million people (43% of all deaths) die from CVD each year (Whelton *et al.*, 1995). These cardiovascular diseases include coronary artery disease, congestive heart failure, hypertension, stroke, etc. Hypertension, or high blood pressure, is the major modifiable risk factor for CVD (Burt *et al.*, 1995). Nearly one-quarter of Americans have hypertension and the prevalence is especially high (> 54%) in people 60 years and older (Burt *et al.*, 1995). Hypertension is currently considered to be a sustained diastolic blood pressure greater than 90 mmHg, or a sustained systolic pressure above 140 mmHg. Hypertension is the most critical risk factor for stroke and is considered to play a major role in the pathogenesis of many other diseases. Current pharmacological treatment of hypertension has been shown to decrease the risk of CVD. Still, the epidemic of blood pressure related CVD compels the research community to search for a better understanding of the mechanisms involved in hypertension.

B. Types of hypertension

The exact mechanism of most forms of hypertension is unknown. This is complicated by the probability that hypertension is a multifactorial disease involving many physiological disturbances. Hypertensive individuals are generally classified as

having either primary (essential) or secondary (non-essential) hypertension, depending on whether or not a specific cause or mechanism can be determined.

1. Primary hypertension

The majority of hypertensive patients (80-90%) are classified as suffering from primary hypertension. There is no known single cause of this disease but a variety of pathologic factors have been implicated: increased sympathetic nervous system or renin angiotensin system activity, abnormal insulin sensitivity, decreased renal function, alterations in vascular structure, abnormal lipid metabolism, genetic predisposition, etc. (Hunt and Williams, 1994; Julius, 1994).

2. Secondary hypertension

The prevalence of secondary hypertension is thought to vary between 10-20%, and it is classified as such when a single cause can be identified. Some major causes of secondary hypertension that have been identified include: mineralocorticoid excess (Gordon *et al.*, 1994), renal artery stenosis (Aristozabal and Frohlich, 1993) and chronic renal failure (Weidmann and Beretta-Piccoli, 1983).

II. Chronic Renal Failure

A. Background

An accelerated, progressive decline in renal function over time is termed chronic renal failure (CRF). CRF is not a disease entity in itself, but rather a clinical condition resulting from a number of pathologic processes that can lead to derangement and insufficiency of renal excretory and regulatory function. After diabetes (33%), hypertension (28%) is the second leading cause of CRF (Whelton *et al.*, 1992). End-stage renal disease (ESRD) results from decades of CRF. The prevalence, morbidity, and

mortality of ESRD is increasing in the last 30 years, especially due to the aging population. In 1991, the Federal ESRD program, which accounted for 93% of all renal disease patients, spent $6.6 billion on approximately 165,000 enrollees. The enrollment is predicted to reach 250,000 patients by the year 2000 (Eggers *et al.*, 1989). The average annual cost of one year's therapy for each patient was $29,000. Dialysis and/or renal transplantation are usually the therapeutic endpoints for the continual loss in renal function.

It is generally observed that once a significant decline is renal function has been initiated, regardless of the original insult to the kidney, there results a progressive deterioration in function that ultimately leads to total kidney failure. A major concern is that there is a long "silent period" from the initiation of kidney damage until the appearance of clinical, biochemical, or laboratory markers of the disease.

B. Experimental chronic renal failure

A variety of experimental animal models have been utilized to simulate the deterioration of renal function observed in CRF. The fawn-hooded rat is a genetic model of spontaneous glomerulosclerosis. In these rats, systemic hypertension develops along with glomerular hypertension leading to a decline in renal function (Simons *et al.*, 1991). Experimental models of diabetic nephropathy are of great interest because approximately 30% of patients with insulin-dependent diabetes mellitus develop renal failure and require dialysis, kidney transplantation or ultimately die. Experimental diabetic nephropathy is commonly induced by subtotal reductions in kidney mass (25%) and administration of streptozotocin (Chen *et al.*, 1992).

The most prominent model of CRF, the reduced renal mass (RRM) model, has been mainly employed in rats and dogs. It has been demonstrated experimentally and is also observed clinically that reductions in total kidney mass need to exceed 50% to induce renal insufficiency. The RRM model of CRF has been accomplished experimentally by two methods that are not equivalent. The excision method of RRM involves the partial surgical excision of both poles of one kidney and the removal of the contralateral kidney. This procedure effectively results in 66-83% ablation of total renal mass. The remnant renal tissue undergoes functional and morphological adaptation so as to maintain excretory function. Typically these RRM rats remain healthy for months except when the animals are placed on a high sodium intake, where hypertension rapidly develops and renal deterioration ensues (Ylitalo, 1976). The ligation method of RRM involves ligating 2 of the 3 renal arteries followed by contralateral nephrectomy. The ligation method is an inappropriate model of most clinical CRF because hypertension quickly develops (days to weeks). It is thought that the ligation method can induce pockets of ischemia leading to increased renin release; these abnormalities do not reflect the clinical course generally observed in human CRF (Meyer and Rennke, 1988).

1. Physical and metabolic changes in RRM

Early on the rats appear healthy and normal upon gross examination. Polydypsia and polyuria are early indicators of loss of renal concentrating ability. With continued deterioration of kidney function, uremic toxins accumulate in the blood, and the whole animal shifts into a catabolic state. In the final stages of RRM, there is weight loss due to muscle wasting, accompanied by edema and blood volume expansion due to decreased fluid excretory capacity and loss of plasma proteins.

Renal and cardiovascular abnormalities induced in the RRM model mimic the changes observed with deterioration in renal function over time in CRF. These include, but are not limited to: progressive rises in serum creatinine (Scr); blood urea nitrogen (BUN); and urinary protein excretion (Upro). Also observed are a decline in glomerular filtration rate (GFR), creatinine clearance (Ccr), and hematocrit. These laboratory indices are commonly monitored to assess renal function in both humans and animals.

2. Mechanisms of experimental chronic renal failure

a. Hemodynamic factors

CRF and RRM result in the permanent loss of nephrons. The decreased excretory function results in declining GFR and leads to body fluid volume excess. Structural and functional adaptations in the surviving nephrons produce hyperfiltration as partial compensation (Hostetter *et al.*,1981). This adaptive increase in single nephron glomerular filtration rate (SNGFR) maintains total kidney GFR in the short-term. These same adaptations also, however, contribute to the development of further glomerular injury. Most investigators in the field agree with the experimental evidence of Brenner and colleagues indicating that increased intraglomerular pressure is mainly responsible for the progressive injury to the remaining nephrons (Brenner *et al.*, 1985). Dietary protein restriction has been reported to preserve renal morphology and slow the deterioration of renal function in various models of experimental renal disease (*e.g.* Hostetter *et al.*, 1986; Diamond *et al.*, 1987) and in human CRF (Klahr *et al.*, 1994). The beneficial effects of protein restriction are thought to be associated with a reduction in glomerular hydrostatic pressure and a blunting of compensatory renal growth (Nath *et al.*, 1986). On the other hand, urinary excretion of protein increases as renal function

declines, presumably as a result of functional and structural damage to the glomerular filter.

b. Hypertrophic factors

The degree of glomerular hyperplasia and hypertrophy following RRM depends on the amount of renal mass removed and the age of the animal, but recent studies show that hypertrophy predominates after subtotal nephrectomy (Heeg *et al.*, 1989). Hypertrophic stimuli (*i.e.* increased sodium and protein intake, glucocorticoids, growth factors) have been found to accelerate glomerulosclerosis and overall renal deterioration (Norman *et al.*, 1987). Dramatic hypertrophy of all nephron segments in the renal stump occurs due to the structural and metabolic adaptations needed to maintain excretory capacity. Some investigators have hypothesized that stretching of the glomerular basement membrane due to glomerular hypertrophy and enhanced glomerular capillary pressure is the cause of the deleterious consequences to the glomeruli of RRM (Kleinknecht *et al.*, 1995).

3. Factors affecting the progression of renal lesions

a. Protein intake

Increasing dietary protein in rats with RRM has been shown to enhance the progression of renal lesions leading to decreased survival in the absence of hypertension (Kleinknecht *et al.*, 1995). Low protein diets (7%) have been demonstrated to protect the remnant kidney but these regimens are at the expense of undernutrition and growth defects (Salusky *et al.*, 1981). Increases in protein intake are thought to activate the renin angiotensin system (RAS) thereby contributing to hypertension and renal deterioration (Puller *et al.*, 1986; Rosenberg *et al.*, 1990). Other investigators have forwarded the

hypothesis that calorie restriction, irrespective of whether or not protein is restricted, can retard growth and prevent the development of end-stage pathology in the RRM model (Tapp *et al.*, 1989). Protein and calorie restriction are still being investigated in RRM models, but the application to human CRF seems of limited benefit.

b. Sodium intake

Several authors have shown that sodium restriction has protective effects on renal function in the RRM model (Hout *et al.*, 1983; Koletsky *et al.*, 1959; Lax *et al.*, 1992) even when no changes in BP were observed (Daniels *et al.*, 1990).

c. Coagulation abnormalities

Early studies in RRM focused on the possibility that inhibition of blood coagulation slows the progression of hypertension and renal deterioration (Purkerson *et al.*, 1976; Zoja *et al.*, 1989). Intraglomerular thrombosis and platelet aggregation have been suggested to play a role in glomerular dysfunction in RRM. Warfarin and heparin administration have been shown to retard the progressive increase in hypertension and renal deterioration following RRM (Purkerson *et al.*, 1982; Olson, 1984). Additional reports have shown that inhibition of platelet aggregation by the thromboxane synthesis inhibitor, OKY 1581, prevented cardiac hypertrophy and hypertension in RRM rats (Purkerson *et al.*, 1984). These data support a role of platelet aggregation and glomerular thrombosis in the pathogenesis of RRM and suggests that inhibition of blood coagulation prevents the development of hypertension and the progression of renal failure. The influence of coagulant system activation on hypertension and renal deterioration in RRM is still being investigated, although the emphasis of most recent research has focused on hemodynamic factors.

C. Chronic renal failure and hypertension

1. Background

There exists a close relationship between CRF and systemic hypertension. High BP is often an initiator of renal insufficiency leading to ESRD. High BP also acts as a promoter of renal damage in patients with established kidney disease, *e.g.* diabetic nephropathy. All levels of untreated hypertension are associated with declining renal function (Shulman *et al.*, 1989), but most BP related renal disease can be attributed to mild hypertension or even high normal BP (Whelton *et al.*, 1992). There is a beneficial effect on renal function in CRF with BP reduction acutely and chronically (Gansevoort *et al.*, 1994; Lebovitz *et al.*, 1994). For example, Upro is usually increased in CRF, but is stabilized or even decreased by antihypertensive drug therapy.

Conversely, impairment of renal function almost always causes some elevation in BP, but the etiology of hypertension in CRF is complex and probably multifactorial. Some of the potential causes of hypertension in CRF are: body fluid volume excess; increased sympathetic nervous system activity (SNA); alterations in humoral factors; structural cardiovascular changes; or some combination of these. The most common explanation is that a loss of functioning nephrons causes a decrease in sodium and water excretion, which leads to increased body fluid volume and elevated BP. According to the "pressure-natriuresis" theory, elevated BP restores renal fluid excretion back to normal levels (Cowley *et al.*, 1992).

2. Hypertension development in experimental CRF

a. Ligation method

BP increases immediately following renal artery ligation due to the exaggerated secretion of renin. Within weeks there is observed a severe hypertension (SBP > 180 mmHg) that is associated with volume expansion. Production of the RRM model by the ligation method does not produce elevations in BP that are influenced by salt intake (Kleinknecht *et al.*, 1995). Mortality usually ensues within 6 months after the partial ligation due to cardiovascular disease resulting from progressive increases in BP and uremia.

b. Excision method

Three stages of hypertension can be recognized in the excision RRM model (Gretz *et al.*, 1993). The first stage is characterized by a short period (*i.e.* days to weeks) of acute renal failure accompanied by sodium retention and volume expansion due to reduction in renal excretory function. Compensatory mechanisms, such as cellular hypertrophy and glomerular hyperfiltration, result in a steady improvement in renal function occurring during this phase such that increases in BP or proteinuria are not commonly observed (Gretz *et al.*, 1993).

This is followed by a long stable phase (weeks to months) with gradual progression of BP, proteinuria, and other signs of renal deterioration. This gradual increase in BP can be exacerbated by increasing NaCl intake (Langston *et al.*, 1963). The overall rate of increase in BP and decrease in renal function in this phase is dependent on sodium intake, dietary protein and on the original amount of renal mass

removed. Therapeutic interventions effective in slowing the onset of terminal renal failure are usually implemented during this phase.

The final terminal phase is characterized by gross edema and uremia, very high elevations in BP, and a generalized somatic wasting that ultimately ends in renal failure and death (Koletsky and Goodsitt, 1960). Effective treatment of hypertension during this malignant phase may be totally different than in previous phases and little can be done to slow the rapid deterioration in renal function.

3. Sodium status

The degree of hypertension in advanced renal failure is frequently related to excessive sodium chloride ingestion. Koomans reported that BP increased during elevated salt intake in a variety of patients with different degrees of renal insufficiency (Koomans *et al.*, 1982). The BP increase tended to be larger in the patients with a greater loss of kidney function. In fact, this "salt-sensitivity" of BP rose exponentially with the decline in function. It is known that salt retention with extracellular fluid volume expansion can result in a raised cardiac output (CO) and elevated total peripheral resistance (TPR), both of which are usually increased in ESRD (Textor *et al.*, 1981). Salt restriction lessens the accumulation of sodium and water in CRF patients, thereby decreasing plasma volume overload, and decreasing BP (Bakris and Gavras, 1993). Dietary salt restriction is commonly initiated in human CRF to alleviate "volume-dependent" hypertension. Low-salt diets have played an integral role in the treatment of hypertension in CRF patients for over 30 years.

4. Sympathetic nervous system activity

An increased sympathetic nervous system activity (SNA) may contribute to hypertension and progressive renal deterioration in patients with CRF. The kidneys are innervated with two main types of sensory receptors: the renal baroreceptors which increase firing in response to changes in renal perfusion pressure; and the renal chemoreceptors which may be stimulated by ischemic metabolites or uremic toxins (Dibona, 1982). These receptors are linked to the sympathetic centers in the central nervous system (CNS) through renal afferent pathways (Faber and Brody, 1985). Converse *et al.*,(1992) reported that in patients with CRF, there exists a elevated sympathetic activity, which may be due to an afferent signal from the failing kidney. Accordingly patients with bilateral nephrectomy had lower rates of sympathetic discharge than CRF patients with native kidneys, and this was accompanied by lower BP's. Many studies have implicated functional abnormalities in the sympathetic nervous system in the hypertension observed in RRM. Elevated plasma levels of norepinephrine have been observed in RRM rats on a high sodium intake, and epinephrine synthesis blockade with SK&F 64139 resulted in a fall in BP (Dipette *et al.*, 1982). Campese and colleagues reported preventing the progression of renal disease and hypertension by renal afferent denervation in RRM rats (Campese *et al.*, 1995a: Campese *et al.*, 1995b). These studies provide evidence that neurogenic factors may play an important role in renal deterioration and hypertension in RRM rats.

5. Hormonal factors

a. Aldosterone

A variety of hormonal factors also have been implicated in the hypertension associated with CRF. Plasma aldosterone levels are increased in humans with CRF (Mitch and Wilcox, 1982) as well as in RRM rats drinking saline compared to sham rats under the same conditions (Chi *et al.*, 1986). It has been shown that elevated plasma aldosterone levels can cause hypertension in dogs particularly under conditions of high salt (Pan and Young, 1982). But previous work in our lab demonstrated that hypertension observed in RRM rats (excision model) is not dependent on an elevated plasma aldosterone concentration (Kanagy, 1991).

b. Na,K-adenosine triphosphate inhibition

Some investigators have suggested that endogenous Na,K-adenosine triphosphate (Na,K-ATPase) inhibitors may play a role in hypertensive CRF. The Na,K-ATPase enzyme is found ubiquitously throughout the cells of the body but is most abundant in the kidney tubules, where it is provides the energy for active sodium reabsorption from the glomerular filtrate (Lingrel *et al.*, 1994). Hout *et al* in 1983 found that hypertensive RRM rats had decreased Na,K-ATPase pump activity in vascular smooth muscle and cardiac cells. The cardiac glycosides (*i.e.* digoxin) are known to inhibit Na,K-ATPase pump activity, produce vasoconstriction and increase cardiac contractility (Vatner *et al.*, 1971). It is thought that an endogenous sodium pump inhibitor exists, preliminarily described as ouabain, and is released in response to volume expansion, which contributes to increases in BP (deWardener, 1990). Yamada *et al.*, in 1994 reported that an antibody to ouabain lowered BP in hypertensive RRM rats on a high sodium intake.

Hamlyn and colleagues have reported that long term ouabain administration produces greater degrees of BP increase in RRM rats, varying proportionately with the amount of kidney mass removed (Yuan *et al.*, 1993). The sustained elevation in BP observed during ouabain administration supports the possibility that endogenous inhibitors of the Na,K-ATPase pump have a pathogenic role in RRM hypertension.

c. Arginine vasopressin

The role of arginine vasopressin (AVP) in hypertension of CRF is uncertain. Vasopressin secretion is directly related to plasma osmolality. AVP's primary action is to increase water permeability of the principal cells in the collecting ducts of the kidney. High plasma AVP concentrations cause both renal vasoconstriction and glomerular mesangial cell proliferation. These abnormalities can eventually result in renal deterioration and increased systemic BP. Yet in 1993, Yamada and co-workers reported that even with relatively high plasma concentrations in CRF patients, AVP does not participate in hypertension. Oral administration of an AVP-V_1 receptor antagonist, OPC-21268, did not result in any BP changes in 7 hypertensive CRF patients. Likewise in hypertensive RRM rats, treatment with this AVP antagonist produced only a minimal decrease in established hypertension (Gavras, 1982). In contrast, in two different experimental models of renal failure, deoxycorticosterone-salt (DOCA-salt) and adriamycin-induced nephropathy, Okada reported that combined therapy of OPC-21268 and the AVP-V_2 selective receptor antagonist, OPC-31260, prevented hypertension development and the progression of renal injury (Okada *et al.*, 1994). To date, the relative importance of increased AVP plasma levels in renal pathophysiology and hypertension in the RRM model remains to be clarified.

d. Natriuretic peptides

CRF is associated with expansion of extracellular fluid volume, and this volume overload is thought to elicit increases in circulating concentrations of natriuretic peptides. Elevated atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) plasma concentrations have been reported in patients with CRF (Rascher *et al.,* 1985; Totsune *et al.,* 1994). ANP secretion is increased in response to the distention of the atria which occurs during plasma volume expansion in CRF. ANP increases urinary sodium excretion by increasing GFR, inhibiting sodium reabsorption by the medullary collecting duct, and indirectly by inhibiting renin and AngII-induced aldosterone secretion (Vander, 1991). These actions of ANP serve to regulate total body sodium and fluid homeostasis. ANP has also been reported to play a role in the adaptive hemodynamic and excretory responses observed in the RRM model under normal and high sodium conditions (Zhang *et al.,* 1994; Brandt *et al.,* 1989). It has been shown that rats subjected to RRM excrete elevated amounts of sodium and water per remnant nephron as a compensatory response to overall reduced excretory capacity (Zhang *et al.,* 1994). Enhanced ANP secretion may promote the compensatory increase in sodium and water excretion per individual nephron. Increases in plasma ANP levels in RRM have been shown to follow rather than accompany the development of hypertension, and the increased plasma concentrations reported are not sufficient to effect BP (Brandt *et al.,* 1989). It seems unlikely then that ANP plays a major role in the maintenance of RRM hypertension. Currently, the focus of most research on the hormonal basis of hypertension in RRM is on angiotensin II (AngII) and endothelin-1 (ET-1).

III. Renin-angiotensin system

A. Hormonal renin angiotensin system

1. Synthesis-cascade

The RAS is of major importance in sodium and water homeostasis, but also influences a plethora of other physiological functions. The RAS is usually described in terms of its synthesis cascade (Figure 1). Angiotensinogen is synthesized in the liver and released into the bloodstream. Granular cells of the juxtaglomerular apparatus in the kidney secrete the peptidase, renin, into the bloodstream in response to decreased renal perfusion pressure or altered sodium chloride delivery. Renin, the rate-limiting enzyme of the RAS, cleaves circulating angiotensinogen to the decapeptide angiotensin I (AngI). AngI is then further cleaved by angiotensin converting enzyme (ACE) to form the octapeptide AngII. ACE is a non-specific protease, found primarily in vascular endothelial cells of the lungs, which degrades other peptides in addition to cleaving AngI. AngII is the principally active component of the RAS. AngII acts upon two main types of angiotensin receptors designated as angiotensin II type 1 (AT_1), and angiotensin II type 2 (AT_2) (Wong *et al.*, 1990). These receptors are located throughout the body in a wide variety of tissues. It is believed that AT_1 receptors mediate most of the physiological effects of AngII. A wide variety of proteases such as neutral endopeptidase terminate the actions of AngII (Poulsen and Jacobsen, 1993). A few of the peptide metabolites of AngII, *i.e.* angiotensin (1-7), angiotensin III, angiotensin (3-8), may also exert physiological effects, but their relative importance is questionable and needs to be determined.

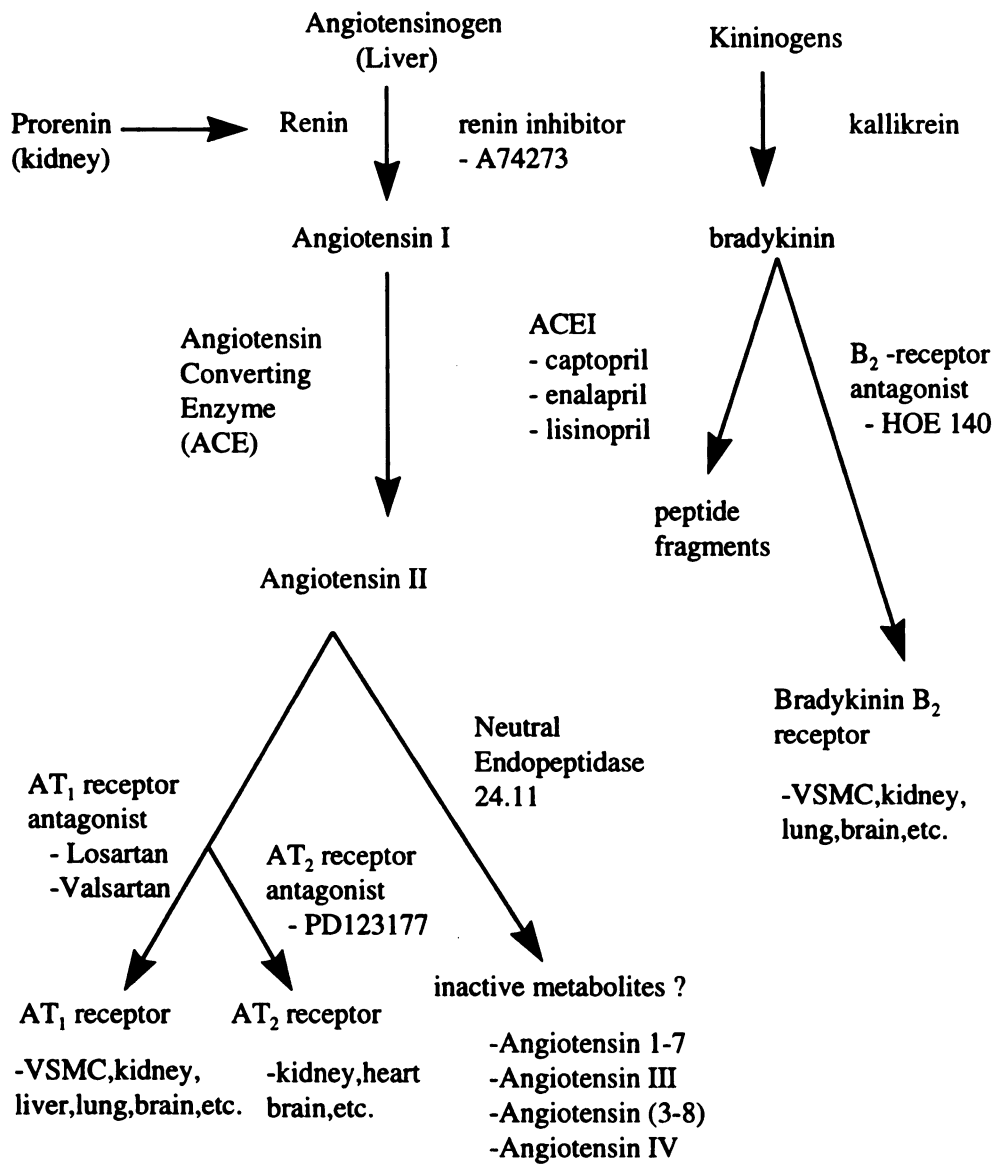


Figure 1: Renin angiotensin synthesis cascade

2. Physiological actions

a. Hemodynamic actions

Most of AngII physiologic actions serve to increase BP and/or the renal retention of sodium and water. Thus it is not surprising that salt deficit and hypotension are the two most potent stimuli for RAS activation. AngII induces contraction of blood vessels, causing an increase in TPR which results in an elevated BP. AngII also is a growth factor, and may be involved in vascular hypertrophy or remodeling, which can cause long-term increases in TPR and result in hypertension (Griffin *et al.*, 1991).

b. Heart

AngII tends to slightly increase the force of heart contractions and heart rate by its facilitory actions on sympathetic outflow (Garrison and Peach, 1990). As blood volume increases due to the renal actions on AngII, increases in left ventricular preload result and therefore augments cardiac output. It must be kept in mind that in intact animals, increased circulating concentrations of AngII increase systemic BP and baroreflex discharge but these actions may initiate reflex vagal activity sufficient to slow the heart.

c. Central nervous system

The brain contains all components of the RAS and AngII may serve as a neurotransmitter or neuromodulator at many sites within the CNS. In addition, circulating AngII can gain access to the brain through the circumventricular organs and elicit cardiovascular responses. The central and peripheral actions of AngII on the nervous system include: stimulation of drinking behavior and AVP release, increasing SNA, and enhancing norepinephrine release from sympathetic nerve terminals (Vander, 1991).

Hypovolemia is known to stimulate the RAS. This stimulation is associated with a compensatory increase in water intake and sodium appetite to which renin from both the kidney and brain may contribute. AngII is the most potent dipsogenic substance yet discovered and it stimulates drinking activity by a direct action on the CNS (Epstein *et al.*, 1970). The effect of AngII on stimulating thirst has also been observed clinically. In patients with severe renal disease exhibiting high plasma renin concentrations intractable thirst was relieved following bilateral nephrectomy (Brown *et al.*, 1969). AngII has also been implicated in a centrally mediated increase in sodium appetite. Circulating AngII is not thought to be a powerful stimulus to sodium appetite, but i.c.v. AngII has been shown to induce increased sodium chloride intake in rats when maintained under sodium replete conditions (Fitzsimons, 1993).

d. Endocrine systems

i. Aldosterone

AngII stimulates the synthesis and release of aldosterone from the adrenal cortex which then acts on the collecting duct in the kidney to cause retention of sodium. The synthesis and release of aldosterone is enhanced under conditions of hyponatremia and suppressed during sodium replete conditions following the inverse relationship between RAS activity and sodium intake.

ii. Arginine vasopressin

The role of AngII in the regulation of AVP secretion was first described by Bonjour and Melvin who demonstrated that i.v. infusions of AngII increase plasma AVP concentrations (Bonjour and Malvin, 1979). It is currently believed that high levels of exogenous AngII that border on the supraphysiological are required to cause increases in

AVP release (Brooks and Malvin, 1993). The receptors that mediate AngII effects on AVP secretion are located in the brain and AngII is thought to utilize the circumventricular organs to gain access to these regions. AVP and the RAS are linked by a negative feedback mechanism. In opposition to the stimulatory action of AngII on vasopressin secretion, it is well established that AVP inhibits renin release (Brooks and Malvin, 1993).

iii. Atrial natriuretic peptide

ANP appears to be a physiological counterpart to activation of the RAS because ANP opposes the majority of actions elicited by AngII and aldosterone. These include: inhibition of AngII-mediated modulation of glomerular filtration, antagonism of AngII-induced proximal tubule sodium reabsorption, attenuation of the vasoconstrictor effects of AngII, suppression of aldosterone secretion and antagonism of aldosterone-mediated sodium reabsorption from distal nephrons (Richards and Nicholls, 1993). Changes in sodium and water status that induce activation of the RAS elicit reciprocal decreases in ANP activity. For example, plasma ANP concentrations rise in proportion to increasing salt intakes whereas the RAS is suppressed.

e. Kidney

As described above, renin released from the granular cells of the juxtaglomerular apparatus in the kidney is the rate-limiting step in the RAS which results in the production of AngII. The control of renin secretion is quite complex. A variety of hormonal and nervous system imputs influence renin secretion: (1) intrarenal baroreceptors, (2) tubular sodium or chloride delivery to the macula densa, (3) renal sympathetic nerves, and (4) AngII (negative feedback) (Vander, 1991). Stimulation of

renin release and activation of the RAS ultimately leads to increases in AngII and aldosterone release which both cause significant effects within the kidney.

i. Renal hemodynamics

The overall renal effect of AngII is to increase renal vascular resistance (RVR) and consequently decrease renal blood flow (RBF) due to direct vasoconstriction of the renal vasculature, which is quite sensitive to the peptide. These responses are observed at plasma levels that have little effects on systemic arterial pressure. The renal vascular responses to AngII depend partly on total body sodium status and are affected by the inverse relationship between RAS activity and sodium intake (Hollenberg *et al.*, 1974).

ii. Glomerular function

Increases in circulating AngII cause decreases in GFR due to both renal hemodynamic effects and direct contraction of mesangial cells. Mesangial cell contraction results in a decrease in glomerular capillary filtration coefficient (K_f) (Dickinson *et al.*, 1963). The decreased K_f decreases the glomerular filtration surface and this leads to reductions in GFR. Changes in afferent and efferent arteriole resistance greatly influence GFR. Micropuncture studies have shown that AngII disproportionately increases efferent resistance resulting in increased glomerular capillary hydraulic pressure (Dickinson *et al.*, 1963). Some investigators have implicated an increased glomerular pressure as the cause of renal deterioration in CRF (Meyer *et al.*, 1987).

iii. Tubular function

One of the important renal actions of AngII is to promote sodium reabsorption from the proximal tubule, but the single most important controller of sodium reabsorption is aldosterone (Vander, 1991). Synthesis and release of aldosterone from the adrenal

cortex is stimulated by AngII (Vander, 1991). The principal cell in the cortical collecting duct is acted upon by aldosterone to stimulate sodium reabsorption. Reflex pathways exist to keep sodium balance within a very tight range. Baroreceptors in the kidney and the carotid sinus, sensitive to changes in extracellular sodium and plasma volume, regulate GFR and sodium reabsorption. The tubuloglomerular feedback loop involves detection of increased sodium concentrations by the macula densa which generates a signal to ultimately decrease GFR, thereby decreasing sodium retention. Activation of fluid-retaining mechanisms resulting in water and sodium volume expansion play a part in the long-term actions of AngII on BP regulation. It should be noted that when circulating AngII levels are high enough to raise systemic pressure, an AngII mediated pressure-natriuresis counteracts the sodium retaining actions of the peptide in the proximal tubules and collecting ducts. When the pressure-natriuresis relationship is impaired, AngII leads to excessive sodium retention, volume expansion and hypertension.

B. Tissue renin angiotensin system

Recent evidence suggests that in addition to the classical endocrine RAS, there exists a "local RAS" that is thought to act in an autocrine or paracrine fashion and is differentially regulated from the circulating RAS (Campbell, 1987). These angiotensin generating systems have been described in many organs in the body including: brain, heart, kidney, adrenal, and blood vessels (Phillips, 1993). Tissues in these organs have been shown to contain mRNA for all the various components of the RAS: angiotensinogen, renin, ACE, etc. Plasma derived renin of kidney origin, however, is the major source of vascular renin and is considered to be the main regulator of vascular AngII production (Dzau and Re, 1994: Kato *et al.*, 1993). Changes in activity of these

local systems are not thought to influence plasma AngII concentrations. Campbell and others have promoted the viewpoint that the circulating RAS provides homeostatic responses to acute changes in BP and fluid and electrolyte status (Campbell, 1987). In contrast the local RAS may affect BP regulation by exerting more of a tonic influence in the tissues where they exist (*i.e.* regulation of vascular tone). Even though the existence of local RAS were described over a quarter of a century ago, their physiological and pathophysiological relevance remains to be defined.

C. Inhibition of the renin angiotensin system

The RAS can be inhibited at several different points in the synthesis cascade. Generally speaking the RAS is suppressed under conditions of high salt intake (HS) or increasing cumulative sodium balance. This may also be true but probably to a lesser extent under conditions where water intake is inappropriately elevated. The RAS also can be inhibited by pharmacological intervention. For example, beta-adrenoceptor blockers decrease renin secretion (Vander, 1991). Direct inhibitors of renin have also been developed (Kleinert *et al.*, 1992). But the drugs used most commonly to impair RAS activity are the ACE inhibitors, *e.g.* captopril, lisinopril and enalapril. Competitive, reversible angiotensin II receptor antagonists are available for both the AT_1 (AT_1RA) (*i.e.* losartan, and EXP3174), and AT_2 (AT_2RA) (*i.e.* PD123177) receptors.

1. Angiotensin converting enzyme actions

As mentioned above, ACE is a non-specific protease that can act on a variety of substrates besides AngII such as: bradykinin (BK), enkephalin, and neurotensin (Skidel and Erdos, 1993). ACE is responsible for inactivation of the vasodilator BK (Figure 1), therefore some of the hypotensive effects of ACEI have been proposed to be due to the

accumulation of endogenous BK (Williams and Hollenberg, 1977). BK causes relaxation of VSMC but its role under basal conditions is considered to be minor (Carretero and Scicli, 1993). Under pathological conditions, low sodium intake, or when degradation is inhibited, BK either directly or via various intermediates may cause: diuresis, natriuresis, antiproliferative and antihypertrophic actions, antithrombotic and fibrinolytic effects (Carretero and Scicli, 1993). There are many published reports on the potential significance of BK in cardiovascular control. Unger and colleagues reported that chronic administration of a bradykinin B_2-receptor antagonist, HOE 140, partially attenuated the antihypertensive effect due to the ACEI ramipril in two-kidney, one-clip hypertensive rats (Bao *et al.*, 1992). Chen demonstrated similar findings in a dissimilar model of hypertension (DOCA-salt). They showed that the reduction in BP due to the ACEI captopril was abolished acutely by HOE 140 administration (Chen *et al.*, 1996). There is much experimental evidence to refute the role of BK in mediating the hypotensive response of ACEI. Plasma kinins are reportedly unchanged or only moderately increased after ACEI administration (Carretero and Scicli, 1988), yet it has been shown that kinin concentrations need to be elevated approximately 20X normal values to cause acute decreases in BP in some forms of experimental hypertension (Salgado *et al.*, 1986). Kohzuki reported in SHR that co-administration of cilazepril (ACEI) and HOE 140 induced no changes in BP other than those associated with cilazepril alone (Kohzuki *et al.*, 1995). A powerful argument that many investigators cite is the lack of an increased hypotensive response to enalapril when compared to AngII receptor antagonist administration (Siegl *et al.*, 1995; Okada *et al.*, 1995). These studies suggest that the hypotensive effects due to ACEI derive from inhibition of AngII formation only. There

still exists a good deal of controversy over the mechanisms responsible for the BP lowering effects of ACEI, however, and further investigation is warranted.

D. Renin angiotensin system and hypertension

1. Background

The notion that the RAS is involved in hypertension evolved from work done by Goldblatt in the 1930's. Goldblatt theorized that essential hypertension was caused by the release of a renal pressor substance in response to renal artery constriction. This pressor substance was later characterized as renin and there is now unequivocal evidence that the RAS participates in the pathogenesis of hypertension.

It is generally agreed that essential hypertension is multifactorial, therefore abnormalities of the RAS may only play a partial role in BP elevation. Hypertensive patients are sometimes classified into two groups based on their plasma renin concentrations. Patients with low-renin hypertension are considered to have plasma renins that are low for their level of salt intake (Swales, 1993). Generally low-renin hypertensives exhibit volume expansion and do not respond well to antihypertensive therapy directed at inhibiting or blocking the RAS. High-renin hypertensives have plasma renin levels above the normal range expected at their level of salt intake. These patients often have increased SNA and cardiac outputs (Esler *et al.*, 1978). Antihypertensive therapy aimed at inhibiting the RAS in these patients usually has beneficial effects when compared to low-renin hypertensives on the same therapy. These classifications are based on historical perspectives, and may not provide an accurate description of patients in light of recent studies involving the development of more

selective antagonists, better biochemical measurements and the discovery of local tissue renin angiotensin systems.

2. AngII induced hypertension

Chronic i.v. administration of low doses of AngII causes the development of a sustained hypertension in normal rats (Kanagy *et al.*, 1990). The degree of increase in BP depends mainly on the infusion rate of AngII. In this model of hypertension, elevation of BP is completely reversed upon discontinuation of exogenous AngII infusion. It has been demonstrated that AngII-induced hypertension is initially dependent on direct vasoconstriction due to the fast pressor effect of AngII, but chronic increases in BP are due to the slow pressor effect (Brown *et al.*, 1981).

a. Fast pressor effect of AngII

Large infusion rates of AngII (*i.e.* > 30 ng/kg/min) administered parenterally in experimental animals and humans elicit large, rapid increases in BP referred to as the fast pressor effect of AngII. BP is increased within seconds to minutes and this effect lasts only as long as the peptide is administered (Brown *et al.*, 1981). Upon discontinuation of AngII infusion, BP returns to normal values within minutes. Tachyphylaxis occurs and BP gradually falls if these high doses of AngII are continuously infused for several days (Dickinson and Lawrence, 1963). It is now well established that the mechanism of the fast pressor effect of AngII is direct contraction of vascular smooth muscle cells via AT_1 receptors resulting in vasoconstriction (Brown *et al.*, 1981).

b. Slow pressor effect of AngII

The slow pressor effect (SPE) of AngII occurs when low amounts of the peptide (*i.e.* < 10 ng/kg/min) are infused which do not invoke a fast pressor response but cause

BP to gradually rise over several days (Brown *et al.*, 1981). Tachyphylaxis is not observed to the SPE and upon discontinuation of AngII infusion, BP returns to pre-infusion levels only after a prolonged period (*i.e.* hours to days). Several theories have been proposed, but the exact mechanism(s) of the SPE are still being investigated.

One mechanism proposed for the development of the SPE is vascular remodeling and/or hypertrophy. These structural changes can occur throughout the vasculature and may contribute to the increase in total peripheral resistance that is commonly observed in hypertension (Heagerty *et al.*, 1993). Yet it is generally thought that hypertrophy and remodeling of vascular tissue requires weeks to months to develop, which does not correlate well with the SPE that is apparent within days (Lundgren, 1974). Accordingly, vascular remodeling may play a role in BP regulation over much longer periods of time, but its influence on initiation of the SPE is not supported by the current experimental evidence.

AngII is known to elicit physiological responses from interactions with receptors within the central nervous system (CNS); therefore, these cardiovascular centers may be an important site of action for the SPE. Circulating AngII can interact with the CNS via receptors in circumventricular organs outside the blood brain barrier to augment sympathetic tone (Lappe and Brody, 1984). Luft and coworkers in 1989 demonstrated that SNA was increased in rats receiving chronic low dose AngII infusions. Blockade of the sympathetic nervous system (Yu and Dickinson, 1971) and ablation of the area postrema (Fink *et al.*, 1987), a circumventricular organ, have been reported to prevent the development of the SPE in rats. On the contrary, other investigators have demonstrated that an enhanced slow pressor response to AngII in spontaneously hypertensive rats

(SHR) was still intact after sympathectomy (Li and Jackson, 1989). The relative role of the CNS on the SPE is still a matter of investigation and controversy.

The SPE has been proposed to result from AngII-induced increases in total body sodium and fluid volume (DeClue *et al.*, 1978). Salt loading was shown to increase the magnitude of the SPE of AngII, whereas salt restriction diminished this effect (Cowley and DeClue, 1976). Our laboratory has found that subpressor rates of AngII infusion do not affect sodium balance or increase plasma aldosterone concentrations, thereby arguing against sodium retention being responsible for the SPE.

Although no one of the aforementioned possible mechanisms discussed appears to be totally responsible for the SPE, it is quite probable that each mechanism plays a role and that their additive effects are needed for the expression of the SPE.

3. AngII involvement in other forms of hypertension

Since in the majority of hypertensive patients there is no definable cause, a great deal of research has focused on developing experimental models of essential hypertension.

a. Spontaneously hypertensive rat

The SHR was derived from selective breeding by Aoki in 1963 (Okamoto and Aoki, 1963). This genetically hypertensive strain is the most widely used experimental model of hypertension. Increased BP develops very early and is accompanied by left ventricular hypertrophy, increased SNA and nephrosclerosis (Kurtz *et al.*, 1995). The involvement of the RAS in SHR has been investigated extensively in recent decades. The majority of these studies demonstrate that inhibition of the RAS with ACEI and AT_1RA

prevents and reverses the progression of hypertension and renal damage in SHR (Kohara *et al.*, 1993; Cachofeiro *et al.*, 1995).

b. Goldblatt renal hypertension

A well studied model of renal hypertension referred to as Goldblatt hypertension is produced by constriction of one or both of the renal arteries with adjustable silver clips (Goldblatt *et al.*, 1934). The procedure that most closely resembles human renovascular hypertension is the two-kidney, one-clip (2K1C) model where both kidneys are present, but one renal artery is clipped and partially occluded. This procedure causes a dramatic increase in renin secretion from the affected kidney (Martinez-Maldonada, 1991) and renin inhibitors, ACEI and AT_1RA are effective antihypertensive agents (McMahon *et al.*,1995; Wallace and Morton, 1984; Thurston, 1994). Thus, this model of hypertension is referred to as "renin-dependent".

c. Transgenic models of hypertension

The causes of essential hypertension are still poorly defined, but genetic factors are known to play an important role. Techniques have recently emerged that allow for the overexpression or deletion of specific genes in experimental animals. Transgenic animals have new genetic material incorporated into their genome through microinjection into germ cells. Ganten and coworkers were the first to overexpress the renin gene in rats (Mullins *et al.*, 1990). Fulminant hypertension resulted and heterozygous animals developed systolic BP of up to 250 mmHg at 10 weeks of age. Other genes influencing BP are currently being identified so that manipulations of the genomes will permit investigators to examine the effect of these alterations *in vivo*. The generation of laboratory animals expressing candidate genes involved in cardiovascular disease may

provide for further investigation of regulatory mechanisms of the gene products and possible pathophysiological consequences of their abnormal expression.

E. Renin angiotensin system and chronic renal failure

1. Renin angiotensin system activity in chronic renal failure

The contribution of the RAS to hypertension associated with human CRF has been studied for many years. Many investigators have demonstrated the efficacy of ACEI treatment in arresting the progression of hypertension and the deterioration of renal function in human CRF. The beneficial effects of ACEI treatment have been reported in many types of renal failure, *i.e.*; hypertensive non-insulin dependent diabetes mellitus (Lebovitz *et al.*, 1994), diabetic nephropathy (Mulec *et al.*, 1994), and non-diabetic CRF (Becker *et al.*, 1994). Additionally, Gansevoort *et al.*, in 1994 reported a lowering of BP and a decrease in urinary protein excretion with the AT_1RA, losartan, in hypertensive patients with renal disease. Thus the RAS, specifically AngII, is involved in both the hypertension and the renal deterioration observed in human CRF and experimental RRM.

2. Other antihypertensive regimens

A variety of antihypertensive agents have been shown to have some protective actions against CVD and renal deterioration in CRF patients. What has been more difficult to demonstrate with these medications is a slowing of renal deterioration independent of BP lowering effects. ACEI, on the other hand, have been shown to exert beneficial effects on the kidney independent of BP lowering effects (Kasiske *et al.*, 1993; Liou *et al.*, 1995; Mann *et al.*, 1990). When compared to ACEI, most other antihypertensive regimens elicit undesirable effects that may be detrimental. For example, thiazide diuretics act in a beneficial way to cause sodium excretion and reduce

peripheral vascular resistance but they tend to induce lipid abnormalities and their efficacy is greatly reduced in patients with renal impairment (National High Blood Pressure Education Program, 1991).

Beta-adrenoceptor blockers are capable of lowering BP effectively and to the same extent as ACEI in CRF. But many studies have concluded that ACEI slow the progression towards end stage renal disease and prolong kidney survival better than beta-blockers, probably through mechanisms in addition to antihypertensive effects (Hannedouche *et al.*, 1994). The beta-blockers also are associated with some detrimental side effects in CRF patients. For example this class of antihypertensives may induce carbohydrate intolerance and exacerbate diabetes mellitus. Propranolol has been reported to cause a 10-20% reduction in GFR (Vulpis *et al.*, 1991).

The calcium channel blockers (CCB) cause moderate reductions in systemic BP in CRF patients. But in experimental models of renal failure this BP decrease is accompanied by renal afferent arteriole dilation, thereby permitting an increased glomerular capillary pressure to persist (Tolins and Raij, 1990). This lack of effect on glomerular hypertension is less effective in preventing hemodynamically-mediated progressive glomerular injury. It has recently been hypothesized that non-hemodynamic effects of CCB's may play a role in slowing renal deterioration. CCB's have been shown to inhibit mesangial cell proliferation and the generation of inflammatory mediators by endothelial cells (Shultz and Raij, 1989; Tolins *et al.*, 1989). Zucchelli and colleagues studied the progressive rate of renal insufficiency in 142 hypertensive patients over four years and found that both CCB and ACEI possess a renoprotective effect that is no greater with either treatment (Zucchelli *et al.*, 1992).

3. Renin angiotensin system activity in RRM model

a. Ligation vs. excision method of RRM

The majority of studies investigating the role of the RAS in RRM have utilized the ligation method. As previously mentioned, the ligation method is not the best model for the study of hypertension in CRF, because the resultant pockets of ischemia that are produced cause exaggerated release of renin and a rapid increase in BP. The hypertension is associated with high intrarenal renin concentrations and is not affected by changes in sodium intake (Kleinknecht *et al.*, 1995), unlike human CRF.

With the excision method there is little change in BP during the first weeks and BP rises progressively only as renal deterioration develops. The hypertension is associated with very low renin concentrations and is directly proportional to the level of salt intake (Kleinknecht *et al.*, 1995). Because of these differences, extrapolations of data from one model to the other are hazardous and a closer examination of the experimental evidence directed towards the proper model of RRM hypertension is warranted here.

b. RAS in ligation method of RRM

The evidence for the involvement of the RAS in the ligation method of RRM is extensive. There is an increased tissue renin content, renin mRNA and renin synthesis in the ligated kidney (Correa-Rotter *et al.*, 1992). In the systemic vasculature it has been shown that there is increased tissue RAS activity (Kuczera *et al.*, 1990). ACEI have been widely reported to slow progression of hypertension and renal deterioration in the ligation method (*e.g.* Anderson *et al.*, 1985; Brunner *et al.*, 1989). This is in spite of studies demonstrating that activity of the circulating RAS is not elevated (Smith *et al.*, 1992). Since inhibition of AngII production is the main action of ACEI, it is likely that a

reduction in AngII activity is responsible for the beneficial effects of ACEI (*e.g.* Lafayette *et al.*, 1992; Pelayo *et al.*, 1990). This suggests that in RRM either local tissue formation of AngII is enhanced or there is an increased responsiveness to circulating AngII.

ACEI have also been reported to reverse established hypertension in this model (Meyer *et al.*, 1987; Brunner *et al.*, 1989). Reversal of established hypertension more closely resembles the clinical setting of CRF in human patients where diagnosis and treatment usually occur well after development of significant renal disease. Both CCB (Dworkin *et al.*, 1993) and ACEI (Katsumata *et al.*, 1990) have been shown to attenuate glomerular hypertrophy and thereby reduce glomerulosclerosis in RRM but many investigators have argued that ACEI posses unique beneficial effects on ameliorating functional and structural damage to the glomerulus (Jackson *et al.*, 1988; Brunner *et al.*, 1989; Tolins *et al.*, 1990). It seems clear from the literature that ACEI may have therapeutic advantages in RRM attributable to mechanisms independent of systemic blood pressure reduction.

Pharmacological studies using AT_1RA's have shown that systemic blockade of AngII receptors lowers BP and limits glomerular injury in RRM (Lafayette *et al.*, 1992; Pollock *et al.*, 1993). These newly developed antagonists have recently been compared to ACEI in RRM. In a study by Lafayette, losartan lowered BP and protected the kidney from further deterioration in RRM but not to any greater extent than that observed with enalapril treatment (Lafayette *et al.*, 1992). When the investigators combined losartan with enalapril they observed no additional benefit over single administration of either drug. These studies support the idea that reducing AngII activity exerts beneficial

antihypertensive and renoprotective effects in RRM whether it is accomplished by AngII receptor blockade or by inhibition of AngII formation.

c. RAS in excision method of RRM

Evidence supporting the involvement of the RAS in hypertension and renal failure in the excision method of RRM is less convincing. It must be kept in mind that RAS activity in the excision method of RRM is determined in part by the level of salt intake. The inverse relationship between sodium intake and the activity level of the RAS has been reported by Ylitalo and coworkers (Ylitalo *et al.*, 1976). They proposed that excess extracellular levels of sodium observed in RRM exert a negative feedback on the production of angiotensinogen and renin. In RRM rats sodium restriction stimulated the RAS, and excess sodium suppressed it. They observed a decrease in plasma AngII concentration and kidney renin concentration in RRM rats placed on an elevated daily sodium intake of 10-15 mEq. Yet, it has been demonstrated that the development of RRM hypertension in rats on a increased salt intake progresses at a faster rate than in rats on normal or low salt intakes (Douglas *et al.*, 1964). The literature is sparse on the effects of blocking the RAS in RRM under conditions of elevated salt intake. Terzi and coworkers reported that inhibition of AngII formation by enalapril did not affect BP in RRM rats fed a high salt diet (Terzi *et al.*, 1992). Yet Kanagy *et al* in 1993 showed that the AT_1 RA, losartan completely prevented hypertension development in RRM rats on high salt intake (HS). Since CRF patients typically consume elevated amounts of salt, at least until the disease is diagnosed, it seems necessary to evaluate further the role of the RAS in the maintenance of elevated BP in RRM during HS intakes. I specifically intend to

investigate the role of the RAS in reversal of established RRM hypertension when rats are kept on HS.

In RRM rats (excision model) on a normal salt intake (NS), recent studies have shown that prophylactic administration of ACEI prevents the development of hypertension and inhibits the decline in renal function (Ashab *et al.*, 1995; Amann *et al.*, 1993). Reports investigating the reversal of these parameters in established RRM rats maintained on NS are non-existent. Once again, this leads to a gap in our knowledge of the involvement of the RAS in CRF and needs to be investigated further.

Very few reports detail the effects on BP and the RAS of lowering salt intake in RRM. An early report by Ylitalo and colleagues showed that when RRM rats were maintained on a low salt intake (LS), elevations in BP were prevented (Ylitalo *et al.*, 1976). They reported that plasma AngII and kidney renin concentrations were considerably elevated from RRM rats maintained on NS. Others have confirmed Ylitalo's results and recently Terzi tested ACEI in RRM rats under conditions of moderate sodium restriction (Terzi *et al.*, 1992). These rats exhibited lesser degrees of renal damage than groups on HS and did not become as hypertensive. Enalapril treatment decreased BP below normotensive levels in these moderately sodium restricted rats. The protective effects of salt restriction do not appear to be unique to the excision method nor to this model of hypertension. Salt restriction has been shown to prevent glomerular injury in RRM rats prepared using the ligation method and displaying established renal disease (Dworkin *et al.*, 1996; Lax *et al.*, 1992). In these studies, BP still increased when on LS but not to the extent that was observed in RRM rats on NS. In other models of hypertension such as SHR, sodium restriction lowered BP by 15% (Ely *et al.*, 1990). Salt

restriction alone appears to be an excellent therapeutic tool for patients with CRF but care should be taken when extrapolating studies in experimental animals to humans. There are risks associated with dietary sodium restriction and some abnormalities have been observed in experimental animals. These include: an abnormal sensitivity to blood loss, attenuated responses to stress situations, cardiac structural compensations, compensatory increases in SNA as well as enhanced activity of the RAS (Ely *et al.*, 1990).

These studies suggest the involvement of the RAS in the development of RRM hypertension, yet it seems likely that the contribution of the RAS to control of arterial pressure in RRM rats varies with salt intake and the method of RRM. Much of the previous work has looked at inhibiting or lowering the activity of the RAS early on in the progression of CRF. Most of my experimental approach is directed towards determining the contribution of AngII to RRM hypertension under varying conditions of salt intake when the disease is well established.

IV. Endothelin System

A. Background

The endothelins are a family of 21 amino acid peptides consisting of 3 isoforms called endothelin-1, endothelin-2, and endothelin-3. These isoforms are produced in a variety of cell types and are found throughout the body in many tissues. There are specific patterns of isoform expression in individual tissues. Most of the studies have focused on endothelial cell ET-1 because it appears to be the most widely distributed isoform and is most often implicated in cardiovascular control. The lung and the kidney have been shown to be the predominant sites of ET-1 production (Rubanyi and Polokoff, 1994). The production of ET-1 is clearly linked to regulation of transcription of ET

mRNA. A variety of stimuli have been shown to increase message levels for ET including growth factors and cytokines such as thrombin (Emori *et al.*, 1992), TGFβ (Kurihara *et al.*, 1989), and insulin (Hu *et al.*, 1993). Vasoactive substances *i.e.* AngII (Dohi *et al.*, 1992), AVP (Imai *et al.*, 1992), and bradykinin (Marsden *et al.*, 1991) have also been reported to increase mRNA expression in endothelial cells. ET synthesis begins as prepropeptides which are cleaved by a protease into inactive intermediates called big ET-1,-2, and -3. Big ET is activated *via* cleavage by a specific endopeptidase called endothelin-converting enzyme (ECE), forming the biologically active ET-1,-2, and -3 (Opgenorth *et al.*, 1992). In terms of biological activity, ET-1 has been demonstrated to be a 140 fold more potent vasoconstrictor than big ET-1 and the prepropeptide is devoid of vasoconstrictor action (Code *et al.*, 1990). Membrane metalloendopeptidase I (*a.k.a.* neutral endopeptidase and enkephalinase) has been shown to efficiently cleave mature ET-1, rendering the peptide biologically inactive (Sokloovsky *et al.*, 1990). The plasma half-life of ET-1 injected i.v. in the rat is about 60 seconds with the lungs removing approximately 90% of the bolus (Rubanyi and Polokoff, 1994). ET is subject to a high degree of plasma protein binding (> 98%) and serum albumin may act as a "pseudo-receptor" to bind ET and ET receptor antagonists (Wu-Wong *et al.*, 1996)

Of the 3 isoforms, ET-1 is considered to mediate most of the physiologically important cardiovascular effects. Vascular endothelial cells produce only ET-1, and they appear to be the most abundant source of ET-1 *in vivo* (Yanigasawa *et al.*, 1994). Yet, ETs are ubiquitous peptides and their receptors are distributed in almost all tissues (Koseki *et al.*, 1989). These receptors all have 7 transmembrane domains and are coupled to G-proteins. Their locations are tissue specific and their physiologic actions are quite

diverse. ET acts on two pharmacologically and molecularly distinct receptor subtypes identified as ET_A and ET_B receptors. The affinity of the 3 isoforms of ET differs for the ET receptor subtypes. At the ET_A receptor subtype the affinity is as follows: ET-1 > ET-2 > ET-3. At the ET_B receptor subtype each isoform has an equal binding affinity (ET-1 = ET-2 = ET-3). The general consensus, until recently, was that ET_A receptors mediate direct vasoconstrictor actions and ET_B receptors produce vasodilator effects via the release of nitric oxide and cyclooxygenase products. But Seo *et al.*, in 1994 reported that both ET_A and ET_B receptors are involved in vasoconstriction in human blood vessels. Vasoconstrictive ET_B receptors were located on vascular smooth muscle cells. Many others have confirmed the existence of two ET_B receptor subtypes in various animal species. The current classification of ET_B receptor subtypes is in the process of modification where endothelial cell receptors producing vasodilation will putatively be designated ET_{B1} and vascular smooth muscle cell receptors producing vasoconstriction will be designated ET_{B2}. Cardiovascular responses to exogenously administered ET-1 may be difficult to interpret because the relative contribution of the ET_A and ET_B subtypes varies depending on the vascular bed and species studied.

B. Physiological actions of endothelin

1. Hemodynamic actions

ET administered intravenously produces a rapid and transient vasodilation, followed by a profound and long-lasting vasoconstriction (Yanagasawa *et al.*, 1988). It is thought that the long-lasting pressor response to exogenous ET-1 administration is not due to the peptide's prolonged presence in the plasma, but rather to slow dissociation from the receptors. ET-1 is the most potent vasoconstrictor of isolated blood vessels

identified to date (Rubayani and Polokoff, 1994). This vasoconstriction leads to increases in systemic BP which are due to increased total peripheral resistance (Rubayani and Polokoff, 1994). In addition to ET's direct vasoconstrictor effects, the peptide can potentiate the contractile responses to other vasoconstrictors such as norepinephrine and serotonin (Tabuchi *et al.*, 1989). Other potential ways in which ET may act as a regulator of vessel reactivity and vascular tone have not yet been clearly defined.

2. Heart

ET-1 has direct actions on the heart that include positive inotropic and chronotropic effects in addition to prolongation of action potential duration (Rubayani and Polokoff, 1994). The coronary circulation is particularly sensitive to the vasoconstrictor effects of ET (Kurihara *et al.*, 1989). The involvement of ET in the physiological and pathophysiological mechanisms of heart failure is an ongoing area of much research.

3. Central nervous system

ET's are considered to be neuropeptides because they are: localized in the brain, bind specifically in some brain tissues, and i.c.v. injections of ET have been shown to significantly change cardiovascular, respiratory, and neuroendocrine system function (Rubayani and Polokoff, 1994). These i.c.v. injections produce profound vasoconstrictor and pressor responses (Siren and Feurerstein, 1989). Additionally, centrally administered ET-1 activates SNA and AVP release (Matsumura *et al.*, 1991). Even though activation of the baroreflex stimulates ET-1 release into the plasma, i.v. ET-1 does not affect baroreceptor reflex control of heart rate (Knuepfer *et al.*, 1989).

4. Endocrine systems

ET can interact with a variety of hormones at both the level of biosynthesis and the site of biological action.

a. Renin angiotensin system

Much evidence exists for the existence of an interaction between the renin angiotensin and ET systems. ET stimulates aldosterone secretion through direct actions on the zona glomerulosa of the adrenal gland (Rubayani and Polokoff, 1994). Some controversy exists as to whether ET stimulates or suppresses renin secretion. Most studies *in vitro* have found that ET suppresses renin secretion from the kidney (reviewed by Rubanyi and Polokoff, 1994). Generally, *in vivo* studies are in agreement with *in vitro* work and demonstrate that ET administered i.v. or intrarenally suppresses or does not change PRA in dogs, rats or humans. ET has been shown to stimulate AngII production *in vitro* and act synergistically with many of the biological actions of AngII *in vivo*. ET stimulates ACE activity and dose-dependently increases the conversion of AngI to AngII in cultured pulmonary artery endothelial cells (Kawaguchi *et al.*, 1990). Previous work in our lab has demonstrated that ET-1 induced hypertension produced by continuously administered i.v. ET-1 (5.0 pmol/kg/min) could be prevented by co-infusion of captopril (Mortensen and Fink, 1992). Yet captopril administration after ET-1 induced hypertension was established did not produce an antihypertensive result. The mechanisms of the interactions between the renin angiotensin and ET systems are still being elucidated and will require additional investigation.

b. Arginine vasopressin

It is generally considered that ET and AVP act synergistically as vasoconstrictors and ET-1 potentiates the vasoconstrictor action of AVP (Rubayani and Polokoff, 1994). ET given i.v. and i.c.v. has been shown to stimulate AVP release from the neurohypophysis, increase circulating AVP plasma levels, and elevate BP (Nakamoto *et al.*, 1991; Yamamoto *et al.*, 1991).

c. Atrial natriuretic peptide

In general there exists a functional antagonism between ET and ANP in most biological systems. ANP is a vasodilator that causes natriuresis thereby reducing plasma volume and osmolarity. ET is a potent vasoconstrictor that decreases sodium excretion. ET stimulates the release of ANP from atrial myocytes which results in elevations in circulating plasma levels (Rubayani and Polokoff, 1994). Additionally, ANP has been demonstrated to reduce ET production in cell culture (Hu *et al.*, 1992). In a comprehensive study in human patients, ET-1 effectively antagonized the cardiovascular, renal and endocrine actions of ANP (Ota *et al.*, 1992).

5. Kidney

Many cell types within the kidney are known to produce ET and they can act in both a paracrine and an autocrine fashion on different ET receptors. High affinity binding sites for ET are found throughout the kidney although the renal medulla has been reported to contain the greatest density of receptors (Kohan *et al.*, 1996). The vasculature of the kidney seems particularly sensitive to the physiologic effects of endogenous ET.

a. Renal hemodynamics

Systemic and intrarenal infusion of ET increases RVR and decreases RBF resulting from constriction of both afferent and efferent arterioles (Badr *et al.*, 1989). This vasoconstriction is often preceded by a transient renal vasodilation like that observed in the systemic effect of ET, but vasodilatory prostaglandins in addition to NO have been implicated in attenuation of the vasoconstrictor effect of ET in the kidney (Chou *et al.*, 1990).

b. Glomerular function

ET reduces GFR and causes contraction of mesangial cells leading to reductions in K_f and filtration surface area (Badr *et al.*, 1989; Ferrario *et al.*, 1989). ET is also a potent mitogen and activates many cellular signaling pathways in mesangial cells (Simonson *et al.*, 1989). These properties of ET lead to diminished excretory capacity of the kidney.

c. Tubular function

Intravenous infusions of ET decrease sodium excretion partly by decreasing filtered load and partly by increasing aldosterone secretion (Goetz *et al.*, 1988). The decreased diuresis observed in response to ET is thought to be due indirectly to a reduction in RBF and GFR.

C. Inhibition of endothelin system

The development of inhibitors of ET synthesis, ET receptor agonists and ET receptor antagonists (ETRA) has provided pharmacological tools for the identification of multiple receptor subtypes and the physiological responses following receptor activation.

1. Receptor agonists

ET-1 is considered a non-selective agonist for all ET receptor subtypes, but this isoform has greater affinity than the other isoforms at the ET_A receptor. There are no known selective ET_A agonists, but there are several ET_B agonists such as: sarafotoxin 6c (STX 6c), sarafotoxin 6b (STX 6b), and IRL 1620. Most of these agonists have been shown to elicit NO or prostacyclin release from endothelial cells, but may also cause VSMC contraction in some instances.

2. Endothelin converting enzyme inhibitors

Endothelin converting enzyme (ECE) inhibitors have only recently been investigated in disease states where endothelin is thought to play a role. The first ECE inhibitor was phosphoramidon which has been shown to be an effective inhibitor of ET-1 production (Sawamura *et al.*, 1990). In many vascular preparations, inhibition of ET mediated responses by blocking the enzymatic activity of ECE have been demonstrated with phosphoramidon (Fukuroda *et al.*, 1990). Phosphoramidon and similar drugs may not be ideal therapeutic tools because decreased ET production would be expected to result in both decreased ET_A and ET_B receptor activation. The discovery of selective, potent peptide and non-peptide receptor antagonists, however, has facilitated the search for ET involvement in normal functions and disease states.

3. Endothelin receptor antagonists

Selective antagonists exhibiting high affinities (K_d = nM-pM) for the two ET receptor subtypes are currently available.. Two of the most commonly used ET_A receptor antagonists (ET_A RA) are BQ-123 and FR139317. These antagonists have approximately 1000 fold selectivity for ET_A vs ET_B receptors in rat preparations and are thought to bind

to the ET_A receptor subtype very tightly (Doherty *et al.*, 1993). Recently developed non-peptide orally active ET_A RA (*i.e.* PD155080, PD156707, Ro 46-2005) are now being investigated in animals models of disease.

Endothelin subtype B receptor antagonists (ET_BRA) such as BQ-788 and RES 701-1 have been used in some experiments recently, but the results are difficult to interpret because these blockers bind to both endothelial ET_{B1} receptors and VSMC ET_{B2} receptors. The only reported endothelial ET_{B1} receptor antagonist is RES701-1, but recent comparisons of this compound in various animal species suggest that RES701-1 is much less selective for ET_{B1} receptors in rats than in other species (Tanaka *et al.*, 1995).

Additionally, non-selective peptide (*i.e.* PD145065) and non-peptide (*i.e.* bosentan, SB209670, BMS182874) endothelin receptor antagonists (ET_A/ET_BRA) have been developed. Some investigators have theorized that blockade of both receptor subtypes might result in additional benefits over blockade of ET_A receptors alone, and comparisons of efficacy of these selective and non-selective antagonists are currently being evaluated.

All of these antagonists are classified as being competitive and reversible, but their tight binding and long receptor occupation simulates the appearance of a non-competitive irreversible binding situation.

D. Endothelin and hypertension

1. Background

Since its discovery in 1988 by Yanigasawa *et al.*, ET-1's potent vasoconstrictor effects and long duration of action have led many investigators to study its role in hypertension. ET has been postulated to be involved in the pathogenesis of hypertension

because many of its biological actions lead to elevations in peripheral vascular resistance. ET may influence the short-term regulation of BP by direct vasoconstriction, which has been reported in rats (McMurdo *et al.*, 1993) and humans (Sorensen *et al.*, 1994). ET may also act through vascular remodeling as a result of smooth muscle proliferation to influence the long-term regulation of BP (Ohlstein *et al.*, 1992).

ET involvement in mild to moderate hypertension is controversial, but its role in malignant hypertension is supported by convincing evidence. Plasma ET levels are not elevated in most forms of experimental hypertension (Rubayani and Polokoff, 1994). In contrast, when severe or malignant hypertension is accompanied by end-organ damage (*i.e.* arteriosclerosis, renal failure), circulating ET concentrations are consistently elevated (Yokokawa *et al.*, 1991; Luscher *et al.*, 1990).

There may be an increased responsiveness of VSMC to the vasoconstrictor actions of ET in hypertension (Miyauchi *et al.*, 1989; Dohi and Luscher, 1991), but this phenomenon has not been demonstrated unequivocally (Winquist *et al.*, 1989; Dohi and Luscher, 1991). Therefore, this concept requires more investigation. In contrast to the inconclusive results on increased responsiveness to ET, subpressor concentrations of ET have been shown to potentiate the vasoconstriction induced by other agonists in many hypertension models (Tabuchi *et al.*, 1989; Dohi and Luscher, 1991).

ET may activate specific areas of the CNS that can result in increases in sympathetic tone or enhanced release of vasoconstrictor hormones such as AVP or norepinephrine (Vanhoutte, 1993). Both acute and chronic i.c.v. administrations of ET have been reported to elevate arterial pressure in a dose-dependent manner (Ouchi *et al.*, 1989; Nishimura *et al.*, 1991). Additionally, ET injected into the dorsolateral

periaqueductal gray area (PAG) increased BP (D'Amico *et al.*, 1995). These investigators also demonstrated that stimulation of the pressor neurons in the PAG by ET produced an increase in sympathetic tone. Work utilizing ETRA in the CNS has just begun, and much more effort will be needed to characterize the central role of ET in cardiovascular regulation.

As previously mentioned, ET has profound renal effects (*i.e.* decreased RBF and GFR) at concentrations that do not alter systemic hemodynamics. These effects may play a crucial role in pressure-volume regulation and may be extremely important in the development of hypertension. Tomobe and co-workers have shown an increased reactivity to ET in renal arteries from SHR (Tomobe *et al.*, 1988). Slight alterations in pressor responsiveness in the renal vasculature can have profound effects on long term regulation of systemic BP.

2. Endothelin and sodium intake

The influence of sodium intake on the physiologic and pathophysiologic functions of ET are uncertain. Clozel in 1993 demonstrated an increased antihypertensive effect using the ET_A/ET_BRA, Ro 46-2005, in normotensive sodium-depleted squirrel monkeys compared to sodium-replete conditions. In contrast to these results, some investigators have shown that ETRA are more effective in lowering BP in animal models of salt-dependent hypertension than in other types of experimental hypertension (Schiffrin *et al.*, 1995: Doucet *et al.*, 1996). There have been no reports comparing the effects of ETRA treatment on the progression of hypertension and renal deterioration in RRM animals under varying sodium intakes. Since sodium balance plays an integral part in the

development and progression of renal failure in RRM, my experiments were designed to characterize the relationship between ET and sodium intake in this model.

3. ET-1 induced hypertension

Yanagisawa's original work with ET showed that bolus injections of the peptide had long-lasting and potent vasoconstrictor effects (Yanagisawa *et al.*, 1988). Since then it has repeatedly been demonstrated that chronic i.v infusions of ET cause large and sustained increases in BP (Mortensen and Fink, 1991; Yasujima *et al.*, 1991, Wilkins *et al.*, 1993). The pressor effect has been shown to increase in a dose-dependent manner and cease within hours upon cessation of the infusion.

4. Endothelin involvement in experimental forms of hypertension

a. Spontaneously hypertensive rat

ETRA's have only recently been administered in experimental hypertension. Some groups have demonstrated an antihypertensive effect in SHR due to systemic ET_A receptor blockade both acutely over minutes to hours (Douglas *et al.*, 1994; Ohlstein *et al.*, 1993) and chronically over several days (Bird *et al.*, 1995). But studies utilizing blockade of ET formation or blockade of both ET_A and ET_B receptor subtypes have produced conflicting results. Early work by McMahon and colleagues reported that the ECE inhibitor, phosphoramidon, lowered BP in SHR to a greater extent then ET_A receptor blockade with BQ-123 (McMahon *et al.*, 1993). Blocking ET formation would be expected to cause decreased binding of ET to ET_A and ET_B receptor subtypes thereby theoretically lessening ET's influence on VSMC vasoconstrictor actions and on endothelium induced vasorelaxation. Much work by Schiffrin and co-workers with bosentan (ET_A/ET_BRA) has suggested that ET does not play a role in the maintenance of

hypertension or in the vascular hypertrophy in SHR (Li and Schiffrin, 1995). They treated hypertensive SHR for 4 weeks with bosentan and did not observe any changes in BP. The variable antihypertensive efficacy of ET blockade in these studies may be due to differences in the degree of ET_A vs. ET_B subtype blockade. The influence of each receptor subtype on systemic hemodynamics is still being defined and the involvement of ET in SHR is still controversial and will require more investigation.

b. Goldblatt hypertension

In the 2K1C model of Goldblatt hypertension, chronic endothelin receptor antagonism has not been demonstrated to exert antihypertensive effects. Both selective ET_A (Bazil *et al.*, 1992) and non-selective ET_A/ET_B receptor blockade (Li *et al.*, 1996; Schricker *et al.*, 1995) were not associated with hypotensive responses in 2K1C, so ET does not appear to play a major role in this so-called renin-dependent model of hypertension.

Another type of Goldblatt hypertension is the one-kidney, one-clip (1K1C) model which involves clipping of one renal artery and contralateral nephrectomy. The contralateral nephrectomy drastically alters the development of hypertension in 1K1C relative to that of 2K1C. 1K1C hypertension is generally more severe than in 2K1C, and is associated with suppressed RAS activity. This variant of Goldblatt hypertension is thought to resemble the RRM model in that there exists a volume expansion along with expansion of exchangeable body sodium (McAreavey *et al.*, 1984). Schiffrin and colleagues have reported an increased vascular and cardiac ET gene expression in 1K1C rats 2-4 weeks after application of the clip (Sventek *et al.*, 1996). When these investigators administered bosentan to 1K1C rats during this time interval, no

antihypertensive effect was observed (Li *et al.*, 1996). They concluded that even in the presence of increases in ET-1 gene expression, an ET component of BP elevation is not evident in renovascular hypertension in rats.

c. Mineralocorticoid

Excess secretion of mineralocorticoids (*e.g.* aldosterone, deoxycorticosterone) causes an antinatriuresis and kaliuresis which leads to increases in BP (Kenyon and Morton, 1994). In experimental mineralocorticoid-induced hypertension, deoxycorticosterone-acetate (DOCA) is usually administered subcutaneously and accompanied by unilateral nephrectomy and a high-sodium diet. This model of hypertension is associated with suppression of renin activity due to sodium retention (Kenyon and Morton, 1994). Much evidence now exists for the involvement of ET in the DOCA-salt model of hypertension. Suzuki *et al.*, in 1990 reported that vascular ET reactivity was increased in DOCA-salt hypertensive rats. More evidence comes from Schiffrin and colleagues who demonstrated that ET gene expression is enhanced in blood vessels from DOCA-salt rats (Schiffrin *et al.*, 1996). In this experiment, vascular expression of ET was not enhanced in rats treated with DOCA or salt alone, even when BP rose to hypertensive levels. Acute blockade of endothelin receptors with the ET_ARA, BQ-123 and FR139317, decrease BP in DOCA-salt rats over a period of minutes to hours (Okada *et al.*, 1994; Fujita *et al.*,1995). Chronic blockade of endothelin receptors with bosentan (ET_A/ET_BRA) has been shown to partially attenuate the progressive rise in BP observed in DOCA-salt rats (Li *et al.*, 1994). The conclusion from these experiments was that ET activity is increased in DOCA-salt and ET played a role in the maintenance of BP in this experimental form of hypertension.

5. Endothelin involvement in human hypertension

ET was the cause of hypertension associated with an endothelin secreting malignant hemangioendothelioma in humans (Yokokawa *et al.*, 1991). In two reported cases, changes in plasma ET concentrations correlated directly with changes in BP. Other preliminary investigations have reported that single doses of bosentan administered to hypertensive patients produce significant reductions in diastolic BP that are maintained for 24 hours (Warner *et al.*, 1996).

E. Endothelin and chronic renal failure

1. Endothelin activity in chronic renal failure

Conflicting evidence is found in the literature for the involvement of ET in renal disease in humans. It must be kept in mind that the causes of kidney injury are unknown in most CRF cases because the disease is usually not detected until significant renal damage has occurred. In humans with CRF, urinary excretion of ET-1 has been reported to be both elevated (Ohta *et al.*, 1991; Roccatello *et al.*, 1994) and decreased (Saito *et al.*, 1991). Additionally, plasma ET has been reported to be both elevated (Saito *et al.*, 1991; Koyama *et al.*, 1989) and unchanged (Brooks *et al.*, 1991: Totsune *et al.*, 1989). Since pharmacological inhibition or blockade of ET effects have just recently become available, there is a scarcity of information characterizing the role of ET in human cases of CRF and we must currently rely on animals models to advance our understanding of this disease.

2. Endothelin activity in RRM model

In the RRM model in rats, many investigators have found an increase in plasma ET levels (Orisio *et al.*, 1993). Yet other investigators have demonstrated plasma ET levels in RRM rats are the same or even numerically lower than in sham rats (Benigni *et*

al., 1991). The current consensus is that plasma ET concentrations in RRM rats are usually measured to be within the range found in normal rats unless malignant hypertension or end-organ disease is present. Systemic plasma concentrations of ET may correlate poorly with ET-induced pressor effects, however, because: ET secreted from endothelial cells acts mainly on closely apposed vascular smooth muscle cells; circulating ET is rapidly and efficiently cleared by the lungs ($t^{1/2}$ = 1 min); and ET_B receptor stimulation often leads to formation of physiological antagonists such as nitric oxide and prostacyclin (Yanagisawa *et al.*, 1994). Also, most experiments reporting ET levels represent the family of ET's, and not exclusively the biologically active ET-1.

Since there is an abundance of ET synthesized in the kidney and both receptor subtypes are present, work investigating the pathophysiological role of ET in the kidney is of great interest. As mentioned before, ET is a potent mitogen and it promotes growth of mesangial cells. This proliferation can lead to decreases in: filtration coefficient, GFR and functioning of the kidney. Some investigators have proposed that inflammatory diseases of the glomerulus, regardless of the original insult, are associated with activation of ET synthesis (Luscher and Wenzel, 1995).

a. Endothelin activity in the ligation method

RRM rats prepared by the ligation method exhibit an increased renal ET gene expression. This correlates with renal synthesis of ET, measured as increased ET excretion in urine. Expression becomes greater as renal failure progresses (Brooks *et al.*, 1991; Orisio *et al.*, 1993). Urinary excretion of ET appears to be a good marker of renal deterioration in contrast to plasma ET levels, which do not change over the course of the disease (Benigni *et al.*, 1991). Since the development of ETRA's in the last few years,

reports defining ET's involvement in models of RRM hypertension have begun to appear in the literature. Benigni *et al.*, in 1994 reported that FR139317 (ET_ARA) reduced proteinuria, attenuated the progression of hypertension, and slowed renal deterioration in RRM rats. They administered FR139317 once daily by i.p. injection at a dose of 32 mg/kg for 53 days and recorded BP by tail plethysmography. Of particular interest was that Benigni did not demonstrate reversal of hypertension or renal deterioration with the ET_ARA in this study. In 1996, Benigni and coworkers reported that administering bosentan to RRM rats also slows renal deterioration, attenuates progression of hypertension, and reduces proteinuria. They administered bosentan once daily by gavage at a dose of 100 mg/kg for 120 days and recorded BP by tail plethysmography. As in the previous study utilizing FR139317, bosentan did not reverse the hypertension or renal deterioration in these ligated RRM rats but only attenuated their progression. Benigni's report of beneficial results using bosentan in RRM are a little surprising because one would expect that blocking endothelial ET_B receptor mediated release of NO should oppose blockade of ET_A receptor mediated vasoconstriction. These investigators did find that there is up-regulation of the ET_B receptor gene and increased mRNA levels in RRM as the disease progresses. Whether this is a counterregulatory mechanism in response to vasoconstriction or involved in the maintenance of that vasoconstriction is currently being investigated. Not all reports have demonstrated beneficial effects when administering ETRA's in RRM. One group of investigators gave A-127722 to RRM rats and found that prophylactic administration of this ET_ARA immediately following completion of the partial nephrectomy by the ligation method did not prevent hypertension development nor retard renal deterioration (Polakowski and Pollock, 1996).

b. ET activity in the excision method

To date there has been no studies investigating the role of ET in the RRM model utilizing the excision method. Since the excision method of RRM is the appropriate model of CRF, investigations utilizing ETRA's in this model could provide information that is clinical relevant.

From these studies it seems clear that further investigation is needed to understand the roles that each ET receptor subtype plays in renal disease and to determine if optimization of drug therapy can lead to the ultimate goal of reversal of hypertension and renal deterioration in RRM. The second major part of my experimental approach was directed towards determining the contribution of ET to RRM hypertension and renal deterioration, particularly in the excision method and when the disease is well established.

V. Blood Pressure Regulation

The major purpose of my research is to study the mechanism(s) of hypertension associated with CRF (using the RRM excision model). It is important therefore to review current understanding of how BP levels are established. The mechanisms by which BP is regulated in the short-term are not identical to the mechanisms involved in long-term BP control. Rapid alterations in arterial pressure are generally achieved through adjustment of vascular resistance by local tissue mechanisms (myogenic and humoral), and reflexly regulated neural and hormonal systems. These vasoconstrictor effects increase BP over relatively short periods of time, *i.e.* seconds to minutes, in response to a variety of external and internal stimuli. Long-term control mechanisms serve to establish a relatively stable "setpoint" for BP over time periods of weeks to months. The major

means by which this type of regulation is achieved are believed to be: adjustment of body fluid volumes by the kidney; and the development of vascular wall thickening due to smooth muscle proliferation or remodeling. Neural and hormonal signals also might contribute to long-term BP regulation, either through modulating renal function or vascular structure, or by directly affecting cardiac or vascular function.

Hypertension may result from a disorder of short-term or long-term BP control mechanisms. The implication for my work is that a complete evaluation of the possible causes of hypertension in CRF requires investigation of physiological systems involved in both short-term and long-term BP regulation. This will be achieved by examining the effects of acute and chronic therapy with specific pharmacological inhibitors of the renin-angiotensin and endothelin systems.

VI. Specific aims

The overall purpose of my thesis work was to understand the role of hormonal factors in the hypertension that is commonly associated with CRF. My general hypothesis was that hormonal factors, specifically, AngII and ET-1, contribute to the maintenance of hypertension in RRM rats, and that their relative importance differs depending on the dietary intake of salt. I tested the following specific hypotheses in conscious, chronically instrumented rats:

(1) If the RAS exerts short-term control of BP in hypertensive RRM rats, acute ACEI treatment will lower blood pressure over minutes to hours.

(2) If the RAS exerts long-term control of BP in hypertensive RRM rats, chronic ACEI treatment will lower BP over days to weeks.

(3) ACEI lower BP in hypertensive RRM rats only by decreasing the concentration of AngII in the blood.

(4) The level of salt intake influences the antihypertensive actions of ACEI in RRM.

(5) If ET-1 exerts short-term control of BP in hypertensive RRM rats, acute ETRA treatment will lower BP over minutes to hours.

(6) If ET-1 exerts long-term control of BP in hypertensive RRM rats, chronic ETRA treatment will lower BP over days to weeks.

(7) The level of salt intake influences the antihypertensive actions of ETRA in RRM hypertension.

METHODS

I. Animals

Male Sprague-Dawley rats (Sasco-King Animal Laboratories, Madison, WI) weighing between 200-250g were used in all experiments. The rats were housed in clear plastic boxes with woodchip bedding in a climate controlled room with a 12 hour light-dark cycle. Rats were housed two to a box and allowed unlimited access to standard rodent chow (Rodent Laboratory Chow #5001, Purina, St. Louis, MO) and tap water while awaiting surgery.

II. Surgical procedures

A. General

Surgical anesthesia was performed with pentobarbital sodium 45-50 mg/kg i.p. (Nembutal®, Abbott Laboratories, Chicago, IL). Atropine sulfate 0.2mg/kg i.p. (Sigma Chemical Co., St Louis, MO) was also administered prior to all surgical procedures. Methohexital sodium 5-10 mg/kg i.v. (Brevital®, Eli Lilly, Indianapolis, IN) was used for supplemental anesthesia during catheterization. Surgical instruments were sterilized by autoclaving at 200 ^{0}C for 40 minutes. Aseptic procedure was followed throughout all surgical procedures and loss of body temperature was prevented by operating while the rats were on a heating pad. Post-operatively, a 0.5mg/kg subcutaneous dose of butorphanol tartrate (Stadol®, Bristol Laboratories, Princeton, NJ) was given as an analgesic.

B. Reduction of renal mass

A 5/6 reduction of renal mass was accomplished by a two-stage subtotal nephrectomy. The first stage of the procedure involved the surgical excision of approximately 2/3 of the left kidney mass. Under anesthesia, the left flank was shaved and washed with an iodine antiseptic cleanser (Betadine®, Purdue Frederick Co., Norwalk, CT). A lateral incision was made, the kidney was exteriorized, and the renal artery and vein were isolated. The vessels were briefly occluded with a bulldog clamp. While the vessels were occluded, the two poles of the left kidney are surgically excised with a scalpel. The exposed surfaces of the remnant kidney were cauterized and lightly covered with sterile thrombin for topical use (Thrombostat®, Parke-Davis Co., Ann Arbor, MI) to prevent excess bleeding. Total ischemia lasted less than two minutes. After bleeding had completely stopped, the kidney was replaced into the abdominal cavity. Muscle and skin were individually sutured and a prophylactic dose of ticarcillin sodium 40mg/kg s.c. (Ticar®, Smith Kline-Beecham, Pittsburgh, PA) was given. The rats were allowed 7 days for surgical recovery and nephron adaptation before the second stage of the nephrectomy was started. Using the same anesthetic method, the right flank was shaved as described previously and the right kidney was exteriorized. This time, the renal artery and vein were ligated and the right kidney was removed. Antibiotics and analgesia were given as previously described. Kidney weights from both surgeries were recorded to calculate the reduction in renal mass. The sum of the two stages of surgical nephrectomy resulted in approximately a 5/6 reduction of kidney mass with a functional remnant kidney left intact. The rats were housed in clear plastic boxes as described previously while awaiting catheterization.

C. Arterial and venous catheterization

Catheters were constructed of polyvinyl chloride (Tygon®, Microbore) with silicone rubber tubing (Silastic®, Dow Corning, Midland, MI) attached to the intravascular end. Catheters were advanced through the internal iliac artery and vein to the abdominal aorta and vena cava. The catheters were tunneled s.c. along the back and exteriorized at the head. The catheters were fed through a stainless steel spring which was then anchored to the skull using jeweler's screws and dental acrylic. The arterial catheter was filled with a heparinized sucrose solution and occluded when not in use. The venous catheter was attached through a hydraulic pivoting swivel to a 5 ml syringe. This syringe was filled with a NaCl solution which was infused continuously at a rate of 5ml/24hrs (2 mEq Na^+/24hrs) with a Harvard infusion pump. Post surgery, animals were placed in metabolism cages while awaiting experimentation. During the next three recovery days, 40mg/kg ticarcillin sodium i.v. was administered to each rat. This antibiotic regimen was also given as needed during the experimental protocol.

III. Chronic rat model

After catheterization, rats were individually housed in metabolism cages to allow daily monitoring of water intake, urine output and urinary electrolytes. A hydraulic swivel mounted above the cage allowed the animals freedom of movement and unlimited access to sodium-deficient rat chow (Teklad, Madison, WI) and drinking water. In rats receiving continuous i.v. drug treatment, pharmacological agents were added to the intravenous NaCl infusion on the appropriate experimental days.

IV. Hemodynamic measurements

BP was recorded daily for 15-20 minutes between 8:00 am and 12:00 pm from the arterial catheter using a pressure transducer (Model P50, Statham). The pressure signal was run through a digitizer (Stiemke, Madison, WI) and recorded on a polygraph (Model 7, Grass Instrument Corp.). HR was determined directly from the trace of the polygraph. To test the blockade of ACE when administering ACEI, a 50ng bolus of AngI was given. The magnitude of ACE inhibition by ACEI's was estimated from the pressor response to the AngI bolus. Any changes in BP were recorded for up to 30 seconds after the injection. Blockade of endothelin receptors by endothelin receptor antagonists was assessed by inhibition of the pressor and depressor responses to exogenously administered i.v. bolus of ET-1 at 0.5 nmol/kg. Inhibition of depressor responses due to ET_B receptor blockade were recorded over a period of 0-15 seconds. In contrast, changes in pressor response to ET-1 due to ET_A blockade were monitored for 15-20 minutes after the ET-1 bolus and the peak pressor response over that time frame was reported.

V. Fluid and electrolyte measurements

Voluntary water intake (WI) was measured from calibrated cylinders and total WI was calculated by adding the water drank in 24 hours and the volume of the intravenous saline infusion. Urinary output (UO) was collected over 24 hours in a calibrated cup. Water balance (WB) was calculated by subtracting UO from total WI. Urinary sodium (U_{Na^+}) was determined by sample analysis with a flame photometer (Model IL943, Instrumentation Lab.) Total urinary sodium excretion ($U_{Na}V$) was calculated by multiplying U_{Na^+} times the 24 hour UO.

VI. Salt protocols

Experiments involving ACEI and ETRA in RRM rats were designed to compare their effects on BP under differing NaCl intakes. The ACEI and ETRA were tested during 3 distinct periods that represented the extremes of salt intake. High salt (HS) rats drank isotonic saline (0.9% NaCl) starting one day following completion of the reduction in kidney mass or sham operation. Rats were allowed a two week period of time to develop hypertension and renal failure. They were then chronically instrumented with arterial and venous catheters and placed in metabolism cages. After arterial and venous catheterization, all rats were fed sodium deficient rat chow (0.002 mEq Na^+ /gm) and received an additional Na^+ intake of 2mEq/24 hours through the venous catheter. These HS rats remained on the oral and venous saline solutions for the remainder of the experiment, and this resulted in a 5-6 fold increase in daily salt intake (normal: approximately 2 mEq/24hr on standard rat chow). Normal salt (NS) rats were kept on normal rat chow and distilled drinking water for 30 days after partial renal ablation or sham operation, while awaiting catheterization. After catheterization and throughout the rest of the experiment, the rats ate sodium deficient chow and received 2 mEq/24 hr NaCl through the venous catheter. These procedures resulted in a normal salt intake of 2 mEq/24 hr in the rat. The low salt (LS) group of rats were kept on a sodium deficient chow and distilled water for 60 days following completion of the 5/6 nephrectomy or sham-operations while awaiting catheterization. After catheterization, all rats received a dextrose solution through the venous catheter replacing the normal NaCl solution given.

VII. Assays

A. Plasma assays

After the daily hemodynamic parameters were recorded, blood samples were drawn directly from the exteriorized arterial catheter into a syringe containing heparin (5 USP units/ml [32 ug/ml]). Samples were immediately spun in a refrigerated microcentrifuge to separate the plasma, which was then stored frozen at -70^{0}C until assayed. All blood samples were analyzed in the same assay to control for interassay variability.

1. Blood urea nitrogen

Blood samples of 0.7ml were collected in a 1.0ml syringe containing 0.05ml of heparin sodium. Blood urea nitrogen (BUN) was determined by a colorimetric assay using a prepared assay kit, (# 640, Sigma Chemical Co., St. Louis, MO.), involving ammonia production. A plasma sample of 10ul was assayed against an individual standard curve for each assay. Normal rat values for BUN vary from 15-22 mg/dl (Biven *et al.*, 1979).

2. Serum creatinine

Blood samples of 0.7ml were collected in a 1.0ml syringe containing 0.05ml heparin sodium. Serum creatinine (Scr) was determined by a colorimetric assay using a prepared kit (#555, from Sigma) involving the alkaline picrate method. A serum sample of 300ul was assayed against an individual standard curve for each assay. Mean normal rat serum creatinine ranges from 0.4 to 1.0 mg/dl depending on the analytical method used (Biven *et al.*, 1979).

3. Endothelin

Two ml of blood collected into an inhibitor cocktail containing EDTA (2mg/ml) and heparin (5 USP units/ml) was microcentrifuged to separate 1.0 ml of plasma on days differing from other plasma sampling. Blood cells were resuspended in normal saline and injected back i.v. into the rat. Plasma ET-1 $[ET]_p$ concentrations were determined using a solid phase ELISA kit (Parameter, R & D Systems, Minneapolis, MN, USA). ET-1 was extracted from 1 ml of plasma with 1.5 ml of extraction solvent composed of acetone:1M HCL:water (40:1:5). The mixture was centrifuged for 20 min at 3000 rpm at 4° C. The supernatant was dried down with a centrifugal evaporator, the pellet reconstituted in sample diluent and assayed. Optical density readings of unknown samples were plotted against a standard curve of synthetic ET-1 spiked rat plasma samples over a range of 1-113 pg/ml. The recovery from the extraction procedure was 36 ± 3%. The interassay variation was 8.24% and the intra-assay variation was 10.15%. Cross-reactivity with other isoforms was demonstrated to be less than 1%. Assays were performed by Edie Quemby-Brown at Parke-Davis Pharmaceuticals Research Division.

B. Urine assays

Urine samples were taken from 24 hour urine collections into calibrated cylinders. All samples were spun in a refrigerated microcentrifuge to separate any particulate matter and then stored at - 70^0 C until assayed. All urine samples were analyzed in the same assay to control for interassay variability. The urinary values reported were normalized per 100 grams body weight of each rat.

1. Urinary protein excretion

Urine samples of 100ul were collected and protein concentration was determined by a colorimetric assay using a prepared kit (#541, Sigma) involving a modified version of the Lowry method. Urinary protein excretion (Upro) was calculated by multiplying the daily urine output times the protein concentration determined from this assay. Normal rat values for total protein excretion are reported to be < 30 mg/dl (Biven *et al.*, 1979).

2. Urinary creatinine concentration

A urinary sample of 300ul was collected and assayed to determine urinary creatinine concentration (Ucr) using the same assay as used for serum creatinine from Sigma.

3. Creatinine clearance

Creatinine clearance (Ccr) was calculated by the formula UO x Ucr/ Scr.

VIII. Statistics

Data were analyzed using a mixed-design ANOVA. Post-hoc tests included: least significant difference, simple main effects, Dunnetts, t-test, Bonferroni's, and analysis of contrasts. Criterion for statistical significance was a "p-value" less than 0.05. Computer software (Crunch® Version 4) was used for statistical analysis.

EXPERIMENTAL RESULTS

I. Renin angiotensin system in reduced renal mass

A. Acute experiments

1. Bolus i.v. administration of ACEI in RRM and sham rats on high, normal, and low salt intakes.

a. Rationale

The purpose of this experiment was to determine the contribution of the RAS to short-term BP regulation in RRM rats under the three different salt extremes. Since the primary short-term mechanism of BP regulation by the RAS is through direct vasoconstriction, a process which can be initiated or terminated within seconds to a few minutes, acute BP responses were measured to i.v. bolus injections of the ACEI, enalaprilat, in RRM and sham rats. The hypothesis was, if the RAS exerts short-term control of BP in RRM rats during any of these 3 salt intakes, acute ACEI treatment should lower BP within minutes to hours.

b. Protocol

Daily control measurements were taken prior to administration of an i.v. bolus of enalaprilat at 5mg/kg in RRM and sham rats maintained on the 3 levels of salt intake. BP was recorded from 5 minutes up to 24 hours after drug administration.

c. Results

Figure 2 presents BP data from acute treatment with enalaprilat in RRM and sham rats. The top graph illustrates the stratification of BP observed in RRM rats during the different levels of NaCl intake prior to enalaprilat administration. HS rats started off with the highest BP, followed by the NS group of rats. The BP of LS rats was only mildly elevated and these values are not considered to be in the hypertensive range. All the RRM rats maintained on HS and NS had higher resting BP's than the corresponding sham groups during the control measurements. In all 3 groups of RRM rats, BP was not acutely lowered over minutes to hours following enalaprilat administration. NS rats did show a delayed antihypertensive effect at 6 and 24 hours after enalaprilat.

The bottom graph illustrates the hypotensive effect of enalaprilat in sham rats under varying salt intakes. BP was decreased at every time point in each treatment group, but only reached statistical significance in sham rats on a low salt intake, under conditions when the RAS is known to be stimulated. In normal rats, a lower dose (1 mg/kg) of enalaprilat (Figure 3) was shown to significantly inhibit the AngI pressor response for up to 5 hours.

d. Interpretations

Other investigators have demonstrated the salt sensitivity of BP in RRM rats and have observed similar differences in BP (Langston *et al.*, 1963). It was concluded from these results that AngII plays an important role in the maintenance of short-term BP regulation only in normal rats on a low salt intake. It would be expected that the RAS exerts its greatest role under conditions of salt deprivation. What was unexpected was that when RRM rats were kept in a salt depleted state, ACEI treatment did not lower BP

acutely. A possible explanation for the differing hypotensive effect in RRM vs. sham rats at all salt intakes is that RRM rats have lower circulating levels of AngII than the corresponding sham rats. AngII levels were not measured in these experiments, but it is generally agreed that the RRM model is a low renin model of hypertension. In fact, most studies have shown that plasma AngII levels in RRM rats are normal or decreased relative to that of sham rats. (Ylitalo, 1976). Another possible explanation for the lack of an antihypertensive effect in RRM is that the doses used were ineffective in inhibiting AngII formation. Data presented in Figure 3 demonstrate that even at lower doses of enalaprilat (1mg/kg), pressor responses to AngI are inhibited acutely. AngI has little pressor activity in itself and the pressor response to AngI in untreated animals is mainly due to its conversion by ACE to the vasoconstrictor AngII. These data show that the doses used were effective in inhibiting ACE over the course of my experiment, and that the negative results reported are not due to inadequate inhibition of the enzyme.

These data indicate that AngII does not exert a direct vasoconstrictor fast pressor effect in RRM rats to maintain BP. On the contrary, the maintenance of BP in normal rats maintained on a low salt intake appears to be partly dependent on the vasoconstrictor properties of AngII.

Figure 2: Acute i.v. enalaprilat administration in RRM and sham rats on high, normal, and low salt intakes. *Asterisks indicate reductions in MAP from the zero control value.

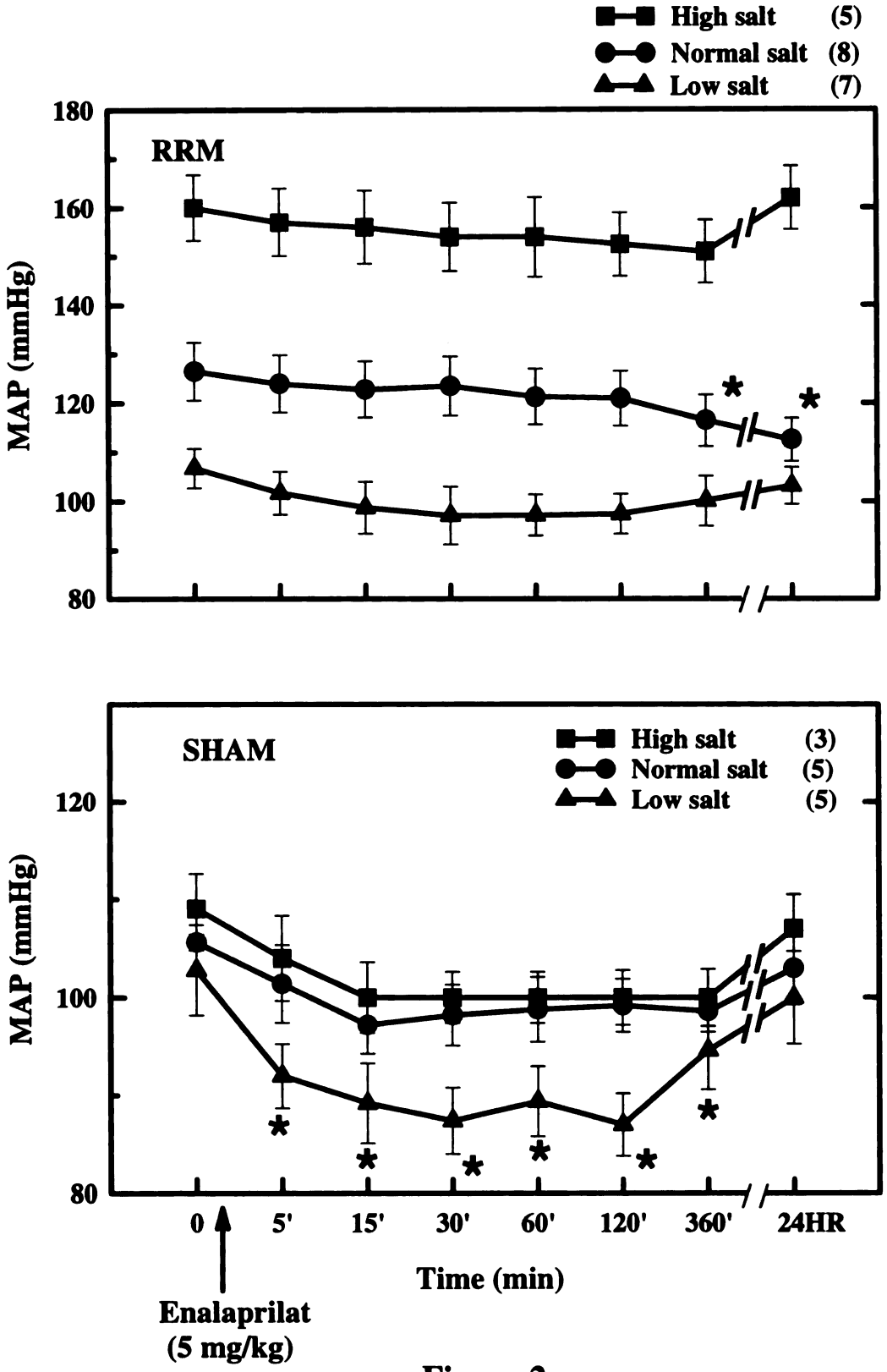

High salt (5)
Normal salt (8)
Low salt (7)
RRM
MAP (mmHg)
180
160
140
120
100
80
*
*
SHAM
High salt (3)
Normal salt (5)
Low salt (5)
MAP (mmHg)
120
100
80
*
*
*
*
*
*
0
5'
15'
30'
60'
120'
360'
24HR
Time (min)
Enalaprilat
(5 mg/kg)

Figure 2

Figure 3: AngI pressor responses in normal rats administered i.v. dextrose or enalaprilat at 1mg/kg. *Asterisks indicate reductions in pressor response from average of control values.

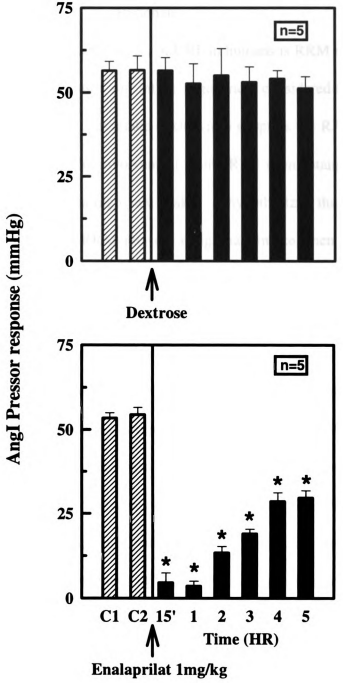

75
50
25
0
n=5
Dextrose
AngI Pressor response (mmHg)
75
50
25
0
n=5
C1 C2 15' 1 2 3 4 5
Time (HR)
Enalaprilat 1mg/kg

Figure 3

B. Chronic experiments

1. Chronic administration of ACEI in hypertensive RRM rats on a high salt intake.

a. Rationale

A clinically relevant model of CRF in humans is RRM rats placed on an elevated NaCl intake. The increased salt intake is generally considered to be detrimental to renal function, and increased salt intake is known to suppress the RAS. This experiment was designed to determine the involvement of the RAS in maintaining chronic hypertension when RRM rats are on a high salt intake. I hypothesized that, if the RAS exerts long term control of BP in RRM rats on a high salt intake, then chronic ACEI treatment should lower BP over days to weeks.

b. Protocol

All rats were subjected to RRM and drank isotonic saline starting one day following completion of the surgery. As previously described in the methods section, these HS rats were allowed a two week period of time to develop hypertension while on increased salt intake. The rats were divided into two experimental groups after catheterization. One-half the rats received enalapril at 250mg/L in the oral isotonic saline solution. The other rats drank only the isotonic saline solution, and they served as the control group. Three days of control measurements were followed by 14 days of enalapril treatment or vehicle during the HS period. Then, distilled water was substituted for the oral saline solution for the last 7 days resulting in a normal salt intake (2mEq/24 hours). Rats in the treatment group still received enalapril during NS intake. BUN

samples and AngI challenges were carried out during control, high, and normal salt experimental periods in both groups.

c. Results

Figure 4 shows BP throughout the 24 days of this experiment. During the 3 control days, BP was slightly elevated and similar in both groups of RRM rats. A progressive increase in BP was seen in both the enalapril and vehicle treated groups during HS. The magnitude of pressure change was not different between groups. Likewise, a similar fall in BP was seen in the two groups after initiation of NS. Moreover, during these last 7 days of the experiment while the rats were on NS no difference in BP between groups was recorded.

Since some investigators believe that a primary mechanism by which AngII acts on long-term BP regulation in CRF is through stimulating tubular retention of water and sodium (Bakris, and Gavras, 1993), water and sodium balance were recorded throughout the experiment. Data on WI, UO, and WB from the vehicle and enalapril treated RRM rats during conditions of HS and NS are shown in Figure 5. Because of the influence of an increased salt appetite in RRM rats, both groups had an increased WI and UO throughout the 14 days of HS administration. These measurements returned to a more normal level immediately after discontinuation of the salt addition in the drinking water.

Figure 6 presents urinary sodium excretion ($U_{Na}V$) and sodium balance (NaB) in the two groups over the course of the experiment. Both groups of RRM rats showed a significant natriuresis during the high salt administration days when compared to the normal salt days. The addition of NaCl to the drinking water resulted in an increased sodium excretion. Inhibition of AngII formation by enalapril did not affect $U_{Na}V$ or NaB

during HS or NS experimental periods. There were no sustained differences in $U_{Na}V$ or NaB between the vehicle and enalapril treated groups throughout the experiment.

Table 1 shows the pressor response to a 50ng i.v. bolus of AngI during control, high, and normal salt days in both the vehicle and enalapril treated RRM rats. The average daily dose of enalapril in this experiment was calculated to be 13.5 mg. A significant decrease in the AngI pressor response was found during enalapril treatment as compared to both the within group control value and the between group non-treated values. This indicated persistent, successful blockade of ACE in enalapril treated rats.

Table 1 also presents BUN values in vehicle and enalapril treated RRM rats during control, high, and normal salt intake periods. Both of the RRM groups had mildly elevated BUN levels throughout the protocol compared to normal values reported in our lab and by others (Kanagy, 1991; Pollock *et al.*, 1993). During NS, elevated BUN values from controls were measured in both groups.

d. Interpretations

From these blood pressure data, it was concluded that when RRM hypertension was allowed to develop in rats on a high salt intake, the RAS played no role in the long-term regulation of BP, even when the rats were subsequently allowed to ingest a more "normal" level of salt. The lack of an antihypertensive effect in enalapril treated rats was not due to inadequate blockade of ACE because pressor responses to AngI during enalapril administration were successfully blocked. These data also suggest that ACEI do not reduce BP by mechanisms other than decreasing AngII formation.

Mildly elevated BUN levels reflect a decreased GFR in both groups of RRM rats and suggests some decrease in renal function occurred. Any progression of renal

deterioration that might have occurred was not measurable during the high salt administration. BUN values have been shown in some studies to progressively increase over time in RRM rats but a two week interval may not be sufficient to clearly demonstrate such a change (Kaufman *et al.*, 1974). Conversely, during NS, clearly elevated BUN values were measured in vehicle and enalapril treated rats. The increased BUN values during the NS in both groups may be explained due to a sudden drop in BP in addition to a slowly progressive deterioration of renal function. Sudden falls in pressure can result in decreases in renal perfusion pressure with resultant decreases in excretion of urea nitrogen. One explanation for the observation that the BUN values in the enalapril treated rats were elevated when compared to those of vehicle treated rats during normal salt intake might be that a larger decrease in glomerular hydrostatic filtration pressure due to vasodilation of the efferent arteriole by enalapril may have caused a larger decline in GFR in the treated rats.

In this experiment, inhibition of AngII formation by enalapril administration did not affect WI, UO or WB. Yet, AngII is known to stimulate drinking behavior under normal conditions. This may be explained due to the low RAS activity normally observed in HS situations. Under conditions of increased sodium intake, the physiological actions of AngII on stimulating drinking behavior would be expected to be minimal. These data suggest that the influence of AngII on WB under conditions of high salt is negligible.

The NaB data agree with the WB data described above in that the influence of AngII on NaB in RRM rats under high salt conditions was negligible. It was concluded

that the RAS does not contribute to chronic water and sodium balance in RRM rats under these conditions.

Chronic experiments involving enalapril in sham rats maintained on HS were not performed because of the lack of an antihypertensive effect in RRM rats under these conditions.

Figure 4: Mean arterial pressure responses to chronic enalapril administration in RRM rats on high salt intakes.

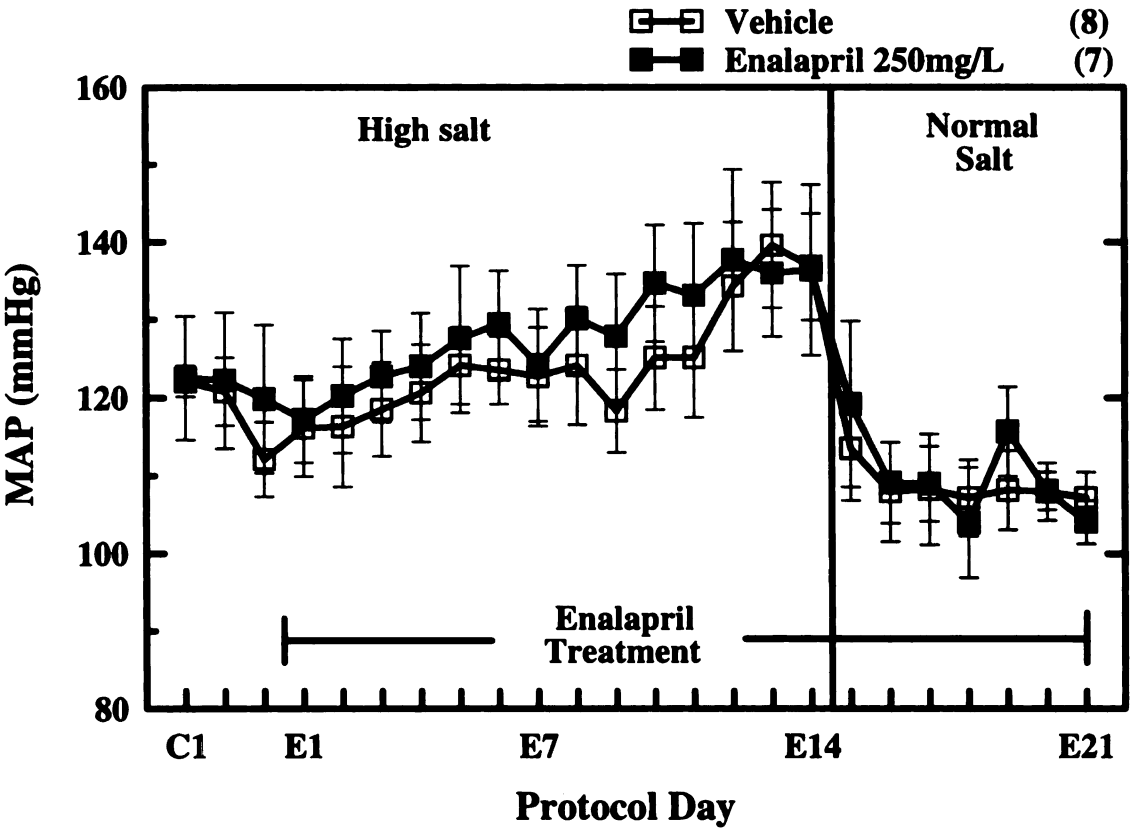

Vehicle (8)
Enalapril 250mg/L (7)
High salt
Normal Salt
160
140
120
100
80
MAP (mmHg)
Enalapril Treatment
C1
E1
E7
E14
E21
Protocol Day

Figure 4

Figure 5: Water intakes, urine outputs and water balances in response to chronic enalapril administration on high salt intakes.

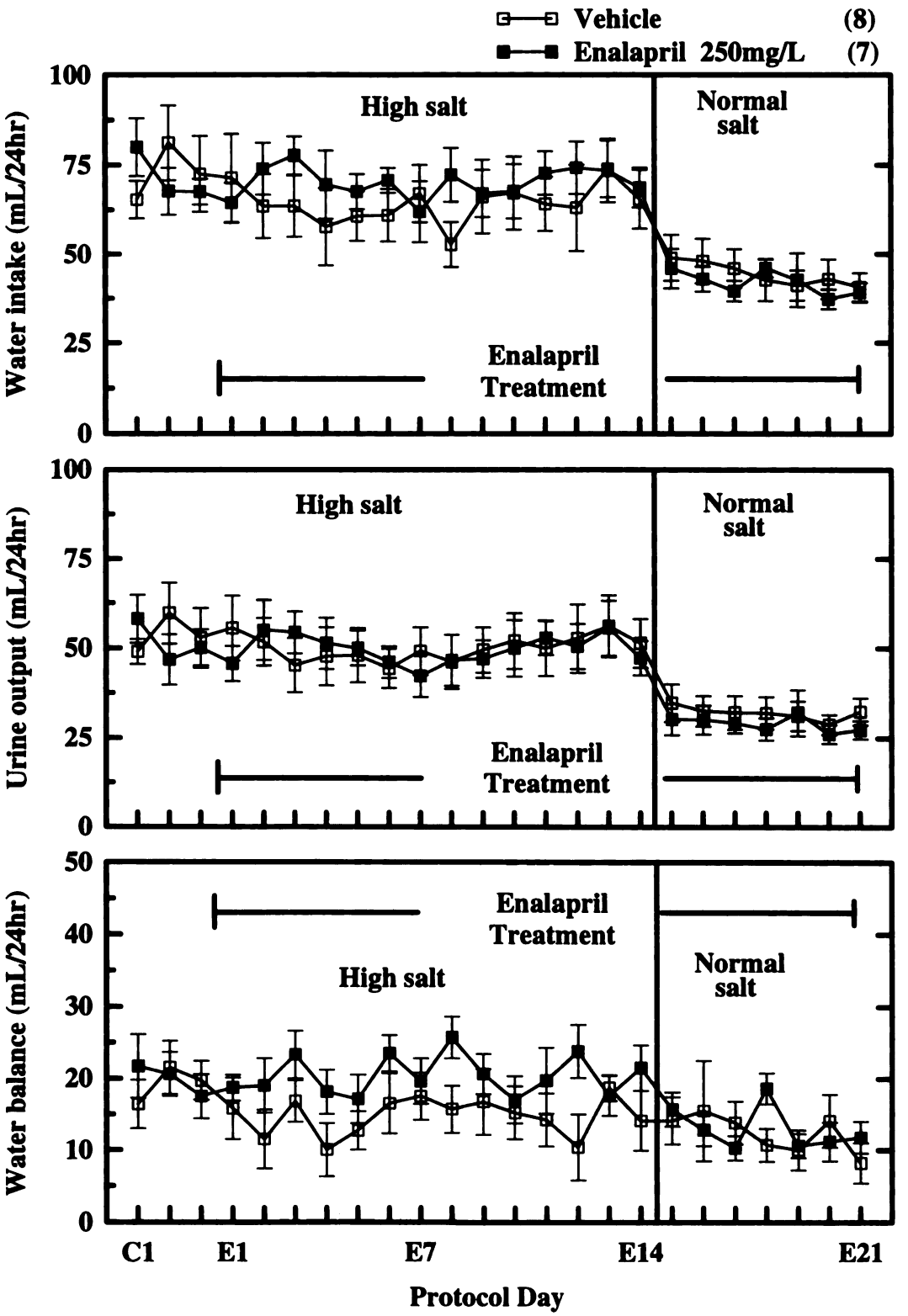

Vehicle (8)
Enalapril 250mg/L (7)
Water intake (mL/24hr)
Urine output (mL/24hr)
Water balance (mL/24hr)
High salt
Normal salt
Enalapril Treatment
C1
E1
E7
E14
E21
Protocol Day

Figure 5

Figure 6: Urinary sodium excretions and sodium balances in response to chronic enalapril administration in RRM rats on high salt intakes. All rats drank an isotonic saline solution and were administered 2 mEq/day NaCL i.v. *Asterisk indicates reduction in sodium balance from the control values.

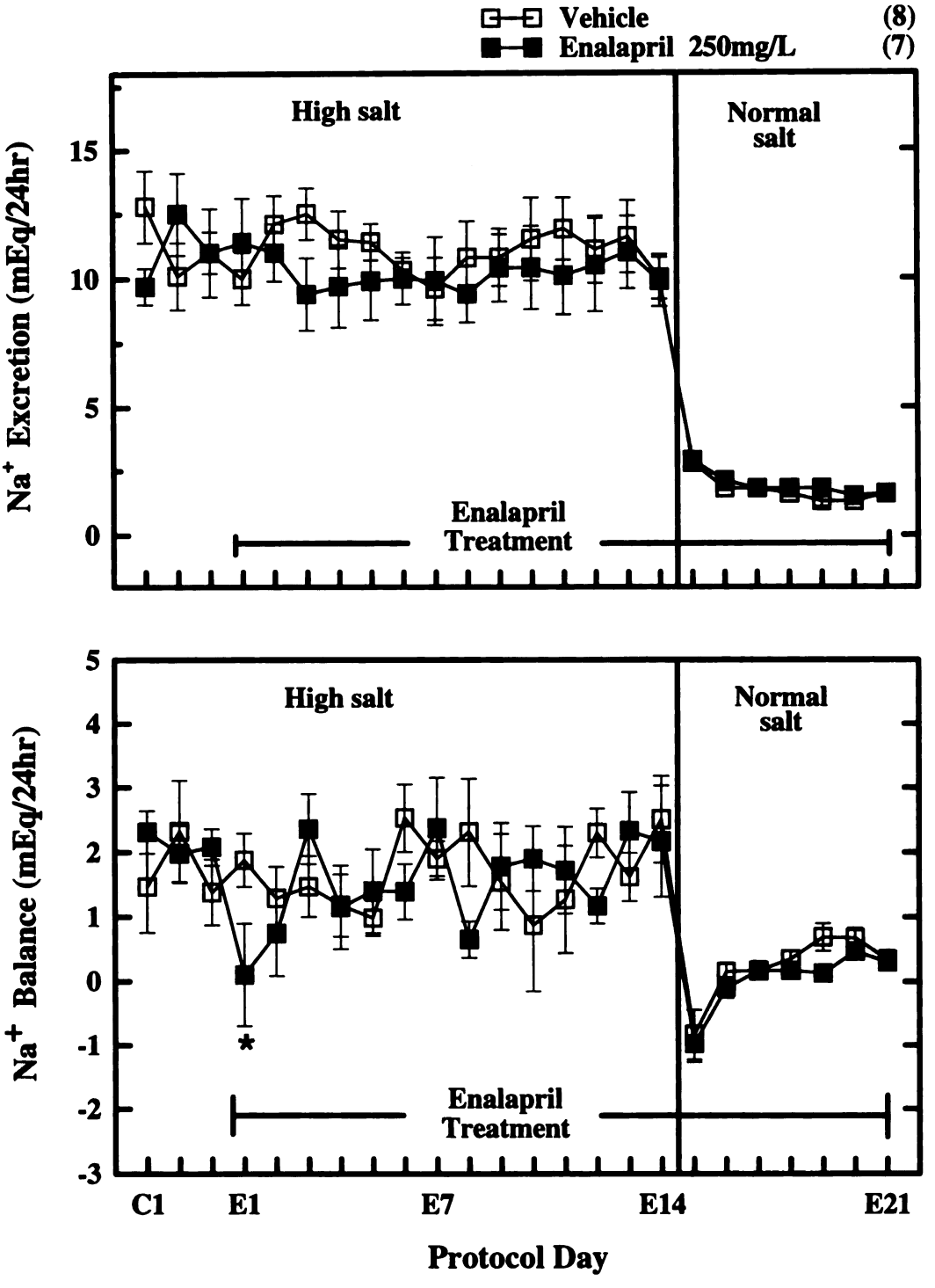

Vehicle (8)
Enalapril 250mg/L (7)
High salt
Normal salt
Na+ Excretion (mEq/24hr)
15
10
5
0
Enalapril Treatment
High salt
Normal salt
Na+ Balance (mEq/24hr)
5
4
3
2
1
0
-1
-2
-3
*
Enalapril Treatment
C1
E1
E7
E14
E21
Protocol Day

Figure 6

Table 1: AngI pressor responses, enalapril dosages and blood urea nitrogen levels in RRM rats maintained on a high salt intake. *Asterisks indicate reductions from within group control and between group vehicle values. †Daggers represent increases from within group control values.

	AngI pressor response to 50 ng iv bolus (mmHg)			Enalapril Dose (mg/day)
	Control (HS)	Treatment (HS)	Recovery (NS)	
Vehicle	34.8 ± 3.5	28.3 ± 3.3	28.0 ± 1.9	0
Enalapril 250mg/L	39.1 ± 1.2	11.2 ± 1.4*	9.2 ± 0.8*	13.5

	BUN (mg/dL)		
	Control (HS)	Treatment (HS)	Recovery (NS)
Vehicle	18.2 ± 1.7	18.2 ± 0.9	22.1 ± 1.0†
Enalapril 250mg/L	21.5 ± 1.3	20.0 ± 1.7	27.7 ± 2.1†*

2. Chronic administration of ACEI in hypertensive RRM and sham rats on a normal salt intake.

a. Rationale

Part of the work described in this thesis was aimed at characterizing how salt intake influences ACEI in RRM hypertension. Given the inverse relationship between dietary salt intake and RAS activity, it is reasonable to assume that the relative importance of the RAS in the pathophysiology of renal failure is larger under conditions of lower dietary salt intake. This issue is particularly important when considering the therapy of CRF, since dietary salt restriction is an established part of the treatment regime (Brown *et al.*, 1971). Thus, the next series of experiments were designed to determine the contribution of the RAS to long-term BP regulation in RRM rats on a lower, more normal salt intake. Based on previously published reports it was expected that ACEI treatment would lower BP in RRM rats on NS. But the mechanism by which this occurs is not fully understood. As mentioned in the introduction of this thesis, factors such as inhibition of local tissue RAS and/or kinin potentiation have been proposed to mediate most or all of ACEI effects on BP reduction. A corollary question addressed in this experiment was: do the ACEI produce antihypertensive effects in RRM rats only through the inhibition of AngII formation?

b. Protocol

The rats were maintained on the normal salt protocol as described in the methods section. After arterial and venous catheterization, RRM and sham rats were divided into four treatment groups: vehicle group, oral enalapril at 250mg/L, and in two groups of rats drinking enalapril at 250mg/L, constant i.v. infusions of 2 and 4 ng/min of AngII were

administered to restore circulating AngII concentrations. The experiment lasted 18 days total: 3 control days, followed by 1 day of i.v. ACEI treatment, followed by 7 days of oral ACEI treatment and ending with 7 recovery days with no treatment. IV ACEI treatment consisted of a 5mg/kg bolus of enalaprilat which was administered to all groups that subsequently received the oral enalapril (250mg/L). The vehicle group of rats served as control, receiving no i.v. or oral ACEI. BUN samples and AngI challenges were carried out during control, treatment and recovery periods in all groups.

c. Results

Figure 7A presents data from chronic BP recordings in RRM rats on NS. Enalapril treatment at 250mg/L in hypertensive RRM rats on NS prevented the continuing rise in BP observed in vehicle treated animals. The chronic treatment data from this experiment suggest that the RAS plays a significant role in long-term BP regulation in RRM hypertension. The known ability of these drugs to influence BP through mechanisms other than inhibition of AngII formation led me to test directly the mechanism of action of enalapril. It was hypothesized that it is only the blockade of AngII formation by enalapril that prevents the progressive rise in BP observed in the vehicle treated rats. To test this hypothesis, enalapril treated rats received continuous infusions of AngII at rates designed to restore normal circulating concentrations of the peptide. If enalapril was preventing hypertension development in RRM rats only by blocking AngII formation, this treatment should fully restore hypertension.

BP data from the groups of RRM rats drinking enalapril at 250mg/L and receiving continuous i.v. replacement AngII at 2 and 4 ng/min is shown in Figures 7B and 7C. Replacement of circulating AngII at 2 ng/min during enalapril treatment restored the

progressive rise in BP observed in the vehicle group during the treatment. Daggers indicate a significant increase in BP from the vehicle group in rats administered replacement AngII at 4 ng/min (Figure 7C). All groups are shown for comparison with no error bars, asterisks, or daggers in Figure 7D.

Figure 8 shows data from chronic BP recordings in sham rats on NS. Enalapril treatment at 250mg/L in normotensive sham rats decreased BP from the 3 control days every day during the treatment (Figure 8A). These data suggest that AngII is a necessary component of long term BP regulation in normal rats on a normal salt intake. It was tested in sham rats if the effects on BP during enalapril treatment could be reversed by continuous infusions of AngII at rates designed to restore normal circulating concentrations of the peptide. An additional group of sham rats given enalapril and administered 1ng/min AngII infusion was incorporated into the study to further characterize the level of replacement AngII needed to restore BP to levels measured in untreated sham rats. Data from 3 different AngII infusion rates in enalapril treated sham rats on NS are shown in Figures 8B-D. Rats administered enalapril and 1 ng/min i.v. AngII still had significantly reduced BP from control levels (Figure 8B). Replacement of circulating AngII at a rate of 2 ng/min i.v. during oral enalapril treatment prevented the reduction in BP recorded in the enalapril only group (Figure 8C). Additionally, this rate restored the normal BP observed in the vehicle group. When the replacement rate of AngII was increased to 4ng/min, BP was again restored to the normal BP observed in the vehicle group while not being elevated into the hypertensive range (Figure 8D). Figure 9 shows all sham groups for comparison of BP with no error bars or asterisks.

The pressor responses to 50ng bolus's of AngI were significantly inhibited in all RRM and sham rats drinking enalapril during the treatment period (Table 2). The significant decrease in AngI pressor responses found during treatment indicates the successful blockade of ACE and the suppression of endogenous AngII formation in all RRM and sham groups administered enalapril.

Table 2 also contains BUN values in sham and RRM rats during control, treatment, and recovery periods. All of the RRM groups had elevated BUN levels compared to sham values. Enalapril treatment alone or with replacement AngII did not alter BUN levels in sham or RRM rats during the treatment period.

There were no measurable differences in WI, UO, or WB in any of the RRM (Figure 10) or sham (Figure 11) rats throughout the experiment when groups were individually compared to their control days. Enalapril treatment alone or with the addition of low infusion rates of AngII, did not affect overall WB in any group of RRM or sham rats. There was an increased WI and compensatory UO in RRM rats when compared to the sham rats in each treatment group.

Figure 12 presents $U_{Na}V$ in all groups of RRM rats over the course of the experiment. The $U_{Na}V$ were generally in the 1-2 mEq/24hr range which correlated well with the i.v. saline infusion administered at a rate calibrated to deliver 2 mEq Na^+/24hr. There were no measurable differences in $U_{Na}V$ in any of the RRM rats when compared to the within group control days, except for the last 3 recovery days in the enalapril 250 mg/L + 4ng AngII group. Enalapril treatment alone or with the addition of low infusion rates of AngII did not affect natriuresis or overall NaB in any group of RRM rats.

Figure 13 shows $U_{Na}V$ in five groups of sham rats on NS. Enalapril treatment alone in sham rats was associated with a significant natriuresis that developed only after several days on enalapril administration. The natriuretic effect of enalapril was reversed by two days after enalapril discontinuation. In fact, the majority of the recovery days were associated with a significant sodium retention. There was no change in $U_{Na}V$ in the vehicle group or any of the groups drinking enalapril and receiving replacement AngII.

d. Interpretations

The data from this experiment support a role of the RAS in long-term BP regulation in RRM rats on NS. The progressive rise in BP observed in non-treated RRM rats was prevented by ACEI treatment; this effect was totally reversed by continuous i.v infusion of AngII at a rate of 2 ng/min. This low replacement rate of AngII was expected to produce systemic peptide concentrations within the physiologic range as has been demonstrated by other investigators (Hall and Brands, 1993). If enalapril was working through mechanisms other than the inhibition of AngII formation, then replacing AngII systemically should have not been able to reverse ACEI full effect on BP. Data from this experiment support the hypothesis that ACEI prevents the progression of hypertension in RRM by blockade of endogenous AngII formation and not by other putative mechanisms.

The data from this experiment also supports the role of the RAS in long-term BP regulation in sham rats on NS. Exogenous AngII replacement at a continuous rate of 1 ng/min partially attenuated the hypotensive effect of enalapril observed in untreated sham rats and 2 ng/min restored the basal level of BP. These data suggest that AngII is a necessary component of BP regulation in normal rats under NS conditions. Data from

this experiment support the hypothesis that ACEI lower BP in normal rats only by blockade of endogenous AngII formation.

One of the experimental strategies was to inhibit the endogenous production of AngII with ACEI. The significant decreases in AngI pressor responses found during the treatment period indicate successful blockade of ACE and the suppression of endogenous AngII formation in all RRM and sham groups administered enalapril. Suppression of endogenous AngII formation facilitated an accurate assessment of the involvement of the RAS through the use of intravenously administered AngII.

The elevated BUN levels in the RRM groups reflect a decreased GFR which suggests RRM groups had a significantly reduced renal function. None of the sham groups exhibited elevations in BUN from normal measurements. There was only a slight progression in BUN levels that did not reach significance over the course of this experiment in RRM receiving no treatment (37.3 $\pm$ 3.5 to 43.0 $\pm$ 3.5 mg/dl). Enalapril treatment alone or with replacement AngII did not alter BUN levels in any group of RRM rats during the treatment period, which suggests a lack of a renoprotective effect of ACEI under these conditions of salt intake and length of investigation.

Enalapril dosages were similar in each of the sham groups and generally less than in any of the RRM groups (Table 2). The increased enalapril consumption in RRM groups was due to an elevated WI. As shown from data above, all of these dosages inhibit AngII formation to a similar extent yet some evidence suggests that ACEI in dosages in excess of those required to lower BP may impart additional benefit to glomerular structure and function (Ikoma *et al*, 1991). This seemed not to be the case in

this experiment because enalapril administration at 250 mg/L did not reverse or change the progression of renal deterioration in RRM rats.

Even though when compared to sham, RRM rats had increased WI and UO, these measures offset each other so as to prevent increases in WB. One of the main results of activation of the RAS is an increased Na^+ and H_2O reabsorption from the proximal tubule (Bakris and Gavras, 1993) therefore inhibition of AngII production would be expected to result in a natriuresis and diuresis. This was not observed during treatment in RRM rats. It must be kept in mind that BP was lower in enalapril treated than untreated RRM groups. The pressure-natriuresis theory dictates that lower BP's cause decreases in sodium and water excretion. This phenomenon may have canceled out any loss of AngII stimulated tubule reabsorption during ACEI administration. Another consideration is that the activity of the RAS is low to normal in RRM and the reduction of an AngII mediated effect due to inhibition of already low plasma AngII concentrations may not exert an effect readily observable using our methods.

The natriuretic response observed in enalapril treated sham rats during the treatment period was most likely due to decreased AngII stimulated tubular reabsorption. This natriuretic effect may even have been greater if it were not for the BP lowering influence on natriuresis. The substantial hypotensive effect of enalapril suggests that activity of the RAS is significant in sham rats on NS. This explains why the natriuretic response to ACE inhibition was measurable in sham rats. The decrease in sodium excretion after withdrawal of enalapril supports the role of AngII in mediating sodium reabsorption.

Figure 7: Mean arterial pressure responses to chronic enalapril administration with and without replacement AngII in RRM rats on normal salt intakes. Panel A depicts vehicle and enalapril only treated groups. Panel B depicts vehicle and group administered enalapril + AngII replacement at 2ng/min. Panel C depicts vehicle and group administered enalapril + AngII replacement at 4ng/min. Panel D depicts all groups with no error bars or significance denotations for overall comparisons. *Asterisks indicate reductions in MAP from the vehicle group. † Daggers denote increases in MAP from the vehicle group.

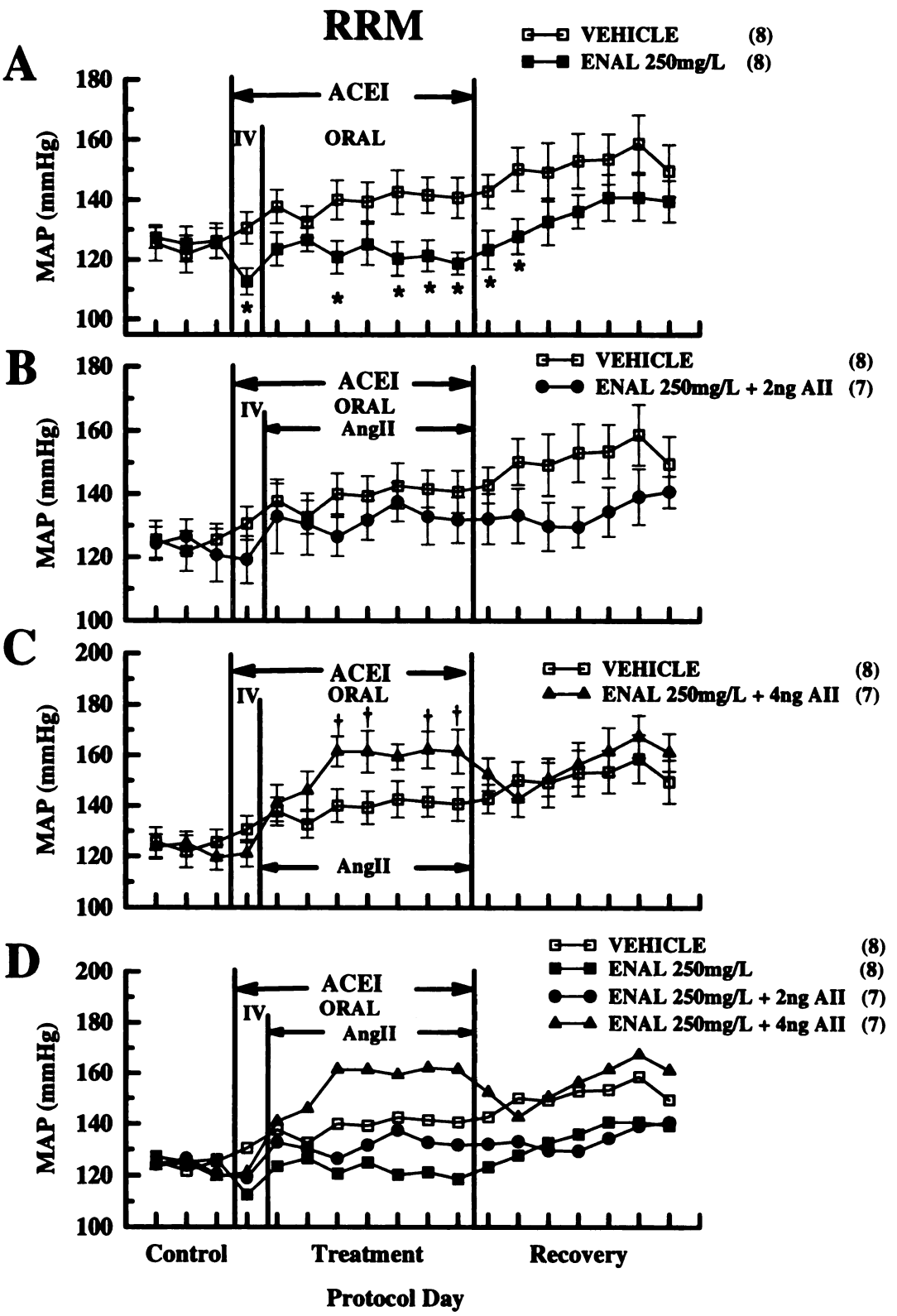

RRM
A
B
C
D
VEHICLE (8)
ENAL 250mg/L (8)
ENAL 250mg/L + 2ng AII (7)
ENAL 250mg/L + 4ng AII (7)
ACEI
IV
ORAL
AngII
MAP (mmHg)
180
160
140
120
100
200
Control
Treatment
Recovery
Protocol Day

Figure 7

Figure 8: Mean arterial pressure responses to chronic enalapril administration with and without replacement AngII in sham rats on normal salt intakes. Panel A depicts vehicle and enalapril only treated groups. Panel B depicts vehicle and group administered enalapril + AngII replacement at 1ng/min. Panel C depicts vehicle and group administered enalapril + AngII replacement at 2ng/min. Panel D depicts vehicle and group administered enalapril + AngII replacement at 4ng/min. *Asterisks indicate reductions in MAP from the vehicle group.

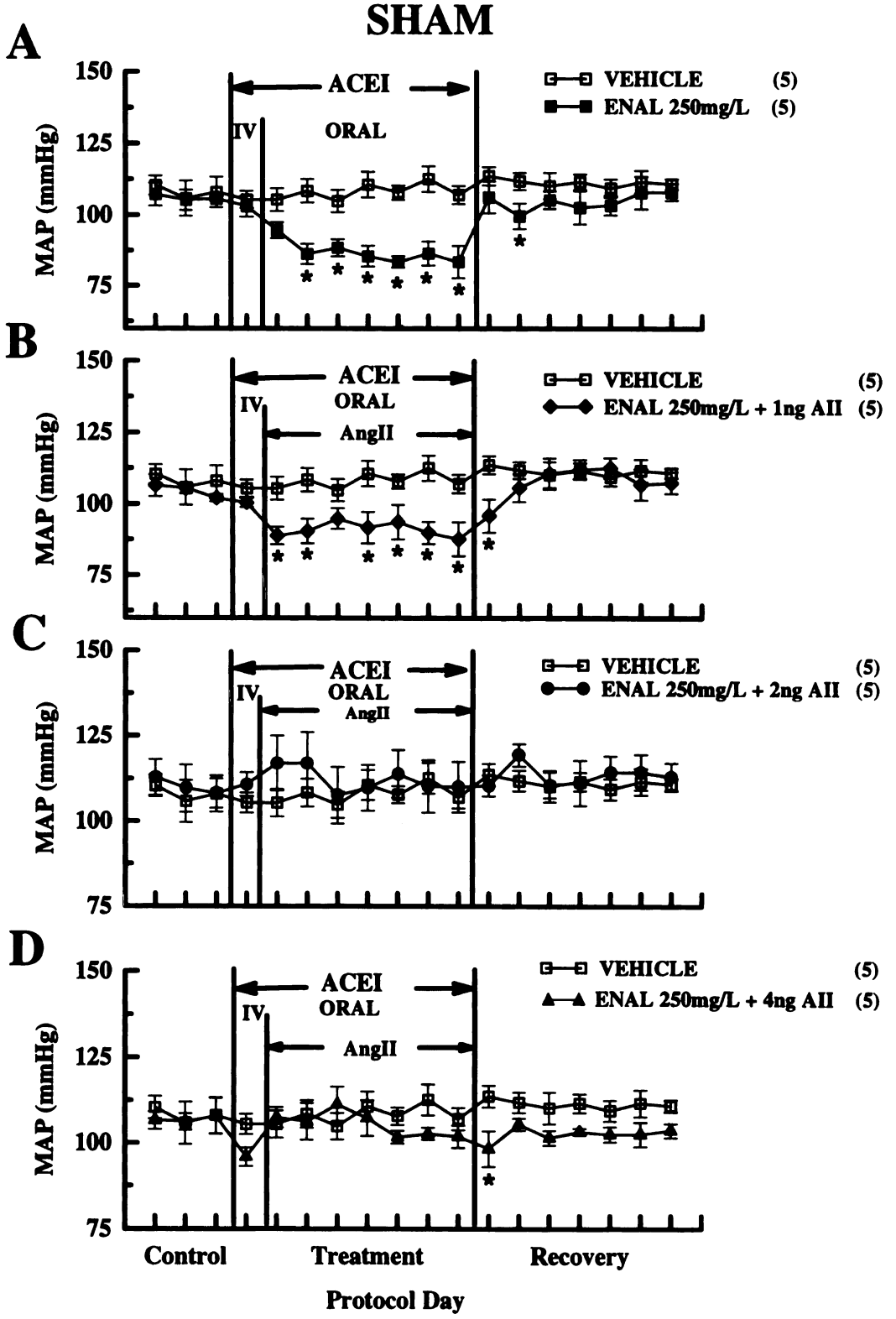

SHAM
A
B
C
D
ACEI
IV
ORAL
AngII
VEHICLE (5)
ENAL 250mg/L (5)
ENAL 250mg/L + 1ng AII (5)
ENAL 250mg/L + 2ng AII (5)
ENAL 250mg/L + 4ng AII (5)
MAP (mmHg)
150
125
100
75
Control
Treatment
Recovery
Protocol Day

Figure 8

Figure 9: Mean arterial pressure responses to chronic enalapril administration with and without replacement AngII in sham rats on normal salt intakes. This graph depicts all groups for overall comparisons with no error bars or significance denotations.

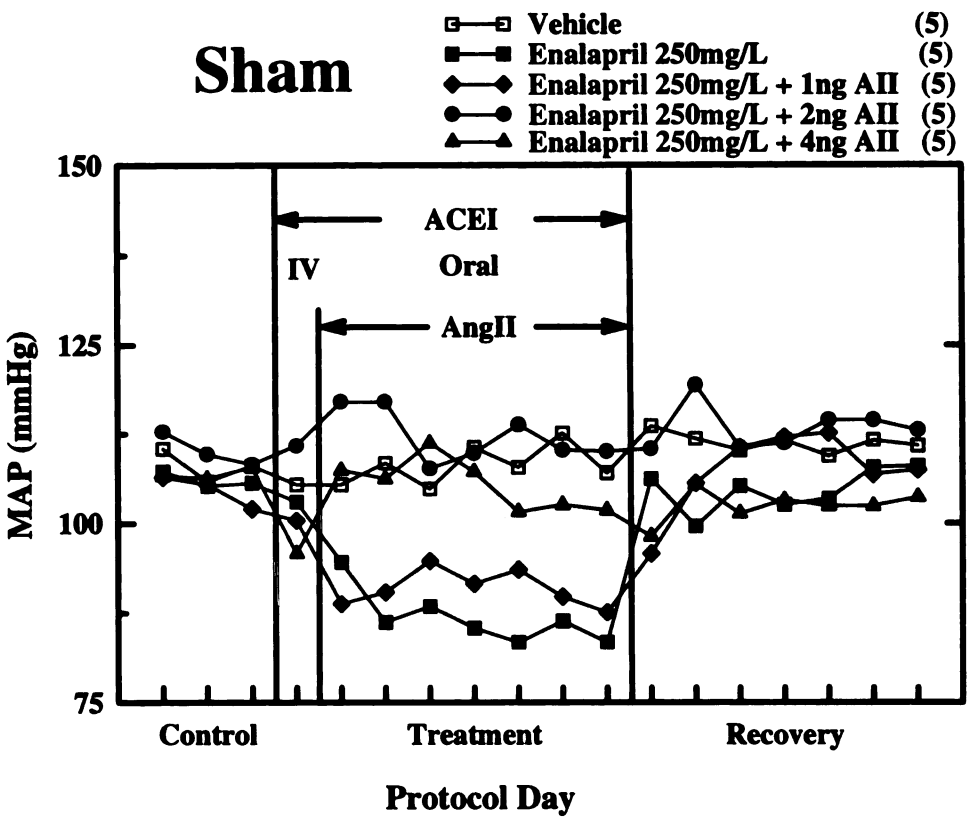

Sham
Vehicle (5)
Enalapril 250mg/L (5)
Enalapril 250mg/L + 1ng AII (5)
Enalapril 250mg/L + 2ng AII (5)
Enalapril 250mg/L + 4ng AII (5)
150
125
100
75
MAP (mmHg)
ACEI
IV
Oral
AngII
Control
Treatment
Recovery
Protocol Day

Figure 9

Table 2: AngI pressor responses, enalapril dosages and blood urea nitrogen levels in sham and RRM rats maintained on a normal salt intake. All RRM blood urea nitrogen values are significantly higher than the corresponding sham values at each time point but are not annotated for ease of comparison. *Asterisks indicate decreases in AngI pressor responses from within group control levels.

Table 2:

	AngI pressor response to 50 ng iv bolus (mmHg)			Enalapril Dose (mg/day)
	Control	Treatment	Recovery	
SHAM				
Vehicle	37.2 ± 7.0	35.6 ± 4.7	37.6 ± 5.2	0
Enalapril 250mg/L	35.4 ± 4.4	9.8 ± 2.3*	35.2 ± 2.8	8.8
Enalapril 250mg/L + 1ng AII	30.4 ± 5.1	6.8 ± 1.3*	35.6 ± 4.8	6.1
Enalapril 250mg/L + 2ng AII	35.2 ± 4.0	7.0 ± 2.0*	33.0 ± 4.4	6.6
Enalapril 250mg/L + 4ng AII	33.0 ± 4.9	9.6 ± 2.5*	29.6 ± 4.7	6.3
RRM				
Vehicle	32.5 ± 3.9	26.1 ± 3.1	32.3 ± 3.2	0
Enalapril 250mg/L	32.7 ± 4.6	5.2 ± 1.1*	31.2 ± 4.9	14.5
Enalapril 250mg/L + 2ng AII	39.7 ± 4.6	7.8 ± 1.3*	35.2 ± 3.1	11.6
Enalapril 250mg/L + 4ng AII	37.7 ± 5.2	10.5 ± 1.3*	29.2 ± 4.9	14.3

	BUN (mg/dL)		
	Control	Treatment	Recovery
SHAM			
Vehicle	12.4 ± 2.1	10.5 ± 1.9	9.0 ± 0.9
Enalapril 250mg/L	17.4 ± 2.3	15.6 ± 2.2	15.5 ± 1.0
Enalapril 250mg/L + 1ng AII	14.3 ± 2.2	12.5 ± 1.5	12.7 ± .04
Enalapril 250mg/L + 2ng AII	19.0 ± 2.1	21.1 ± 2.6	20.5 ± 3.3
Enalapril 250mg/L + 4ng AII	14.9 ± 1.1	11.2 ± 1.1	14.3 ± 1.8
RRM			
Vehicle	37.3 ± 3.5	35.8 ± 3.0	43.0 ± 3.5
Enalapril 250mg/L	40.7 ± 1.9	40.8 ± 5.6	45.2 ± 3.7
Enalapril 250mg/L + 2ng AII	34.5 ± 5.4	28.1 ± 3.9	33.8 ± 5.3
Enalapril 250mg/L + 4ng AII	40.5 ± 4.4	35.5 ± 3.6	42.2 ± 2.8

Figure 10: Water intakes, urine outputs and water balances in response to chronic enalapril administration with and without replacement AngII in RRM rats on normal salt intakes.

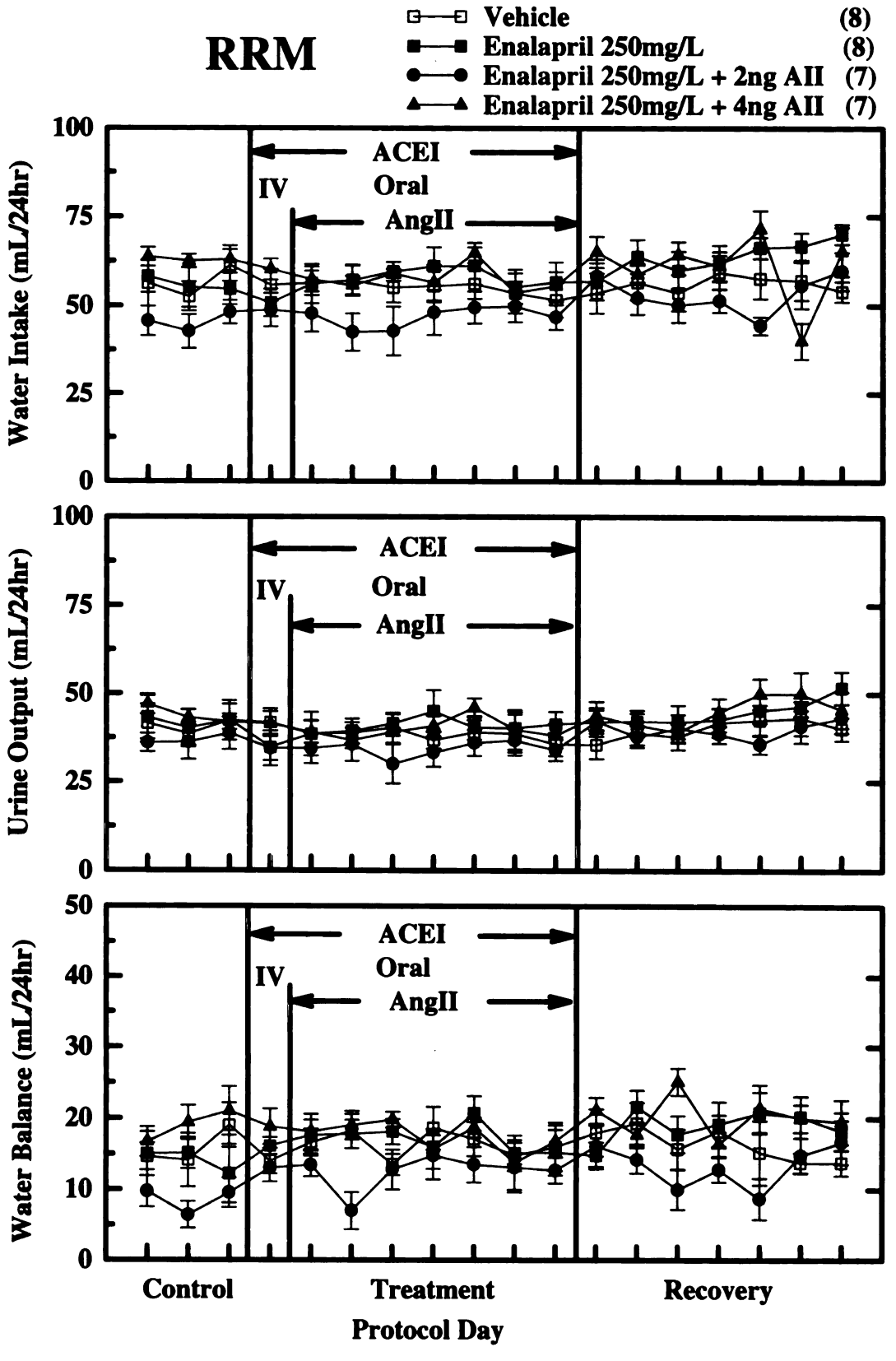

RRM
Vehicle (8)
Enalapril 250mg/L (8)
Enalapril 250mg/L + 2ng AII (7)
Enalapril 250mg/L + 4ng AII (7)
Water Intake (mL/24hr)
Urine Output (mL/24hr)
Water Balance (mL/24hr)
ACEI
Oral
IV
AngII
Control
Treatment
Recovery
Protocol Day

Figure 10

Figure 11: Water intakes, urine outputs and water balances in response to chronic enalapril administration with and without replacement AngII in sham rats on normal salt intakes. *Asterisks indicates reduction in WB from within group control values.

† Daggers denote increases in WB from within group control values.

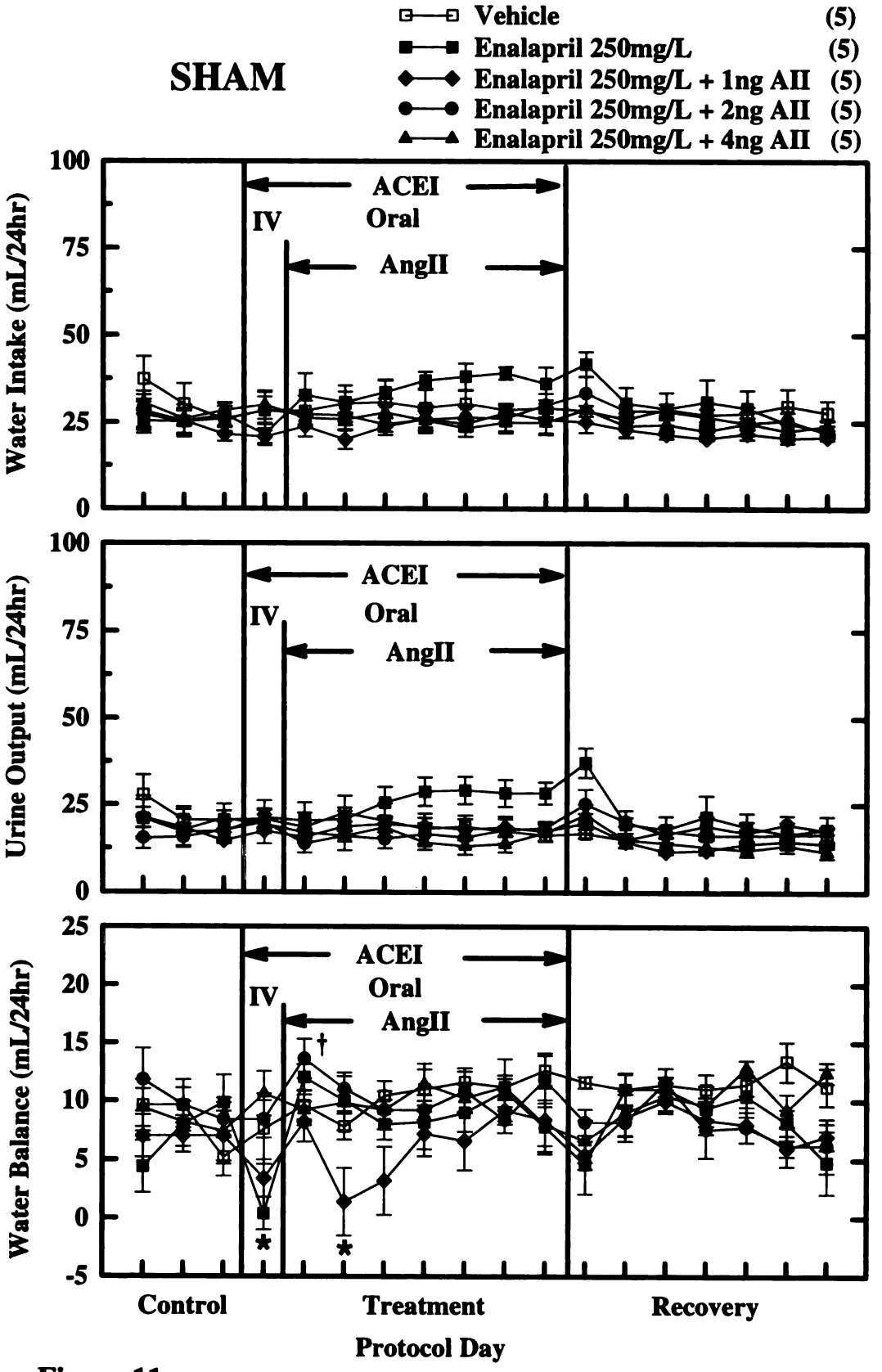

SHAM
Vehicle (5)
Enalapril 250mg/L (5)
Enalapril 250mg/L + 1ng AII (5)
Enalapril 250mg/L + 2ng AII (5)
Enalapril 250mg/L + 4ng AII (5)
Water Intake (mL/24hr)
Urine Output (mL/24hr)
Water Balance (mL/24hr)
ACEI
IV
Oral
AngII
100
75
50
25
0
25
20
15
10
5
0
-5
†
*
Control
Treatment
Recovery
Protocol Day

Figure 11

Figure 12: Urinary sodium excretions in response to chronic enalapril administration with and without replacement AngII in RRM rats on normal salt intakes. All rats were maintained on a fixed sodium intake of 2 mEq/day administered i.v. *Asterisks indicate increases in sodium excretion from within group control values.

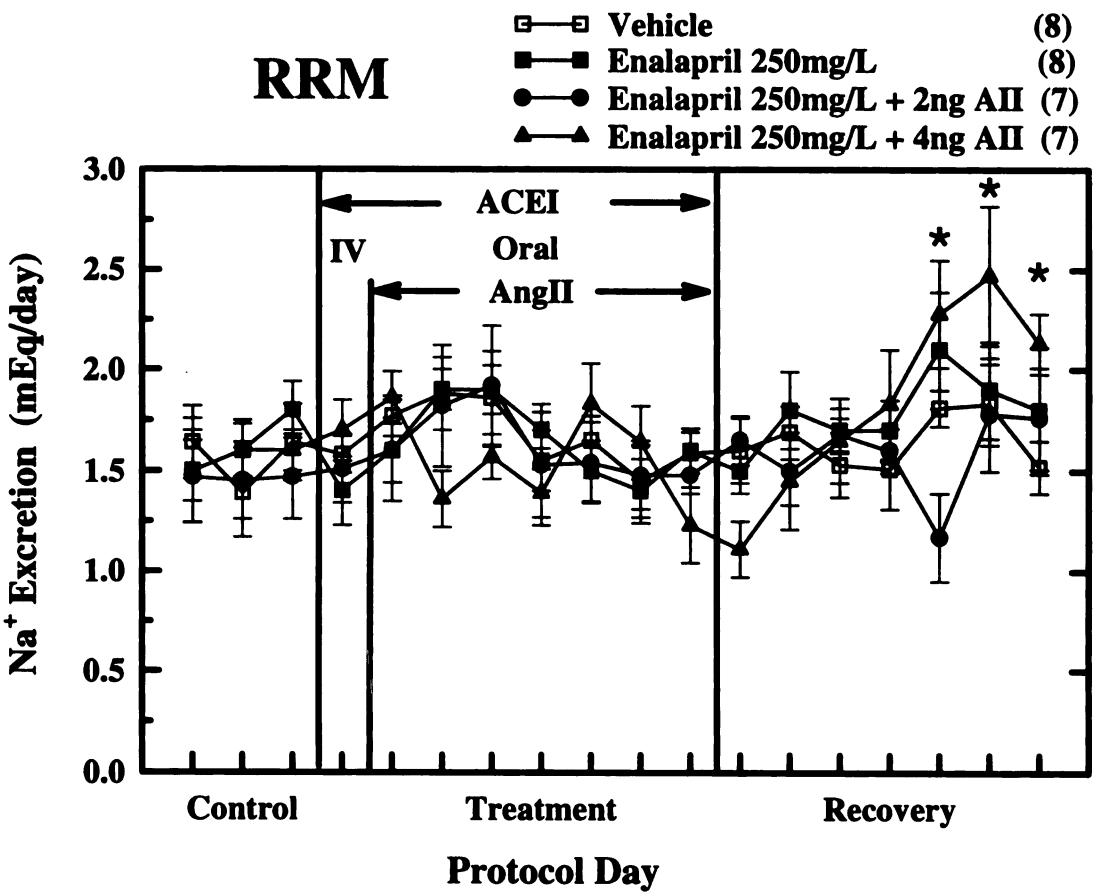

RRM
Vehicle (8)
Enalapril 250mg/L (8)
Enalapril 250mg/L + 2ng AII (7)
Enalapril 250mg/L + 4ng AII (7)
ACEI
IV
Oral
AngII
Na+ Excretion (mEq/day)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Treatment
Recovery
Protocol Day

Figure 12

Figure 13: Urinary sodium excretions in response to chronic enalapril administration with and without replacement AngII in sham rats on normal salt intakes. All rats were maintained on a fixed sodium intake of 2 mEq/day administered i.v. *Asterisks indicate reductions in sodium excretion from within group control values. † Daggers denote increases in sodium excretion from within group control values.

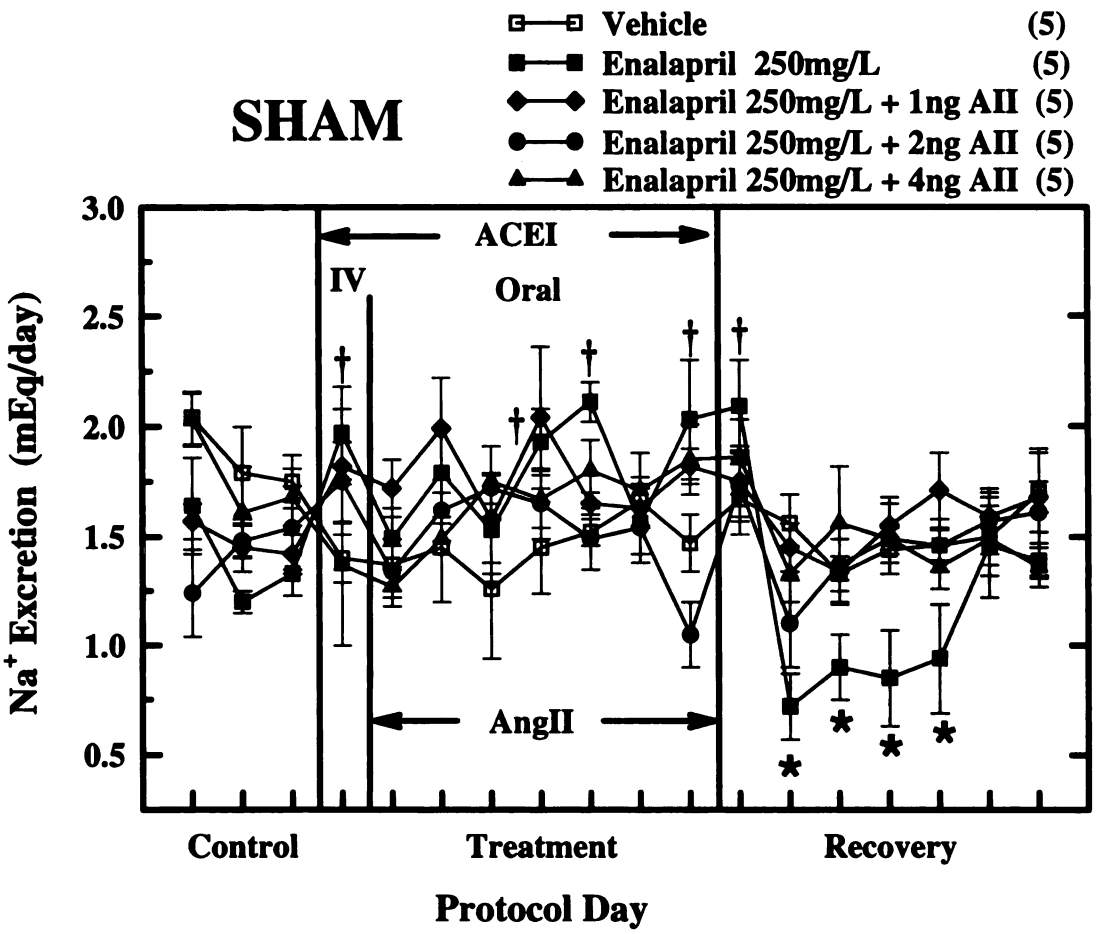

Vehicle (5)
Enalapril 250mg/L (5)
Enalapril 250mg/L + 1ng AII (5)
Enalapril 250mg/L + 2ng AII (5)
Enalapril 250mg/L + 4ng AII (5)
SHAM
ACEI
IV
Oral
AngII
Na+ Excretion (mEq/day)
3.0
2.5
2.0
1.5
1.0
0.5
Control
Treatment
Recovery
Protocol Day

Figure 13

3. Chronic administration of ACEI in RRM and sham rats on a low salt intake.

a. Rationale

Since the inverse relationship between dietary salt intake and RAS activity appears to hold true in renal failure (Ylitalo *et al.*,1976), it was hypothesized that the relative importance of the RAS in the pathophysiology of renal failure is largest under conditions of a dietary intake devoid of sodium. As mentioned earlier, this is a critical point given that dietary salt restriction is an important part of the therapy of CRF in humans. The purpose of this experiment was to confirm the importance of the RAS in long-term BP regulation in RRM rats on a low intake of NaCl.

b. Protocol

In this experimental protocol the same enalapril treatment that was used in the NS experiments was used except now rats were maintained on LS. As described in the methods section, this LS protocol involves sham and RRM rats fed a sodium deficient chow for 8 weeks prior to experimentation. After arterial and venous catheterization, RRM and sham rats were divided into four treatment groups: vehicle group, oral enalapril at 250mg/L, and in two groups of rats drinking enalapril at 250mg/L, constant i.v. infusions of 4 and 10 ng/min of AngII were administered to restore circulating AngII concentrations. The experiment lasted 18 days total: 3 control days, followed by 1 day of i.v. ACEI treatment, followed by 7 days of oral ACEI treatment and ending with 7 recovery days with no treatment. IV ACEI treatment consisted of a 5mg/kg bolus of enalaprilat which was administered to all groups that subsequently received the oral enalapril (250mg/L). The vehicle group of rats served as control, receiving no i.v. or oral

ACEI. BUN samples and AngI challenges were carried out during control, treatment and recovery periods in all groups.

c. Results

Figure 14 outlines BP data from 4 groups of RRM rats on a LS. In Figure 14A, enalapril alone significantly reduced BP from the 3 control days during the treatment period and 2-3 days after enalapril was replaced by distilled water in the recovery period. This hypotensive effect in the recovery period after discontinuation of enalapril treatment in RRM rats was not recorded in sham rats on the same experimental protocol (Figure 15A). Figures 14B and 14C demonstrate that replacement of endogenous AngII at rates of 4 and 10ng/min does not restore the basal level of BP observed in the vehicle group of RRM rats. Figure 14D depicts BP from all groups without error bars or asterisks for comparison.

Figure 15 presents BP data from 4 groups of sham rats on LS. In Figure 15A, enalapril alone significantly reduced BP from the control days during the treatment period. Figures 15B and 15C show that replacement of exogenous AngII at rates of 4 and 10ng/min does not restore the basal level of BP observed in the vehicle group. Figure 15D depicts BP from all groups without error bars or asterisks for comparison.

The pressor responses to AngI were significantly inhibited in RRM and sham groups drinking enalapril during the treatment period (Table 3). The significant decrease in AngI pressor responses found during enalapril treatment indicates successful blockade of ACE and the suppression of endogenous AngII formation in enalapril treated rats.

Data from Table 3 also shows BUN values in sham and RRM rats during control, treatment, and recovery periods. All of the RRM groups had elevated BUN levels

compared to sham values. These elevated BUN levels reflect a decreased GFR which suggests the RRM groups did have decreased renal function. Enalapril treatment alone or with replacement AngII did not alter BUN levels in sham or RRM rats during the treatment period.

Figure 16 shows WI, UO, and WB in RRM rats on LS. In general WI and UO were slightly increased in RRM rats as compared to the sham rats (Figure 17) in each group. WB was not consistently changed from control levels in the groups receiving enalapril alone or with the addition of replacement AngII. Figure 17 presents WI, UO and WB in sham rats on LS. WB was not changed from control levels in the groups receiving enalapril alone or with the addition of any infusion rate of AngII.

Figure 18 shows $U_{Na}V$ in both RRM and sham rats maintained on LS. Urinary sodium excretion was not affected by enalapril administration alone or in combination with AngII replacement in either RRM or sham rats under these sodium deplete conditions.

d. Interpretation

Urinary Na^+ excretion and BUN data confirm that experimental manipulations were successful in achieving the desired effects on salt intake and renal function. The Na^+ excretory rates in both sham and RRM rats (zero to 0.1 mEq/24h) were barely detectable and suggest that interventions aimed at restricting salt intake were successful. Additionally, BUN levels in RRM rats were all elevated after 2 months, even in the absence of hypertension, suggesting that the desired reduction in renal function by partial kidney ablation was accomplished in this experiment.

Since blockade of endogenous AngII formation by enalapril lowered BP in the normotensive rat on a sodium-restricted diet, these results suggest that AngII is required for the maintenance of basal BP under these conditions. Additionally, this experiment supports the role of the RAS in long-term BP regulation in RRM rats on LS. It is interesting that BP in RRM and sham rats was not different prior to treatment. Each group of rats was maintained on a low NaCl diet for two months following subtotal renal ablation. The original expectations were that BP would be elevated in the hypertensive range in RRM rats. A thorough literature search revealed that some investigators have shown an increased BP in RRM rats maintained on low salt intakes (Purkerson *et al.*, 1976; Lax *et al.*, 1992) but most have not (Dworkin *et al.*, 1996; Ylitalo *et al.*, 1976; Dipette *et al.*, 1983). Also unexpected was that the hypotensive effect of enalapril treatment in sham and RRM rats was similar in magnitude in both groups during the treatment period. I anticipated that enalapril would lower BP to a greater extent in sham than RRM rats based on my NS conclusions. Nonetheless, there is some reason to believe that the mechanism of the hypotensive effect of enalapril was different in sham and RRM rats. First, the fall in BP in sham rats was rapid (Figure 15A), but required many days to reach a maximum in RRM rats (Figure 14A). Second, during the recovery period RRM and sham groups exhibited a disparate pattern in restoration of basal BP: the RRM rats had a prolonged hypotensive effect after discontinuation of enalapril as compared to the sham rats. The delayed restoration of basal BP could be due to a lingering inhibition of serum ACE by enalapril. Yet AngI pressor responses were back to control levels during days 2-3 of the recovery period, suggesting that ACE activity was intact during this recovery (Table 3). Another difference between sham and RRM was the

responses to replacement of AngII at 10 ng/min during the enalapril treatment period. Sham rats exhibited less of an hypotensive effect than RRM rats in the first two days of treatment. This difference suggests that higher doses of AngII may be required in RRM rats when compared to sham rats to fully reverse enalapril's hypotensive effects. These results are consistent with the possibility that in sham rats on a very low sodium intake AngII affected basal BP via the fast pressor effect, but that in RRM rats under similar conditions it was the slow pressor effect of AngII that contributed to basal BP regulation. The failure to restore normal BP in sodium-depleted, ACEI treated rats with even a high infusion rate of AngII (10 ng/min) was not unexpected, because both the fast and slow pressor effects of AngII are significantly impaired by sodium restriction (Cowley and McCaa, 1976).

Figure 14: Mean arterial pressure responses to chronic enalapril administration with and without replacement AngII in RRM rats on low salt intakes. Panel A depicts vehicle and enalapril only treated groups. Panel B depicts vehicle and group administered enalapril + AngII replacement at 4ng/min. Panel C depicts vehicle and group administered enalapril + AngII replacement at 10ng/min. Panel D depicts all groups with no error bars or significance denotations for overall comparisons. *Asterisks indicate reductions in MAP from within group control measurements.

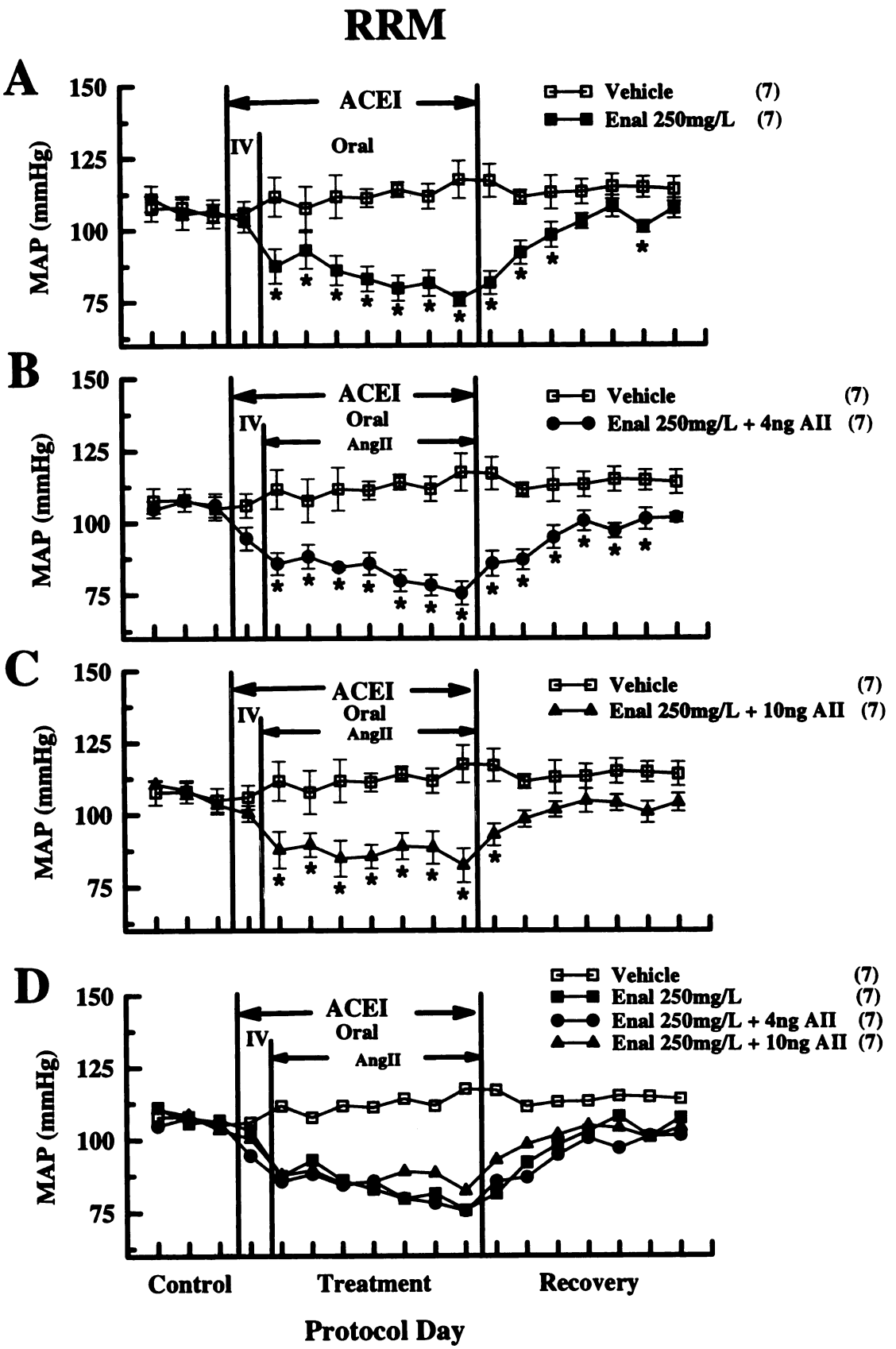

RRM
A
B
C
D
MAP (mmHg)
150
125
100
75
ACEI
IV
Oral
AngII
Vehicle (7)
Enal 250mg/L (7)
Enal 250mg/L + 4ng AII (7)
Enal 250mg/L + 10ng AII (7)
Control
Treatment
Recovery
Protocol Day

Figure 14

Figure 15: Mean arterial pressure responses to chronic enalapril administration with and without replacement AngII in sham rats on low salt intakes. Panel A depicts vehicle and enalapril only treated groups. Panel B depicts vehicle and group administered enalapril + AngII replacement at 4ng/min. Panel C depicts vehicle and group administered enalapril + AngII replacement at 10ng/min. Panel D depicts all groups with no error bars or significance denotations for overall comparisons. *Asterisks indicate reductions in MAP from within group control measurements.

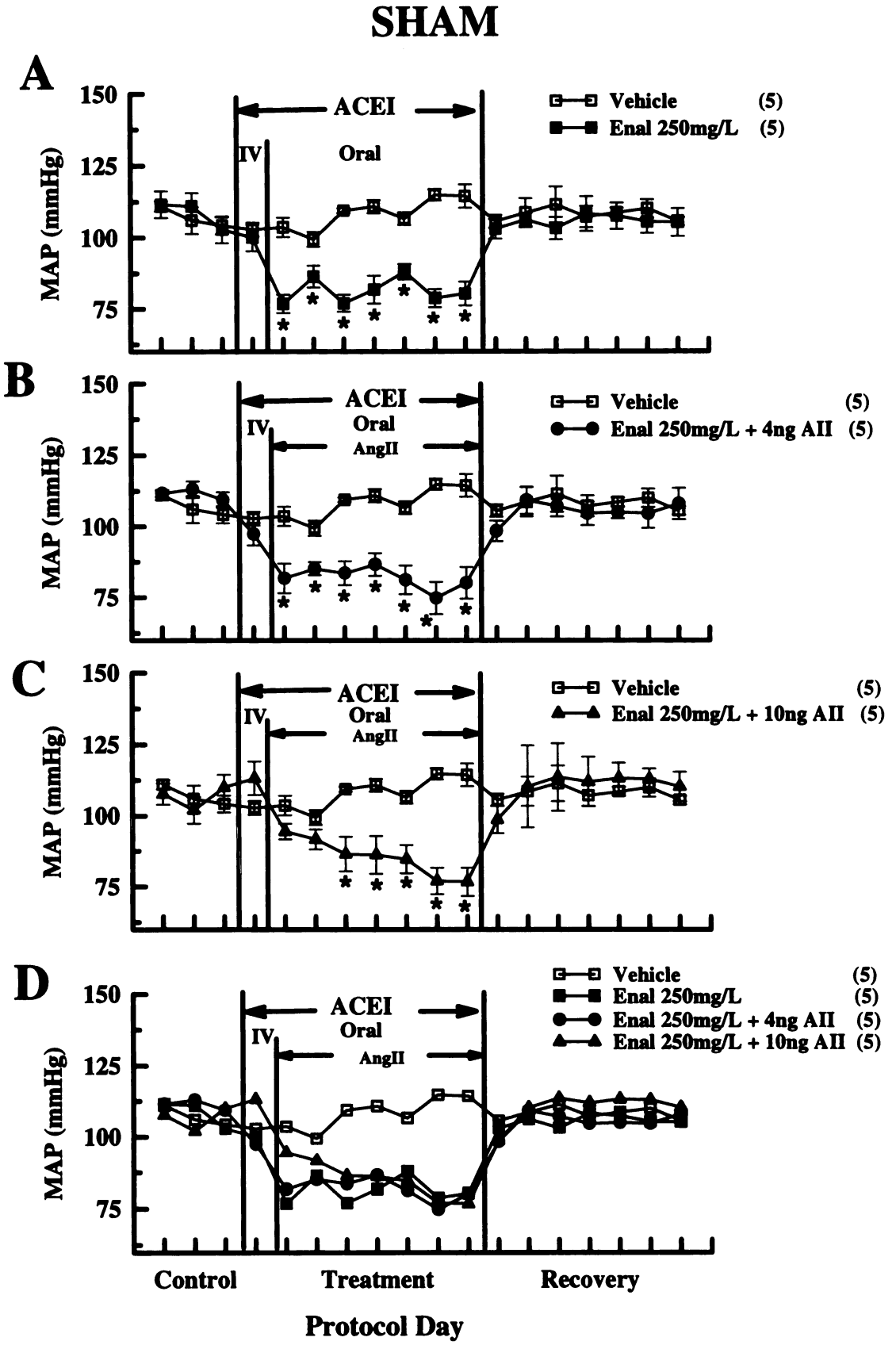

SHAM
A
B
C
D
MAP (mmHg)
150
125
100
75
ACEI
IV
Oral
AngII
Vehicle (5)
Enal 250mg/L (5)
Enal 250mg/L + 4ng AII (5)
Enal 250mg/L + 10ng AII (5)
Control
Treatment
Recovery
Protocol Day

Figure 15

Table 3: AngI pressor responses, enalapril dosages and blood urea nitrogen levels in sham and RRM rats maintained on a low salt intake. All RRM blood urea nitrogen values are significantly higher than the corresponding sham values at each time point but are not annotated for ease of comparison. *Asterisks indicate decreases in AngI pressor responses from within group control levels.

Table 3:

	AngI pressor response to 50 ng iv bolus (mmHg)			Enalapril Dose (mg/day)
	Control	Treatment	Recovery	
SHAM				
Vehicle	27.8 ± 4.4	27.2 ± 4.9	22.8 ± 3.4	0
Enalapril 250mg/L	26.6 ± 3.0	11.6 ± 0.5*	26.0 ± 1.5	6.2
Enalapril 250mg/L + 4ng AII	25.8 ± 1.3	4.6 ± 2.0*	24.4 ± 2.1	6.4
Enalapril 250mg/L + 10ng AII	25.2 ± 4.2	6.2 ± 1.5*	31.4 ± 5.6	7.3
RRM				
Vehicle	31.4 ± 3.1	28.0 ± 3.2	34.7 ± 3.7	0
Enalapril 250mg/L	29.1 ± 1.5	8.1 ± 2.0*	27.7 ± 2.1	9.1
Enalapril 250mg/L + 4ng AII	26.4 ± 2.0	5.2 ± 1.6*	24.5 ± 1.6	9.4
Enalapril 250mg/L + 10ng AII	25.0 ± 4.8	5.4 ± 0.4*	28.7 ± 3.0	7.7

	BUN (mg/dL)		
	Control	Treatment	Recovery
SHAM			
Vehicle	11.8 ± 1.6	10.2 ± 0.4	9.8 ± 0.5
Enalapril 250mg/L	12.7 ± 0.6	14.4 ± 1.3	12.4 ± 0.9
Enalapril 250mg/L + 4ng AII	14.2 ± 2.1	14.0 ± 2.0	16.3 ± 2.4
Enalapril 250mg/L + 10ng AII	16.2 ± 1.6	17.7 ± 0.4	12.2 ± 0.8
RRM			
Vehicle	42.1 ± 6.4	34.2 ± 5.7	40.4 ± 7.7
Enalapril 250mg/L	44.2 ± 5.7	48.2 ± 5.8	44.1 ± 7.6
Enalapril 250mg/L + 4ng AII	38.8 ± 3.1	39.3 ± 2.9	37.6 ± 2.4
Enalapril 250mg/L + 10ng AII	35.8 ± 2.3	43.8 ± 3.3	34.5 ± 1.2

Figure 16: Water intakes, urine outputs and water balances in response to chronic enalapril administration with and without replacement AngII in RRM rats on low salt intakes. *Asterisks indicate increases in water balance from within group control measurements.

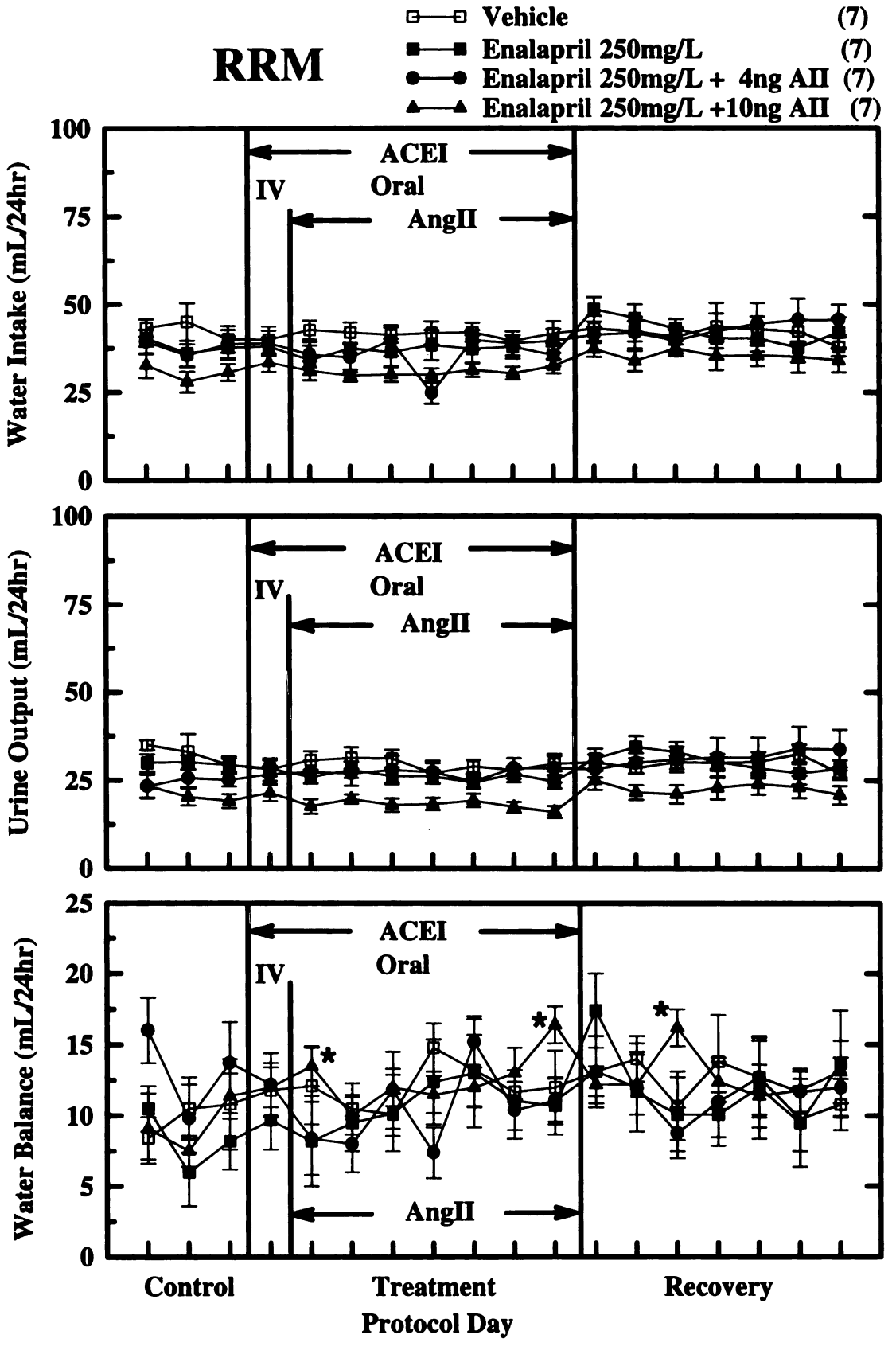

RRM
Vehicle (7)
Enalapril 250mg/L (7)
Enalapril 250mg/L + 4ng AII (7)
Enalapril 250mg/L +10ng AII (7)
Water Intake (mL/24hr)
Urine Output (mL/24hr)
Water Balance (mL/24hr)
ACEI
IV
Oral
AngII
Control
Treatment
Recovery
Protocol Day

Figure 16

Figure 17: Water intakes, urine outputs and water balances in response to chronic enalapril administration with and without replacement AngII in sham rats on low salt intakes.

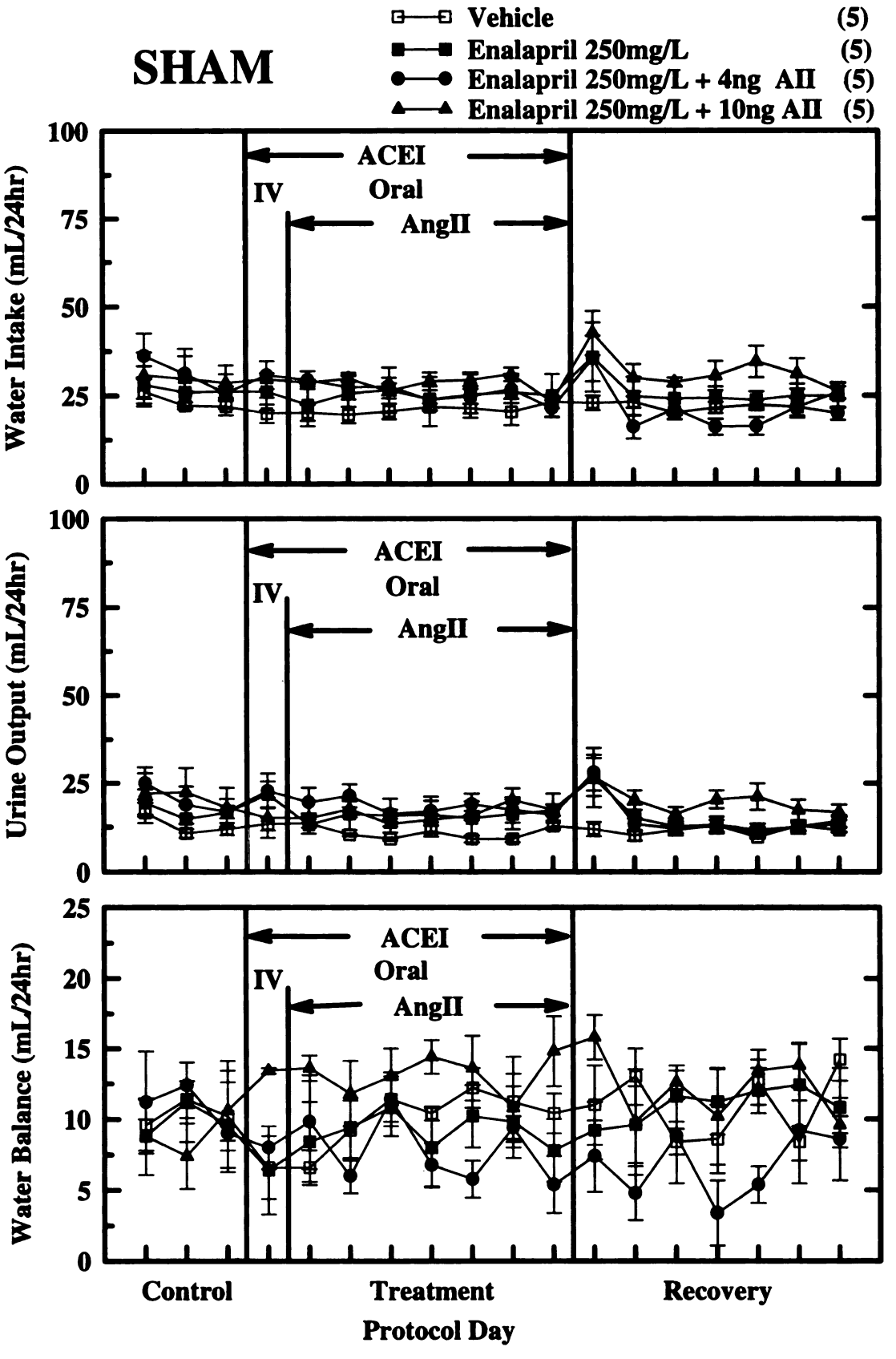

SHAM
Vehicle (5)
Enalapril 250mg/L (5)
Enalapril 250mg/L + 4ng AII (5)
Enalapril 250mg/L + 10ng AII (5)
Water Intake (mL/24hr)
Urine Output (mL/24hr)
Water Balance (mL/24hr)
ACEI
IV
Oral
AngII
Control
Treatment
Recovery
Protocol Day

Figure 17

Figure 18: Urinary sodium excretions in RRM and sham rats administered enalapril with and without replacement AngII on low salt intakes. All rats were maintained on a sodium deficient diet and dextrose i.v. infusion.

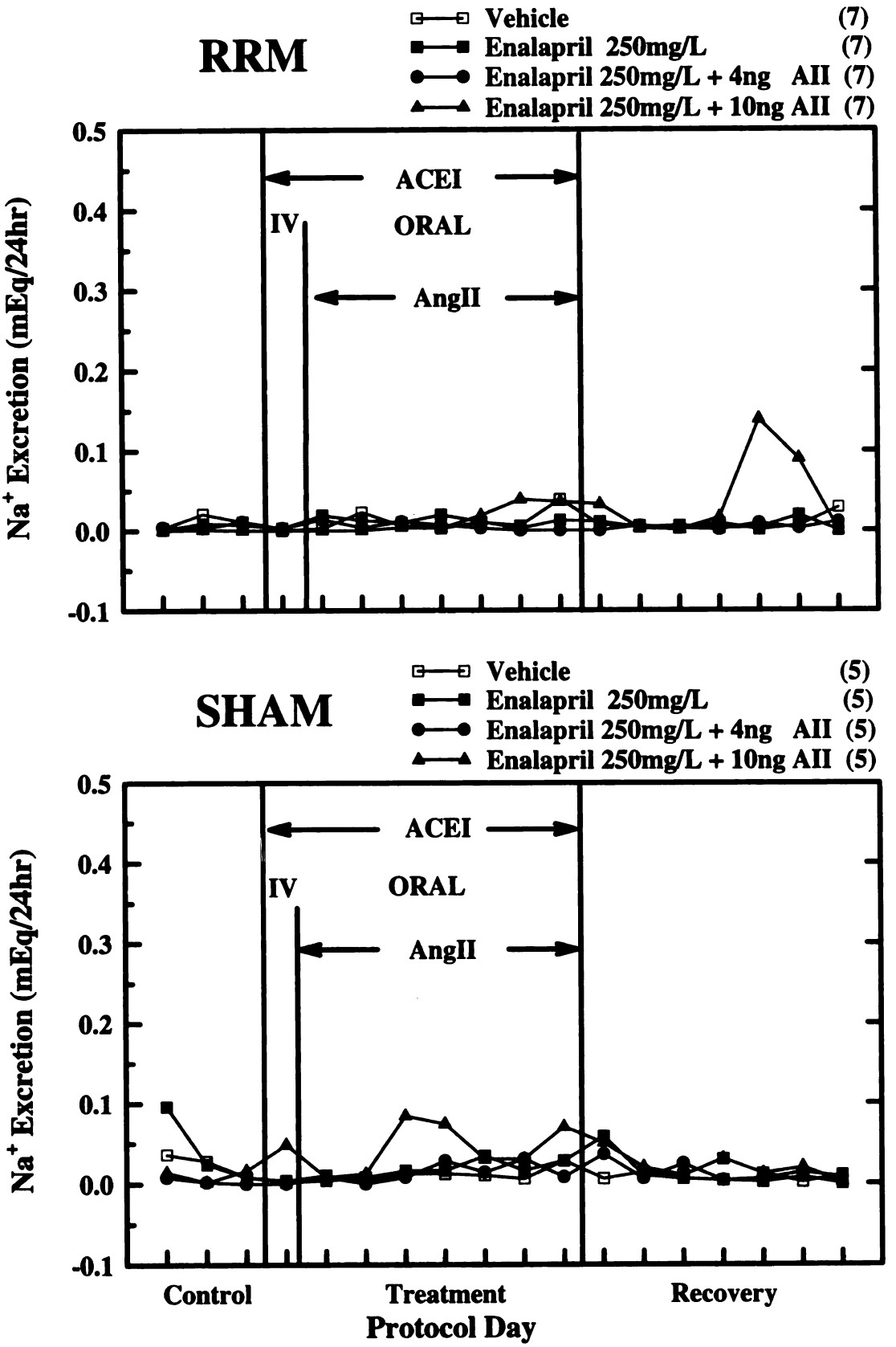

RRM
Vehicle (7)
Enalapril 250mg/L (7)
Enalapril 250mg/L + 4ng AII (7)
Enalapril 250mg/L + 10ng AII (7)
ACEI
IV
ORAL
AngII
Na+ Excretion (mEq/24hr)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
SHAM
Vehicle (5)
Enalapril 250mg/L (5)
Enalapril 250mg/L + 4ng AII (5)
Enalapril 250mg/L + 10ng AII (5)
ACEI
IV
ORAL
AngII
Na+ Excretion (mEq/24hr)
Control
Treatment
Recovery
Protocol Day

Figure 18

II. Endothelin system and reduced renal mass

A. Acute Experiments

1. Acute i.v. administration of an ET_A (PD147953) or an ET_A/ET_B (PD145065) receptor antagonist in ET-1 induced hypertension.

a. Rationale

In this study, the efficacy of PD147953 and PD145065 in reversing the chronic hypertensive response in rats caused by systemic infusion of ET-1 was tested. PD147953 has been characterized as a selective ET_ARA from binding studies in the rat and rabbit (Doherty *et al.*, 1993). PD147953 exhibits 1000 times more selective binding for ET_A verses ET_B receptors in the rat VSMC. PD145065 has been characterized as a non-selective ET_A/ET_BRA from binding and functional studies (Doherty *et al.*, 1993). Previous work in our lab has shown that continuous i.v. administration of ET-1 at 5 pmol/kg/min produces a sustained hypertension in normal rats (Mortensen and Fink, 1992b). The specific aim of this study was to determine if acute i.v. administration of either peptide receptor antagonist would lower BP in this model. The current view is that the endothelial cell ET_{B1} receptor initiates release of nitric oxide and/or prostacyclin upon ET-1 binding, which results in vasodilation. It was expected that a mixed ET_A/ET_BRA would be less efficacious in lowering BP because of blockade of the release of vasodilators.

b. Protocol

Two groups (n=5) of male Sprague-Dawley rats weighing 350-400gm were catheterized for hemodynamic measurements and the continuous i.v. infusion of ET-1 at 5 pmol/kg/min in a saline solution calibrated to deliver a sodium intake of 6.0 mEq/24

hours. The experiment lasted a total of 15 days; 3 control days followed by 7 days of continuously administered ET-1, and ending with 5 recovery days. One-half hour infusions of each ETRA (0.1 mg/kg/min) were administered on four representative days covering each experimental period. BP was recorded at 5`,15`,30`,60`, and 120` after the start of each ETRA infusion on these 4 days.

c. Results

Continuous infusion of ET-1 at a rate of 5 pmol/kg/min in normal rats produced a sustained increase in BP. Data are shown in Figure 19, from experimental days when the antagonists were administered. Thirty minute i.v. administration of PD147953 significantly reduced BP from daily control values between 30-120` on both ET-1 infusion days. This effect was not observed during non-ET-1 infused days (control and recovery). PD145065 significantly reduced BP on protocol day E3 from 15-120` during ET-1 infusion but on day E7 the BP lowering effect did not reach statistical significance. The antihypertensive effect was not observed during non-ET-1 infused days (control and recovery).

d. Interpretations

These results demonstrated that PD147953 and to a less consistent extent PD145065 were able to reversibly inhibit the chronic hypertensive response to exogenous ET-1. The latency of the antihypertensive effect is thought to be due to the difficulty of displacing endogenous ET from its binding sites. ET-1 increases BP mainly by stimulating ET_A receptors. The dose used here was the starting point for additional experiments designed to evaluate the role of endogenous ET-1 in short-term BP regulation in RRM rats under varying salt intakes.

Figure 19: Acute mean arterial pressure responses to ET_A (PD147953) and ET_A/ET_B (PD145065) receptor antagonist infusion in ET-1 induced hypertension. Each antagonist was infused at a rate of 0.1 mg/kg/min for 30 minutes in normal rats (n=5). Infusion lengths are indicated by representative arrows during each protocol day. *Asterisks indicate decreases in pressure from daily control measurement.

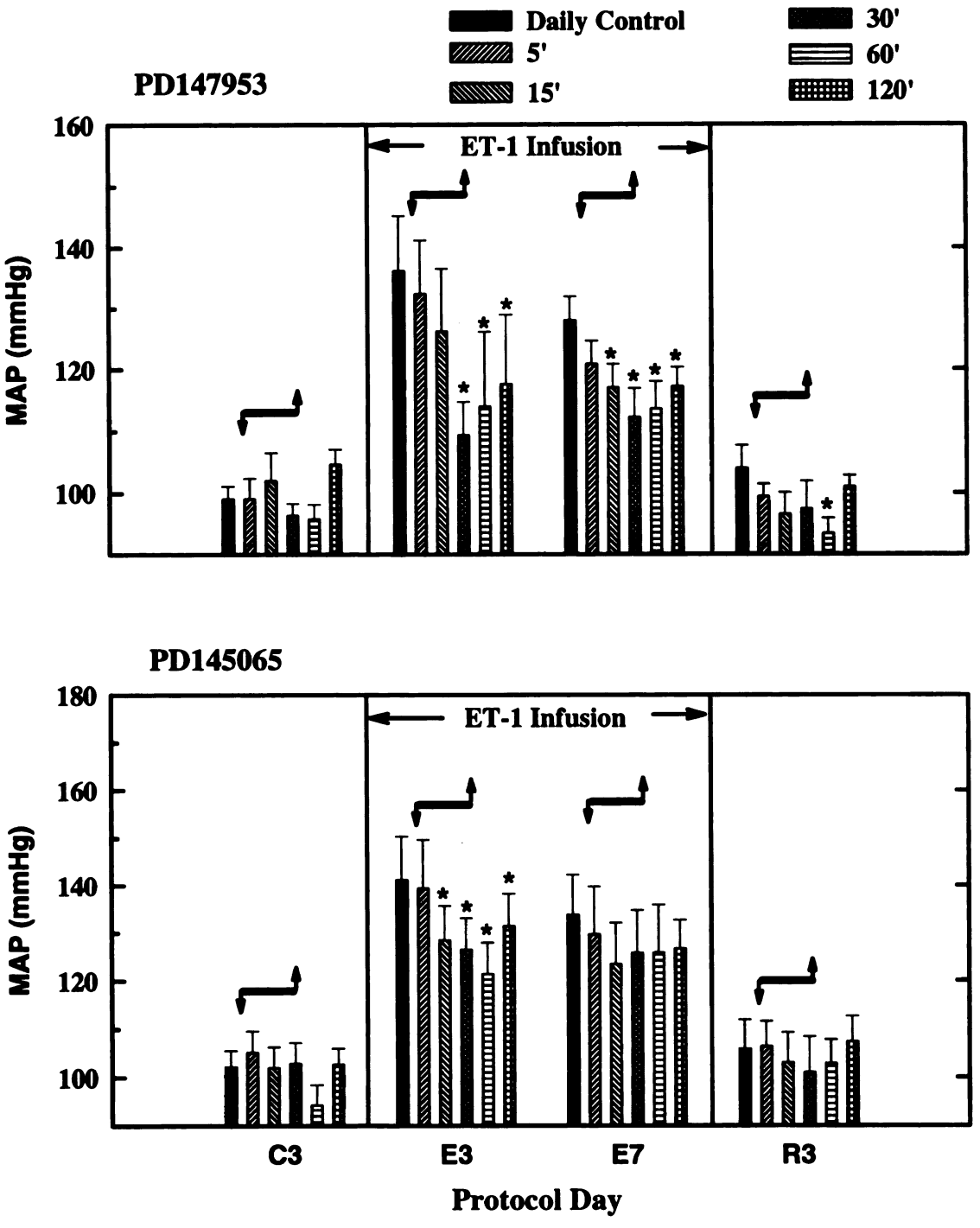

Daily Control
5'
15'
30'
60'
120'
PD147953
ET-1 Infusion
MAP (mmHg)
160
140
120
100
PD145065
ET-1 Infusion
MAP (mmHg)
180
160
140
120
100
C3
E3
E7
R3
Protocol Day

Figure 19

2. Acute i.v. administration of an ET_A (PD147953) or an ET_A/ET_B (PD145065) receptor antagonist in hypertensive RRM and sham rats on a high salt intake.

a. Rationale

ET-1 has the potential to act both acutely and chronically to promote hypertension through a combination of short-term systemic vasoconstriction, and long-term effects on vascular structure, renal function or neural BP control mechanisms. The purpose of this experiment was to define the contribution of ET to short-term BP regulation in RRM rats on a high salt diet. The hypothesis was: if ET exerts short-term control of BP in RRM rats on a high salt diet by direct vascular actions, acute ETRA treatment should lower BP over seconds to minutes, since theses drugs are known to rapidly block the vascular responses to ET (Gardiner *et al.*, 1994). Since the acute antihypertensive effect of the ETRA's in the ET-1 infusion study did not result in the complete normalization of BP, it was decided to add two larger doses of these ETRA in this experiment involving RRM and sham rats.

b. Protocol

Rats were subjected to either RRM or sham operation. All rats drank isotonic saline starting one day following completion of the surgery according to the HS protocol. Control BP measurements were taken prior to the start of twenty minute infusions of each antagonist. PD147953 and PD145065 were infused i.v. at 3 different rates: 0.1, 0.3, and 1.0 mg/kg/min in RRM and sham rats on separate days. BP was recorded from 5 minutes to 6 hours after the start of the antagonist infusions and was taken for the last time 24 hours later. Each rat received both antagonists separated by at least 2 days.

c. Results

Figure 20 presents BP data from acute ET_ARA treatment in RRM and sham rats on HS. The RRM rats all had an elevated BP compared to the sham rats prior to the start of each antagonist infusion. In RRM rats there was a delayed, dose-dependent decrease in BP from 30 minutes to 2 hours after the administration of PD147953. Some initial lowering of BP was observed at each infusion rate but the most pronounced and prolonged effect was observed with the highest infusion rate (1.0 mg/kg/min). In sham rats after 20 minute infusions of the ET_ARA, there were no changes in BP from 5 minutes up to 24 hours at any dose.

BP data are contained in Figure 21 from acute ET_A/ET_BRA (PD145065) treatment in the same RRM and sham rats on HS. In RRM rats, there was a delayed, dose-dependent decrease in BP from 30 minutes to 2 hours after administration of each of the higher antagonist infusion rates (0.3 - 1.0 mg/kg/min). In fact each of these infusion rates produced the same fall in BP in RRM rats. In the sham rats after 20 minute infusions of PD145065, there were no changes in BP from 5 minutes up to 24 hours at any dose.

d. Interpretations

These data show that each ETRA, at the infusion rates used, does not lower BP in sham normotensive rats on HS. These results suggest that ET-1 is not involved in short-term BP regulation in normotensive rats when on HS. On the other hand, the magnitude and the duration of the fall in BP was similar after ET_ARA and ET_A/ET_BRA treatment in RRM rats especially at the 1.0 mg/kg/min rate. These data suggest that ET, acting primarily on the ET_A receptor subtype, contributes to the short-term regulation of BP in RRM rats on HS. The ET_B receptor subtype does not appear important in short-term

regulation of BP in RRM rats. Subsequent studies in RRM used the highest infusion rate from this study to compare the antihypertensive effects of selective versus non-selective endothelin receptor blockade, in rats on lower salt intakes.

Figure 20: Acute mean arterial pressure responses to ET_A (PD147953) receptor antagonist infusions in RRM and sham rats on high salt intakes. The antagonist was infused at three different rates for 20 minutes. *Asterisks indicate decreases in pressure from zero control measurement.

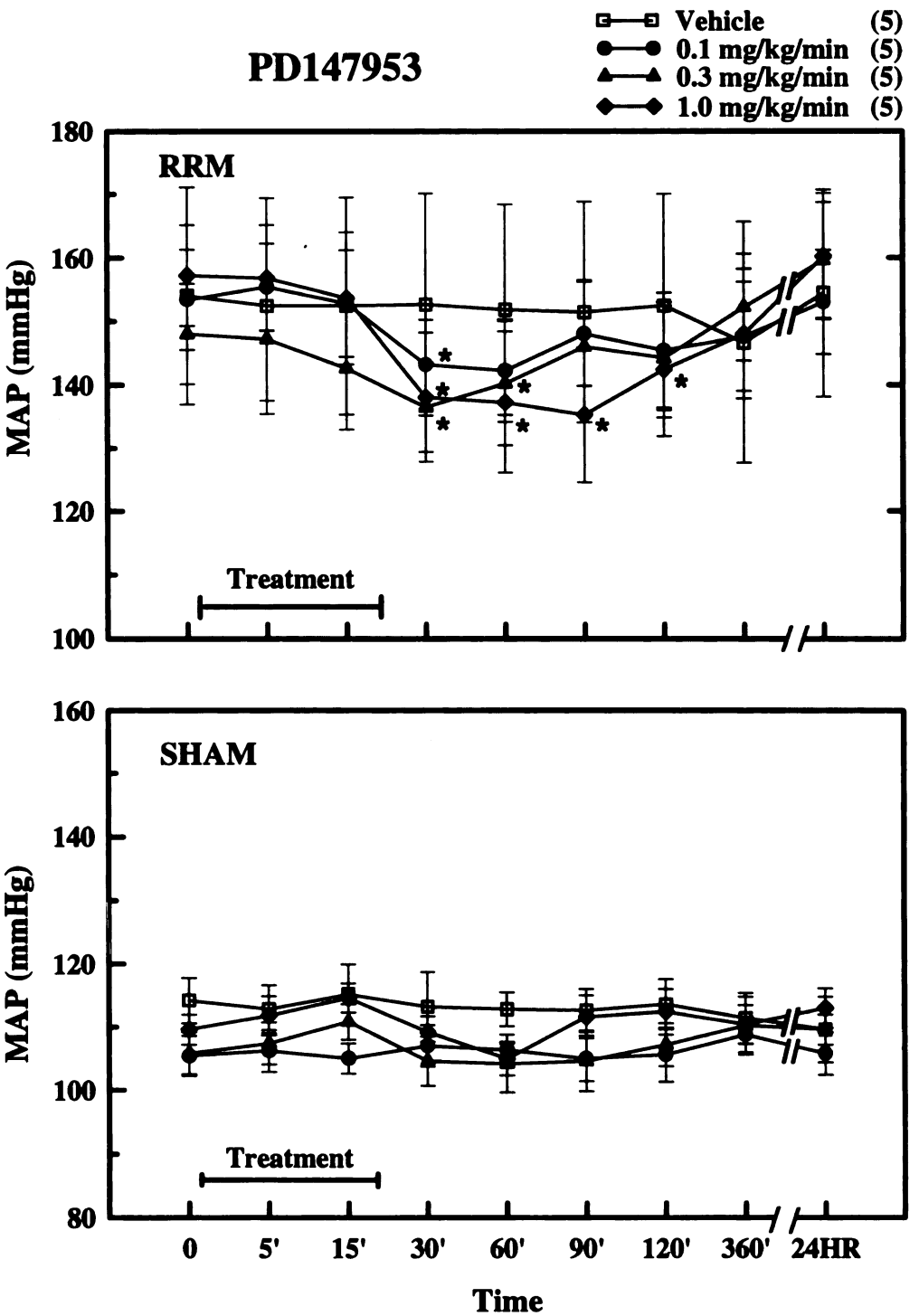

PD147953
Vehicle (5)
0.1 mg/kg/min (5)
0.3 mg/kg/min (5)
1.0 mg/kg/min (5)
RRM
SHAM
MAP (mmHg)
Treatment
Time
0
5'
15'
30'
60'
90'
120'
360'
24HR

Figure 20

Figure 21: Acute mean arterial pressure responses to ET_A/ET_B (PD145065) receptor antagonist infusions in RRM and sham rats on high salt intakes. The antagonist was infused at three different rates for 20 minutes. *Asterisks indicate decreases in pressure from zero control measurement.

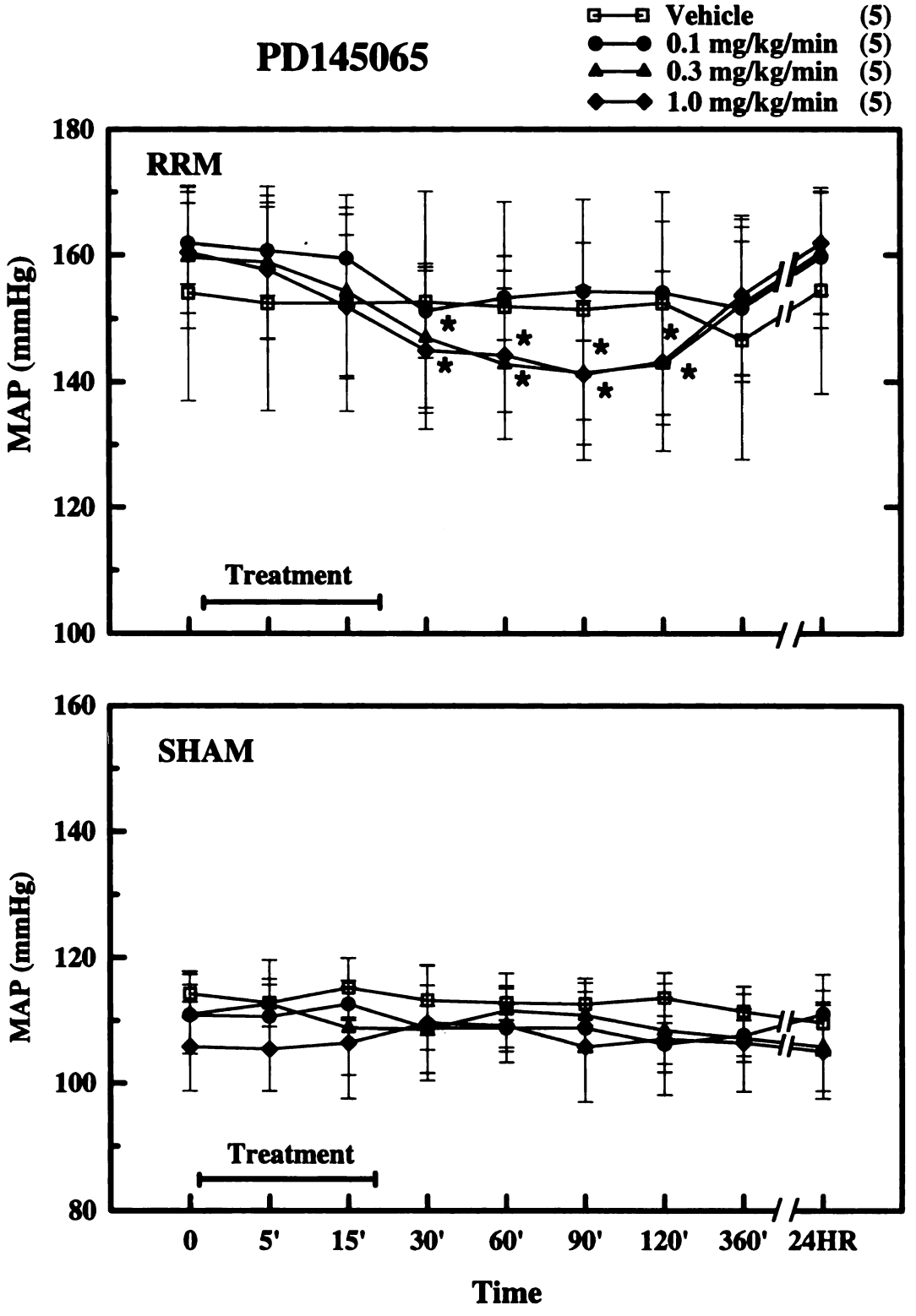

PD145065
Vehicle (5)
0.1 mg/kg/min (5)
0.3 mg/kg/min (5)
1.0 mg/kg/min (5)
RRM
SHAM
MAP (mmHg)
Treatment
Time
0
5'
15'
30'
60'
90'
120'
360'
24HR

Figure 21

3. Comparison of acute i.v. administration of an ET_A (PD147953) or an ET_A/ET_B (PD145065) receptor antagonist, in RRM and sham rats on high, normal, and low salt intakes.

a. Rationale

Currently, the influence of salt intake on the physiological and pathophysiological functions of ET are uncertain. Previous work in our lab demonstrated the salt-dependency of hypertension produced by chronic i.v. infusion of ET-1 in normal rats (Mortensen and Fink, 1992b). Also, an antihypertensive action of ETRA's has been demonstrated in RRM rats on HS. Since sodium balance plays an integral role in the development of hypertension in RRM, the purpose of this study was to characterize the relationship between ET, BP, and sodium intake in this model. In this experiment, the hypothesis was investigated that the contribution of ET to short-term BP regulation in RRM rats was altered by varying salt intake.

b. Protocol

The three levels of NaCl intake described in the methods section were utilized for the acute administration of ET_ARA and ET_A/ET_BRA in RRM and sham rats. Control BP recordings were taken prior to 20 minute i.v. infusions of PD147953 and PD145065 at 1.0 mg/kg/min on separate days. BP recordings were taken acutely from 5 minutes to 24 hours after the start of the infusion.

c. Results

Figure 22 presents BP data following acute PD147953 and PD145065 treatment in RRM and sham rats on a **high salt intake**. These are the same data shown in the last experiment but now I have only reported the 1.0 mg/kg/min infusion results. BP was

severely elevated in RRM rats as compared to sham prior to antagonist administration. Each antagonist caused a delayed decrease in BP in RRM but not in sham rats. The magnitude and the duration of the decrease in BP was similar after ET_ARA and ET_A/ET_BRA treatment.

Figure 23 presents BP data following acute PD147953 and PD145065 treatment in RRM and sham rats on a **normal salt intake**. BP was modestly elevated in RRM rats as compared to sham prior to antagonist administration. Twenty minute infusions of either antagonist did not cause a decrease in BP in RRM or sham rats on NS.

Figure 24 shows BP data following acute PD147953 and PD145065 treatment in RRM and sham rats on a **low salt intake**. As in previous experiments, hypertension was not observed in RRM rats maintained on LS. Therefore at the start of the experiment, BP in RRM and sham groups was not different and within the normotensive range. Neither antagonist caused a decrease in BP in RRM or sham rats on LS. Once again, the lack of a hypotensive effect in RRM and sham rats on LS after ET_ARA and ET_A/ET_BRA administration suggests that ET-1 does not appear important in short-term arterial pressure regulation under low salt conditions.

d. Interpretations

It was concluded from these data that the lack of an antihypertensive effect in RRM or a hypotensive effect in sham rats on NS or LS after ET_ARA and ET_A/ET_BRA treatment suggests that ET-1 does not appear important in short-term arterial pressure regulation under these conditions. The overall conclusion from this set of experiments was that ET contributes to short-term arterial pressure regulation through actions at the ET_A receptor subtype in hypertensive RRM rats only during high salt intake.

Figure 22: Acute mean arterial pressure responses to ET_A (PD147953) and ET_A/ET_B (PD145065) receptor antagonist infusions in RRM and sham rats on high salt intakes. Each antagonist was infused at 1.0 mg/kg/min for 20 minutes. *Asterisks indicate decreases in pressure from zero control measurement.

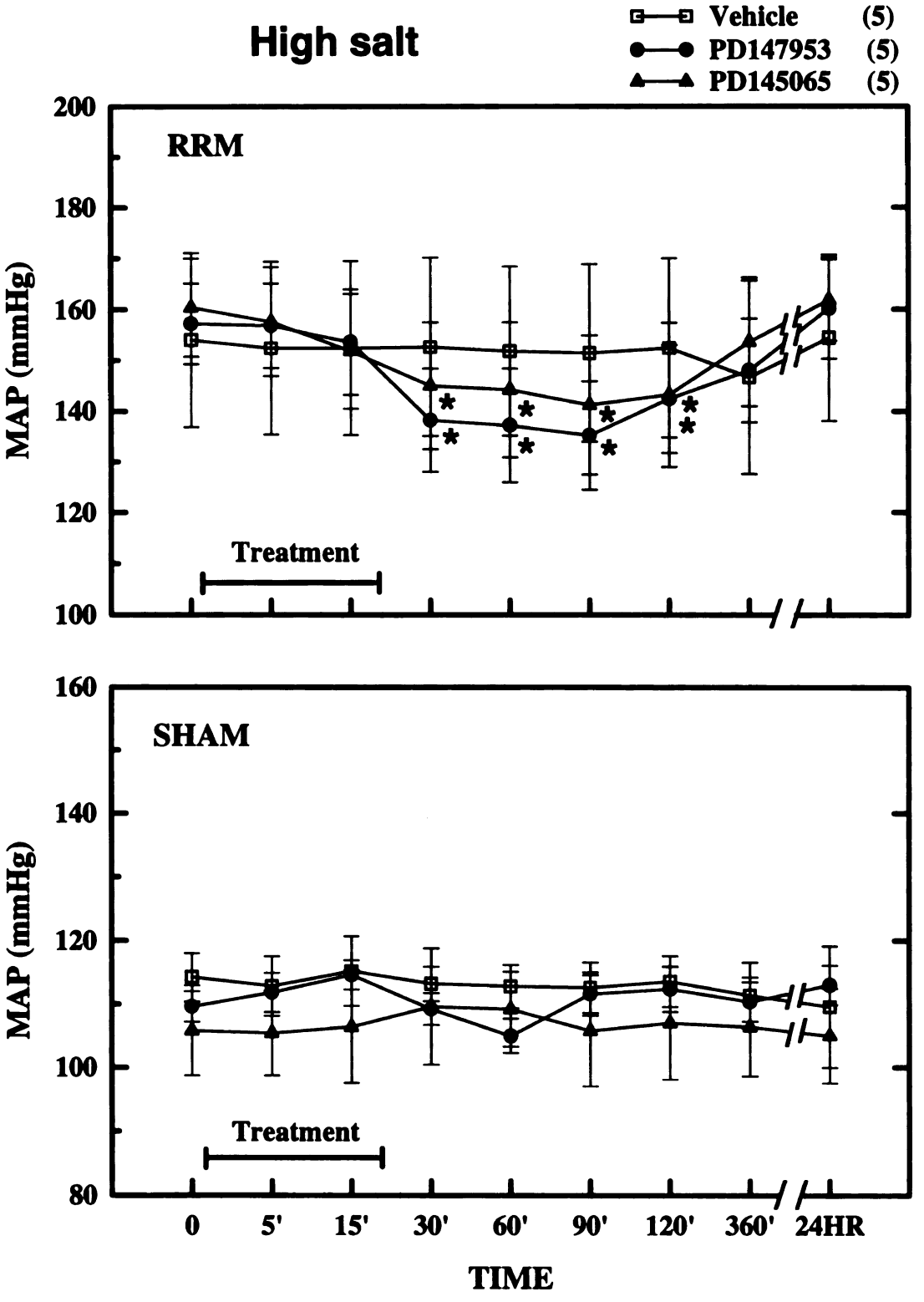

High salt
Vehicle (5)
PD147953 (5)
PD145065 (5)
RRM
SHAM
MAP (mmHg)
Treatment
TIME
0
5'
15'
30'
60'
90'
120'
360'
24HR

Figure 22

Figure 23: Acute mean arterial pressure responses to ET_A (PD147953) and ET_A/ET_B (PD145065) receptor antagonist infusions in RRM and sham rats on normal salt intakes. Each antagonist was infused at 1.0 mg/kg/min for 20 minutes.

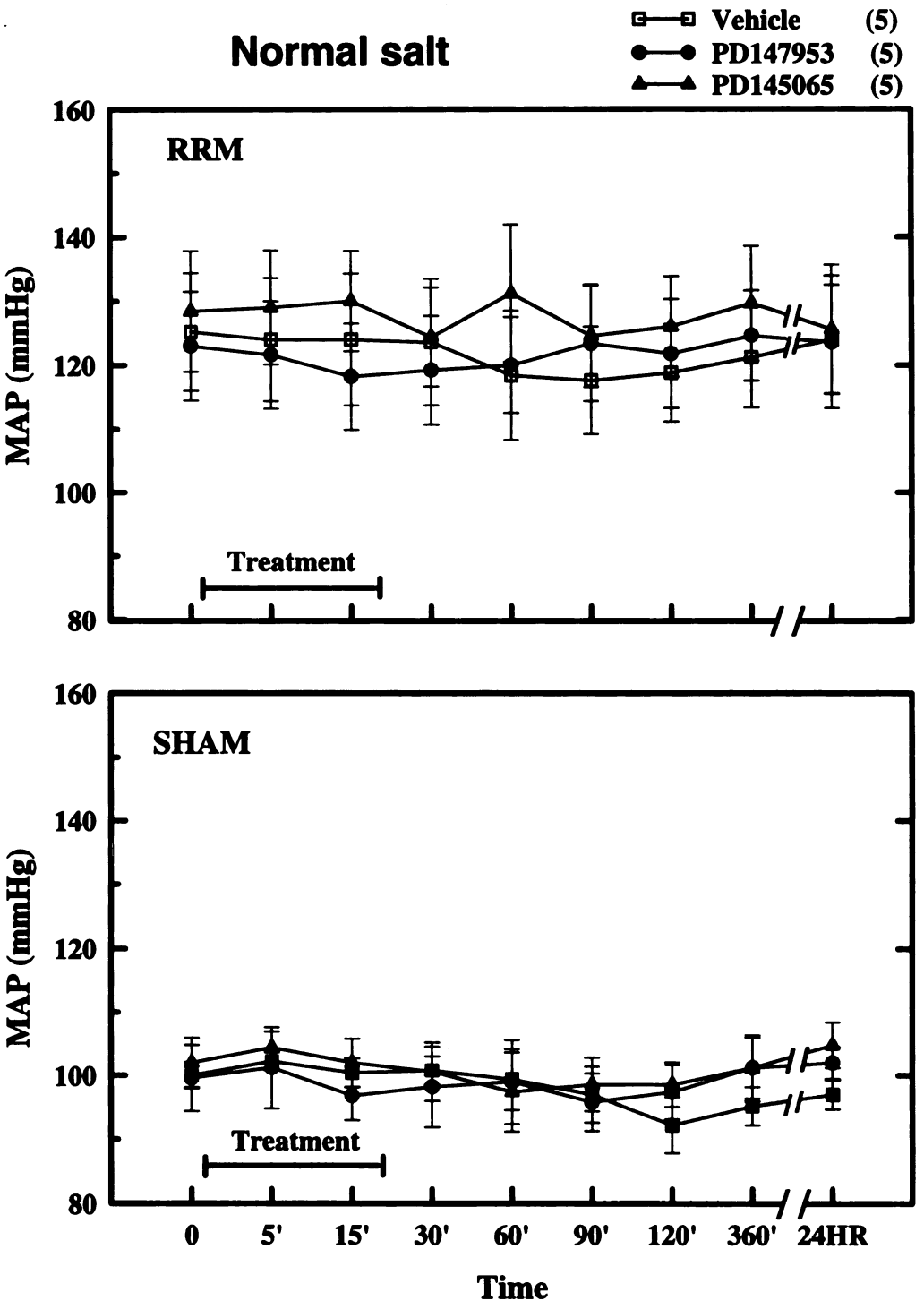

Normal salt
Vehicle (5)
PD147953 (5)
PD145065 (5)
RRM
SHAM
MAP (mmHg)
160
140
120
100
80
Treatment
0
5'
15'
30'
60'
90'
120'
360'
24HR
Time

Figure 23

Figure 24: Acute mean arterial pressure responses to ET_A (PD147953) and ET_A/ET_B (PD145065) receptor antagonist infusions in RRM and sham rats on low salt intakes. Each antagonist was infused at 1.0 mg/kg/min for 20 minutes.

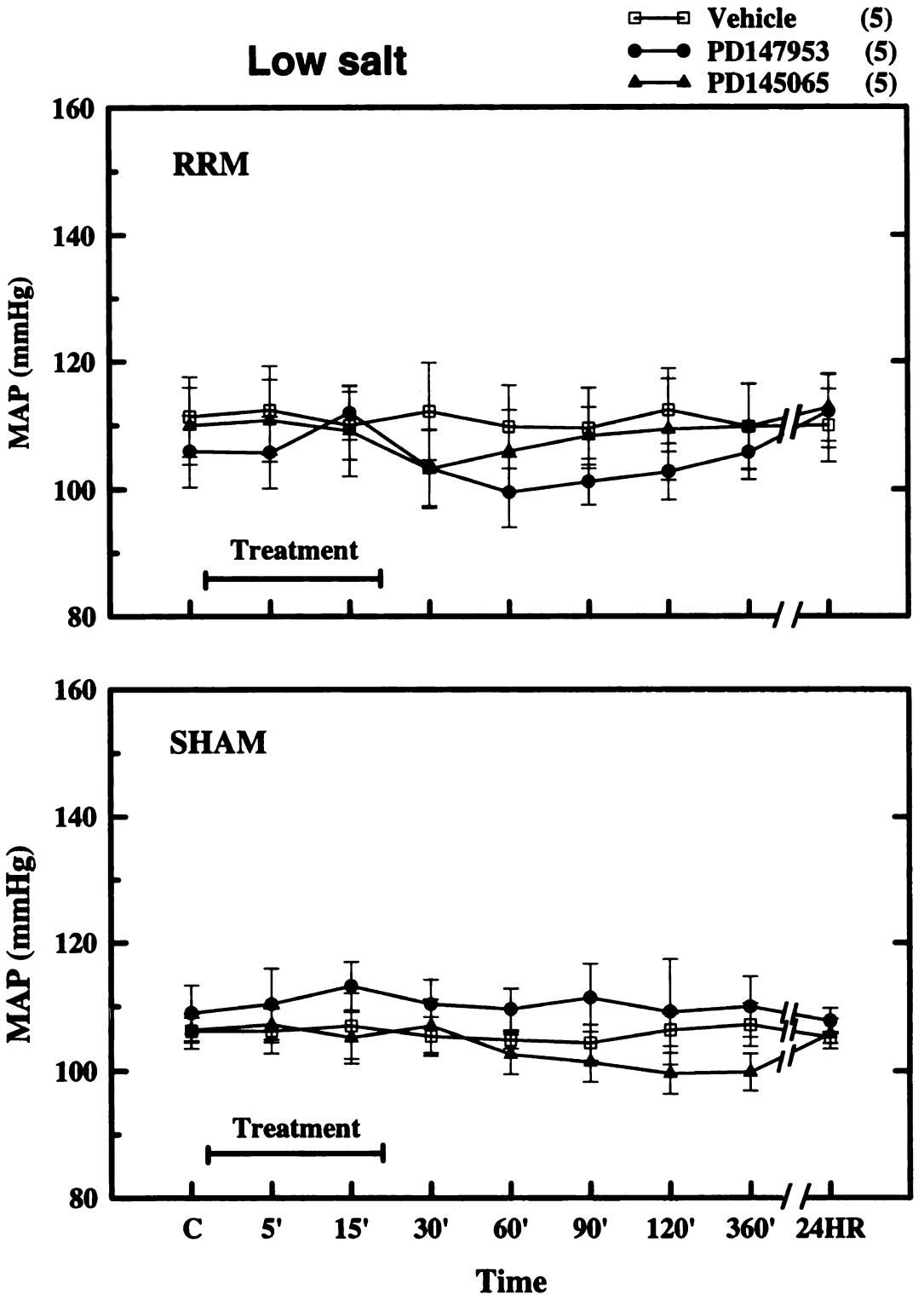

Low salt
Vehicle (5)
PD147953 (5)
PD145065 (5)
RRM
SHAM
MAP (mmHg)
160
140
120
100
80
Treatment
C
5'
15'
30'
60'
90'
120'
360'
24HR
Time

Figure 24

B. Chronic experiments

1. Oral ET_A (PD155080) receptor antagonist treatment in the hypertension induced by continuous i.v. ET-1 infusion.

a. Rationale

One of the main goals of this research was to uncover evidence for a chronic influence of ET-1 on arterial pressure regulation in RRM using ETRA. Therefore, initial experiments were performed to demonstrate the efficacy of ET-1 receptor blockade in reversing long-term cardiovascular effects of ET-1. To this end rats were made hypertensive by continuous i.v. infusion of ET-1 for several days, then treated with the ET_ARA, PD155080 to establish the antihypertensive specificity of this drug and to determine a dose to use in the RRM model. PD155080 is a potent competitive inhibitor of both ET_A and ET_B receptors having IC_{50} values of 7.4 and 4500 nM respectively for each receptor subtype (Doherty *et al.*, 1995). This orally active compound has a bioavailability of 87% and a half-life of 5 hours in the rat so it was a good candidate for chronic administration in these studies (Doherty *et al.*, 1995).

b. Protocol

Normal Sprague-Dawley rats weighing 350-400gm were instrumented with arterial and venous catheters for hemodynamic measurements and continuous i.v. infusions of ET-1. The rats were divided into 4 groups: the vehicle group (vehicle, n=5) was administered an i.v. saline solution calibrated to deliver 6.0 mEq Na^+/24 hours. In another group of rats (ET-1 2.5, n=5) 2.5 pmol/kg/min ET-1 was continuously administered in the i.v. saline solution. PD155080 was given orally to the last two groups of rats that received ET-1 i.v. at 2.5 pmol/kg/min (ET-1 2.5 + PD155080, n=5) or 5

pmol/kg/min (ET-1 5.0 + PD155080, n=5). The experiment lasted a total of 12 days; 2 control days were followed by 10 days of continuously administered saline alone or saline plus ET-1. During ET-1 administration, 3 days of infusion were allowed to establish an increased BP, at which time ETRA treated rats were given PD155080 (25 mg/kg b.i.d.) for 5 days. PD155080 was given orally in powder form mixed with sodium deficient chow in powder form. All rats in the study were given chow twice a day; 5 grams in the morning (9-10 am) and 7.5 grams in the evening (5-8 pm). The experiment ended with two days of recordings after PD 155080 had been discontinued. The 3 groups receiving ET-1 infusions were kept on the infusions these last two days while the vehicle group just received the saline solution.

c. Results

Continuous infusion of ET-1 at a rate of 2.5 pmol/kg/min in normal rats produced a slowly developing, sustained hypertension (Figure 25A). Oral administration of PD155080 for 5 days resulted in a complete normalization of BP. After discontinuation of PD155080, BP returned to hypertensive levels within one day. Continuous infusion of ET-1 at a rate of 5 pmol/kg/min in normal rats produced a rapidly developing, sustained hypertension (Figure 25B). The magnitude of the BP increase was greater in these rats than in rats given 2.5 pmol/kg/min. Oral administration of PD155080 for 5 days resulted in a sustained antihypertensive effect that did not quite reach normotensive levels. After discontinuation of PD155080, BP returned to hypertensive levels within one day. Figure 25B also shows PD155080 administration in rats that received saline only i.v. Saline infusion alone in normal rats did not increase blood pressure. PD155080 resulted in a

slight hypotensive effect during the first 2 days of administration. Upon discontinuation of PD155080, BP rose to mildly hypertensive levels during 2 recovery days.

d. Interpretations

These results show that PD155080 at 25mg/kg b.i.d was able to fully and reversibly inhibit the chronic hypertensive response to exogenous ET-1 at 2.5 and 5.0 pmol/kg/min. Thus, this antagonist dose was utilized in subsequent experiments designed to evaluate the effect of endogenous ET-1 on BP regulation in RRM rats.

Figure 25: Chronic mean arterial pressure responses to ET_A (PD155080, 25 mg/kg b.i.d.) receptor antagonist administration in ET-1 induced hypertension. All rats were administered a saline infusion calibrated to deliver a sodium intake of 6.0 mEq/day through the i.v. catheter. Panel A depicts MAP responses to PD155080 treatment in normal rats continuously infused ET-1 (2.5 pmol/kg/min). Panel B depicts MAP responses to PD155080 treatment in normal rats continuously infused ET-1 (5.0 pmol/kg/min) or saline infusion alone. *Asterisks indicate increases in blood pressure from within group control measurements. † Daggers denote decreases in blood pressure from within group control measurements.

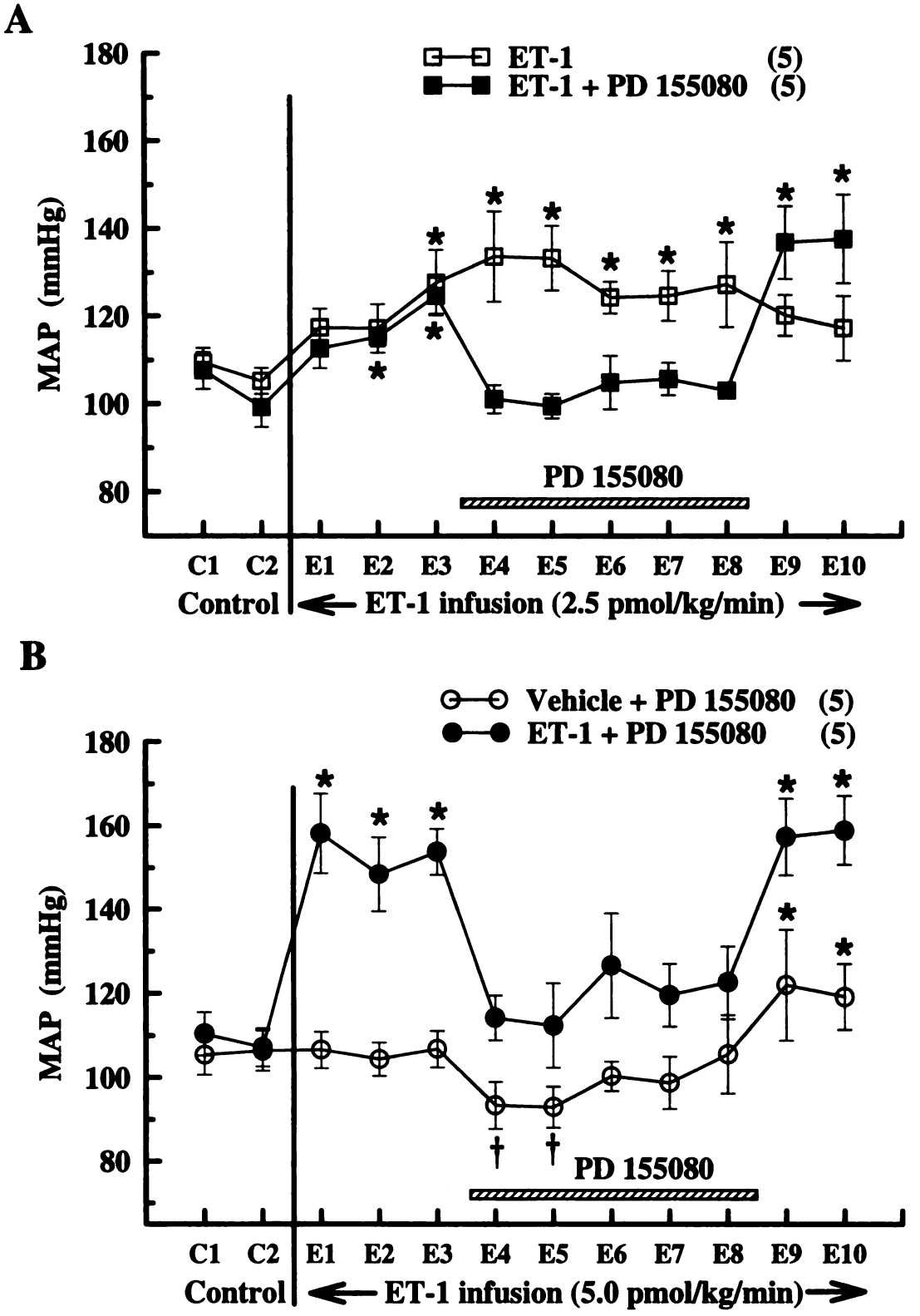

A
ET-1 (5)
ET-1 + PD 155080 (5)
MAP (mmHg)
180
160
140
120
100
80
PD 155080
C1 C2 E1 E2 E3 E4 E5 E6 E7 E8 E9 E10
Control
ET-1 infusion (2.5 pmol/kg/min)
B
Vehicle + PD 155080 (5)
ET-1 + PD 155080 (5)
MAP (mmHg)
180
160
140
120
100
80
PD 155080
C1 C2 E1 E2 E3 E4 E5 E6 E7 E8 E9 E10
Control
ET-1 infusion (5.0 pmol/kg/min)

Figure 25

2. Oral ET_A (PD155080) receptor antagonist treatment in RRM rats on high, normal and low salt intakes.

a. Rationale

Pilot experiments were performed to determine the optimal antihypertensive dose of PD155080 in RRM rats. In addition, the relative importance of ET-1 was tested in the maintenance of BP under varying salt intakes. My previous results with acute administration of peptide ETRA's suggested that RRM rats on higher salt intakes would respond best to chronic endothelin receptor blockade.

b. Protocol

i. High salt, normal salt

In this pilot study, I tested the antihypertensive effect of PD155080 at 100 mg/kg b.i.d. in RRM rats maintained on high and normal salt intakes. Two rats from each group were catheterized and given PD155080 as previously described for 2 days following 2 days of control measurement. BP was recorded for 2 recovery days after PD155080 discontinuation to monitor for reversal of any drug effect.

ii. Low salt

Two rats maintained on LS were catheterized and given PD155080 for 5 days following 3 days of control measurement. They were given PD155080 at the lower dose of 25 mg/kg b.i.d. BP was recorded for 3 recovery days after PD155080 discontinuation to monitor for reversal of any hypotensive effect.

c. Results

Figure 26 shows BP in RRM rats maintained on HS or NS. During the 2 control days, both groups had severe and sustained hypertension. PD155080 administration

substantially lowered BP in both groups with NS RRM rats exhibiting the largest fall in BP. The antihypertensive effects of PD155080 were totally reversed at 24 hours after the discontinuation of ETRA treatment in both groups.

PD155080 lowered BP throughout all 5 treatment days in RRM rats maintained on LS and this hypotensive effect was reversed during the recovery days (Figure 27). But similar to my previous experiments, RRM rats in this experiment did not reach hypertensive levels when kept on LS.

d. Interpretations

The results from these pilot studies suggest that ET is involved in BP regulation in RRM over a period of days. The extraordinary antihypertensive effect of PD155080 in RRM rats maintained on HS and NS actually gave cause for concern. This work has focused on antihypertensive therapies in experimental renal failure, but drastic falls in blood pressure over such a short period of time can actually initiate acute renal failure. Therefore, lower doses of PD155080 were tested in RRM rats to look for the lowest dose necessary to achieve a significant reduction in BP. It was determined from these pilot studies that 25 mg/kg given twice daily was an effective antihypertensive dose in RRM rats. These preliminary experiments established a dosing regimen for further investigation over longer periods of ETRA administration in RRM. From these results it was decided to focus on the involvement of ET in RRM only under NS conditions for a variety of reasons. RRM rats maintained on NS most closely resemble the clinical setting of CRF and results obtained under these conditions would have the most therapeutic relevance. The other conditions represent extremes of salt intake; therefore, experimental pitfalls occurred. An unacceptable degree of mortality (50% at 1 month) was observed in

RRM rats given saline to drink in the HS protocols. Under LS conditions, RRM rats did not become hypertensive over the time frame of investigation (3 months). The other important factor in these investigations was the availability of the newly developed ETRA. PD155080 is not commercially available and was a generous gift from Parke-Davis Pharmaceutical Research, so supplies were limited.

Figure 26: Chronic mean arterial pressure responses to ET_A (PD155080, 100 mg/kg/b.i.d.) receptor antagonist administration in RRM rats maintained on high and normal salt intakes.

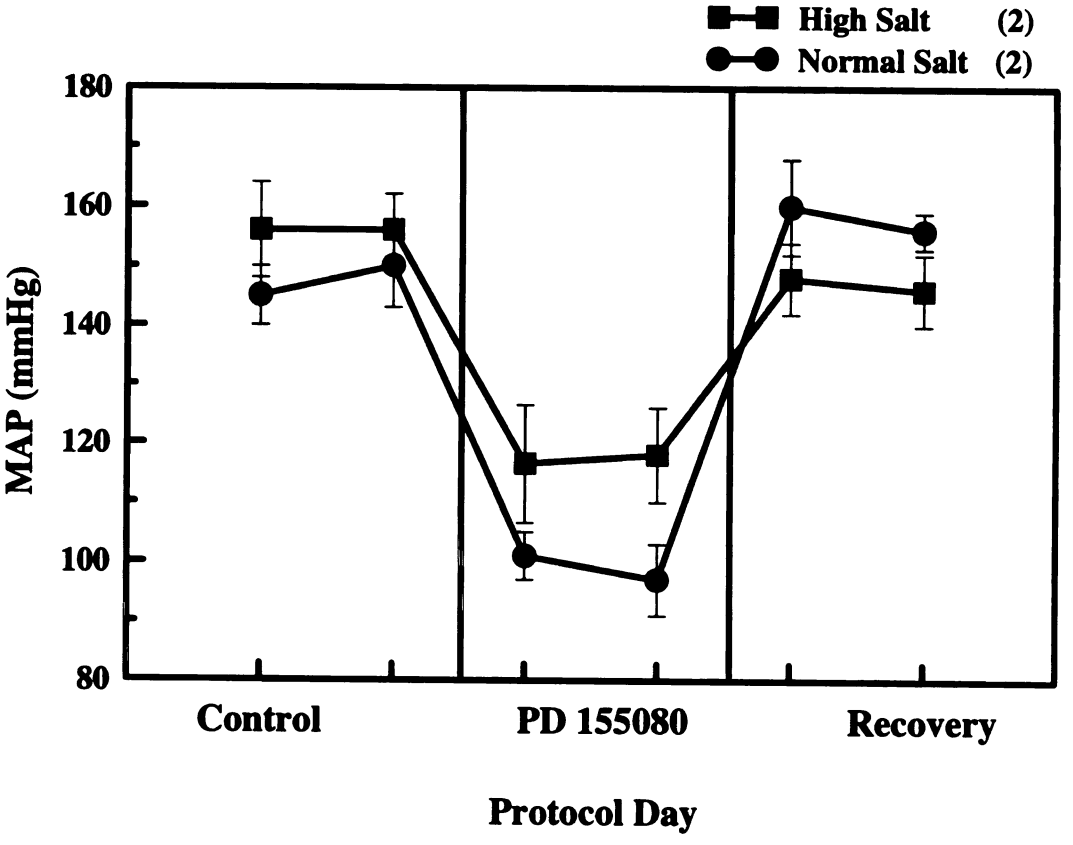

High Salt (2)
Normal Salt (2)
180
160
140
120
100
80
MAP (mmHg)
Control
PD 155080
Recovery
Protocol Day

Figure 26

Figure 27: Chronic mean arterial pressure responses to ET_A (PD155080, 25 mg/kg/b.i.d.) receptor antagonist administration in RRM rats on low salt intakes.

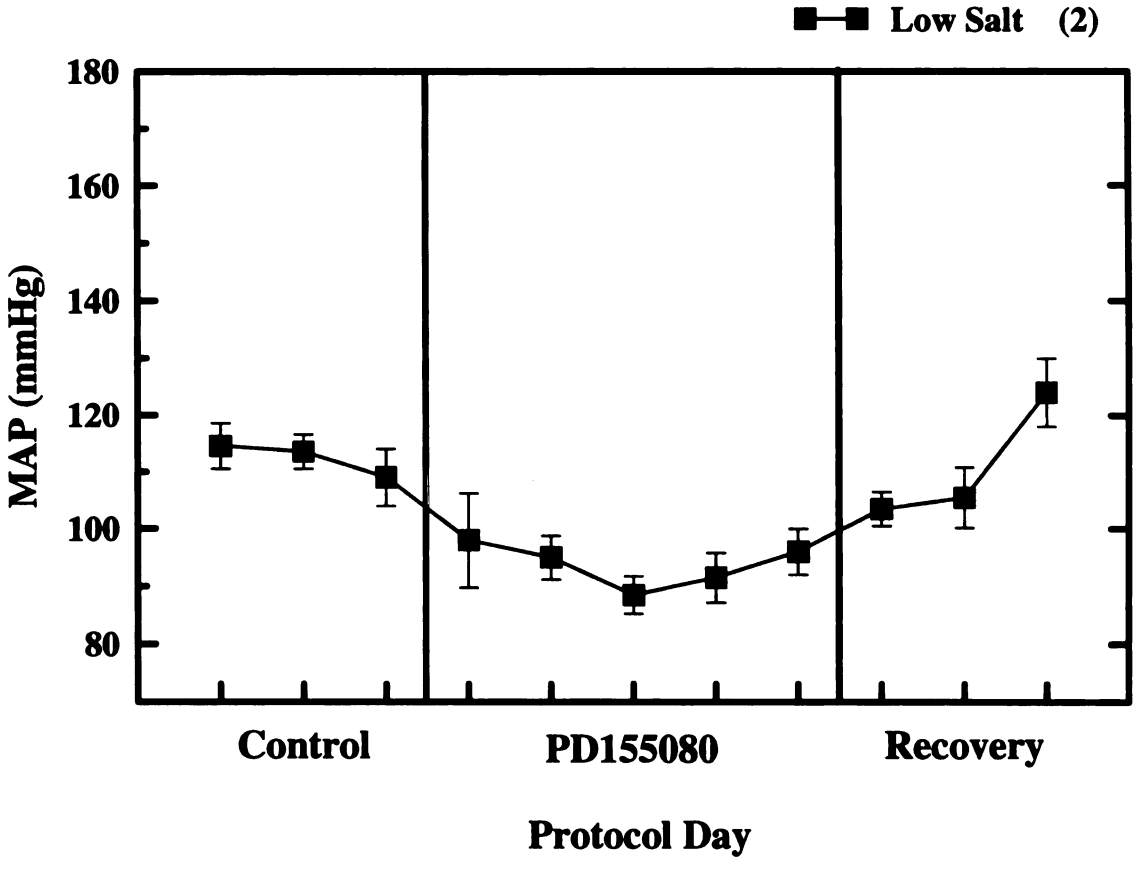

Low Salt (2)
180
160
140
120
100
80
MAP (mmHg)
Control
PD155080
Recovery
Protocol Day

Figure 27

3. Oral ET_A (PD155080) receptor antagonist treatment in established hypertensive RRM rats on a normal salt intake.

a. Rationale

The mechanisms by which ET may play a role in long-term BP regulation are currently speculative. As mentioned before, a clinically relevant scenario would be reversal of hypertension by ETRA administration without worsening renal function. The next experiment therefore was designed to determine the contribution of ET-1 to long term BP regulation in RRM rats one month after renal ablation. The hypothesis was that chronic administration of the oral ET_ARA, PD155080, would lower BP in RRM rats with established hypertension on NS.

b. Protocol

PD155080 (25mg/kg b.i.d.) was administered in powder form mixed into sodium deficient chow in powder form as described previously. The experiment lasted a total of 15 days; 3 control days followed by 7 treatment days and ending with 5 recovery days. Arterial blood was sampled for ET-1 plasma levels and PD155080 plasma concentrations during each of these experimental periods. To assess the degree of endothelin receptor blockade, i.v. bolus's of ET-1 were administered and the level of blockade was estimated from the inhibition of the pressor (mediated by ET_A receptors) and depressor responses (mediated by ET_B receptors). Changes in renal function were monitored by measuring BUN, serum creatinine, urinary protein excretion, and creatinine clearance.

c. Results

Figure 28 presents the effect of PD155080 on BP in RRM and sham rats in NS. Untreated RRM rats demonstrated a sustained, gradually progressive hypertension

throughout the experiment. Hypertensive RRM rats receiving PD155080 exhibited a significant and well-maintained decline in BP throughout the treatment period. This antihypertensive effect was reversed by 24 hours after discontinuation of PD155080. In fact, during the recovery period, BP was significantly higher than during the pretreatment control period. Normotensive sham rats given PD155080 showed a slight, inconsistent hypotensive effect during the treatment period when compared to the BP during the 3 control days.

Figure 29 shows pressor and depressor responses to exogenously administered ET-1 (0.5 nmol/kg) in sham and RRM rats during each experimental period. Depressor responses were unchanged in individual groups from the control period values except for the sham rats given PD155080 (-19.8 mmHg control vs. -13.6 mmHg treatment). Pressor responses during all experimental periods in RRM rats were generally lower than those observed in the sham groups, but PD155080 treatment did not significantly impair acute pressor responses to ET-1 in either group of rats

There were no measurable differences in WB (Figure 30) or $U_{Na}V$ (Figure 31) throughout the experiment in untreated or PD155080 treated sham rats. In RRM rats, the first day of PD155080 administration resulted in a significant decrease in $U_{Na}V$ when compared control days (treatment: 1.22 ± 0.1 vs. control: 1.68 ± 0.2 mEq Na^+/24 hours). A concomitant increase in WB was observed on the first day of PD155080 treatment (treatment: 20.2 ± 3.6 vs. control: 11.0 ± 2.1 ml/24 hours) but this did not reach statistical significance. Upon discontinuation of PD155080 in RRM rats during the first recovery day, a significant increase in $U_{Na}V$ was observed when compared to control days (recovery: 2.20 ± 0.3 vs. control: 1.68 ± 0.2 mEq Na^+/24 hours). This significant

increase in $U_{Na}V$ was coupled with a decrease in WB during the first recovery day, which did not reach statistical significance (recovery: 8.2 ± 2.1 vs control: 11.0 ± 2.1 ml/24 hours

Indices of glomerular function during PD155080 treatment in sham and RRM rats are shown in Table 4. As expected, BUN, Scr and Upro were all elevated and Ccr decreased in RRM rats compared to sham rats. In sham rats, 7 day treatment with PD155080 caused no significant changes in glomerular function. Likewise PD155080 administration in RRM rats did not cause significant changes in glomerular function, despite a strong antihypertensive effect observed during the treatment period.

d. Interpretations

This study showed in RRM rats studied 4 weeks after reduction in renal mass that BP was significantly higher than that of sham rats. But plasma ET-1 concentrations were similar in the two groups (Table 4), confirming an earlier published report (Benigni *et al.*, 1991). ET-1 plasma levels were not significantly different between any groups during the experiment except for a slight elevation in non-treated RRM rats during the recovery period (control: 1.0 ± 0.1 vs. recovery: 1.7 ± 0.1 pg/ml). Nonetheless, one-week treatment with PD155080 caused a significant and sustained decrease in BP in RRM rats, while producing only a modest hypotensive effect in normotensive sham-operated animals. It is noteworthy that PD155080 administration in both sham and RRM groups did elevate plasma ET-1 concentrations from the control levels, but these changes did not reach statistical significance. The difference in BP response was not due to impaired elimination of PD155080 in rats with remnant kidneys, since plasma levels of the drug at the time of BP measurements were similar in both sham and RRM rats (sham: 7.50 ± 1.9

vs. RRM: 6.26 ± 4.1 μg/ml). It is not possible from these data to determine if the synthesis and release of ET-1 from vascular endothelial cells (or other tissues) is increased in RRM rats. Schiffrin and colleagues (Schiffrin *et al.*, 1995; Sventek *et al.*, 1996) reported increased vascular ET-1 gene expression in several models of hypertension in rats, but this has not been investigated in the RRM model. Failure to observe elevated plasma levels of ET-1 in the RRM rats in this study does not rule out increased ET-1 release from endothelial cells, because most of this secretion probably occurs abluminally.

It has been established that release of ET-1 from endothelial cells in the systemic vasculature causes vasoconstriction and smooth muscle cell growth by stimulating ET_A receptors (Rubanyi and Polokoff, 1994). The time course of the antihypertensive response to PD155080 in this experiment was too short for reversal of vascular structural changes. Therefore, inhibition of ET-1 induced vasoconstriction probably accounted for the BP lowering effect of ET_A receptor blockade in the RRM rats. Some of the data though seem inconsistent with this conclusion. For example, measurement of acute BP changes to bolus injections of ET-1 revealed that neither pressor (presumably mediated via vascular ET_A receptors) nor depressor (presumably mediated via endothelial ET_{B1} receptors) responses were significantly inhibited in rats receiving PD155080; yet resting BP in RRM rats was markedly decreased. Since only a single, high dose of ET-1 was used to assess receptor blockade acutely, these results may simply reflect the expected greater difficulty in showing receptor antagonism against higher doses of agonist. An alternative explanation is that endogenous ET-1 raises BP in RRM rats by an action on receptors distinct from those affected by acute i.v. boluses of the peptide, perhaps in the brain,

adrenal gland or other organs involved in BP regulation.

Increased ET-1 gene expression in the kidney and elevated urinary excretion of ET-1 occur in RRM rats, indicating enhanced intrarenal synthesis of the peptide (Orisio *et al.*, 1993). Renal ET-1 causes sodium retention so blockade of renal ET receptors by PD155080 could cause a fall in BP by promoting fluid excretion via the kidney. Recent work suggests, however, that most actions of ET-1 in the rat kidney are mediated through ET_B receptors, making this explanation unlikely (Pollock *et al.*, 1993; Wellings *et al.*, 1994: Qiu *et al.*, 1995). These results also do not support such an explanation in that PD155080 administration in RRM rats was associated with sodium and water retention rather than diuresis and natriuresis.

These findings indicate that ET_A receptor blockade may be an effective therapy for the hypertension associated with CRF. It is obviously important, however, that any such therapy not further impair renal glomerular function. It is therefore noteworthy that the antihypertensive response to PD155080 in RRM rats was not accompanied by any measurable decrease in creatinine clearance, or increase in plasma creatinine, urea nitrogen or urinary protein excretion over the short time-course of this experiment.

Figure 28: Chronic mean arterial pressure responses to ET_A (PD155080, 25 mg/kg/b.i.d.) receptor antagonist administration in RRM and sham rats on normal salt intakes. *Asterisks indicate decreases in pressure from within group control measurements. † Daggers denote increases in pressure from within group control values.

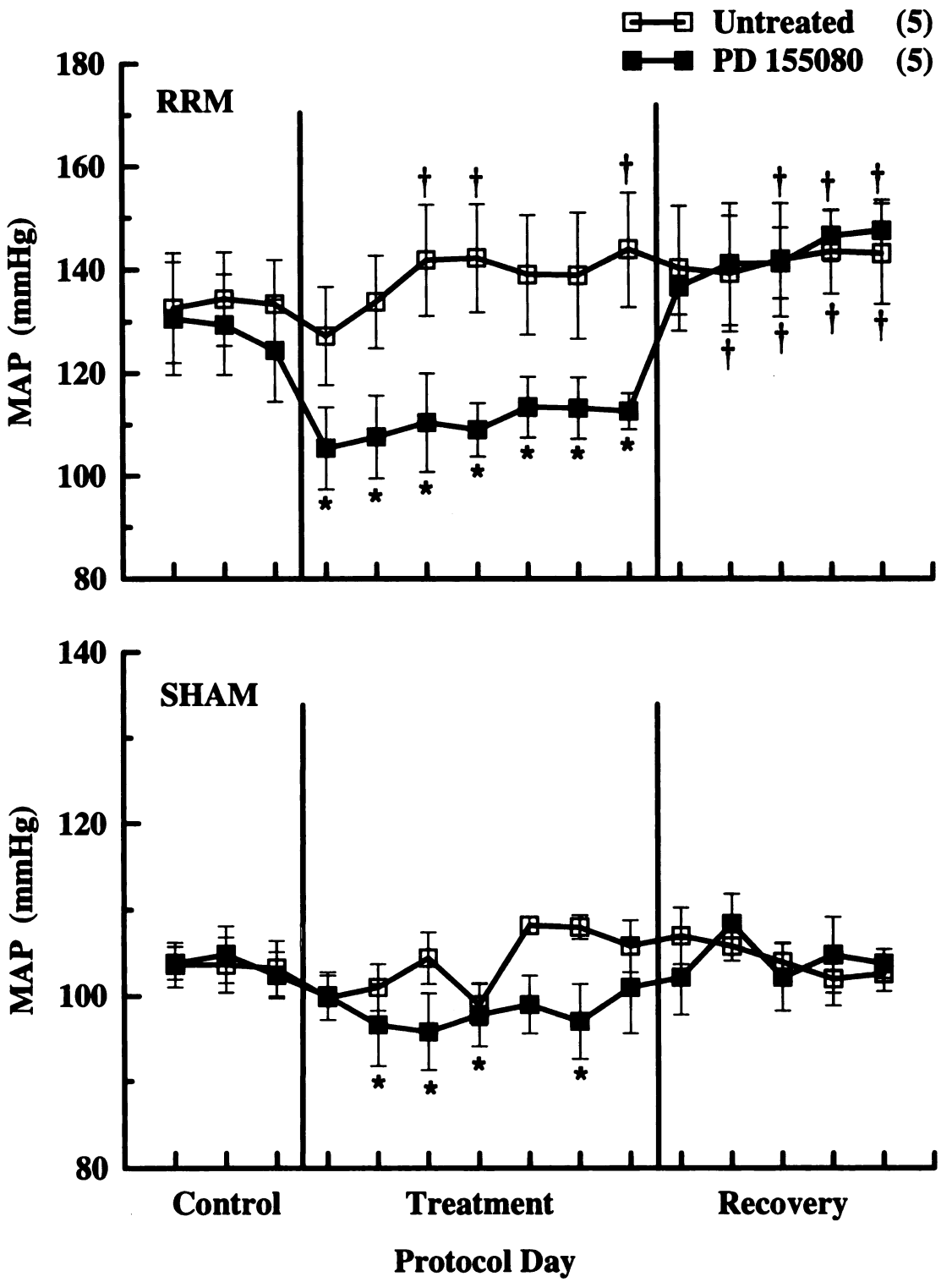

Untreated (5)
PD 155080 (5)
RRM
SHAM
MAP (mmHg)
180
160
140
120
100
80
Control
Treatment
Recovery
Protocol Day

Figure 28

Figure 29: Acute pressor and depressor responses to bolus i.v. ET-1 (0.5 nmol/kg) injections in sham and RRM rats on normal salt intakes during control, treatment and recovery experimental periods. *Asterisks indicate decreases in responses from within group control measurements. † Daggers denote decreases in pressor responses between RRM and sham groups within each individual experimental period.

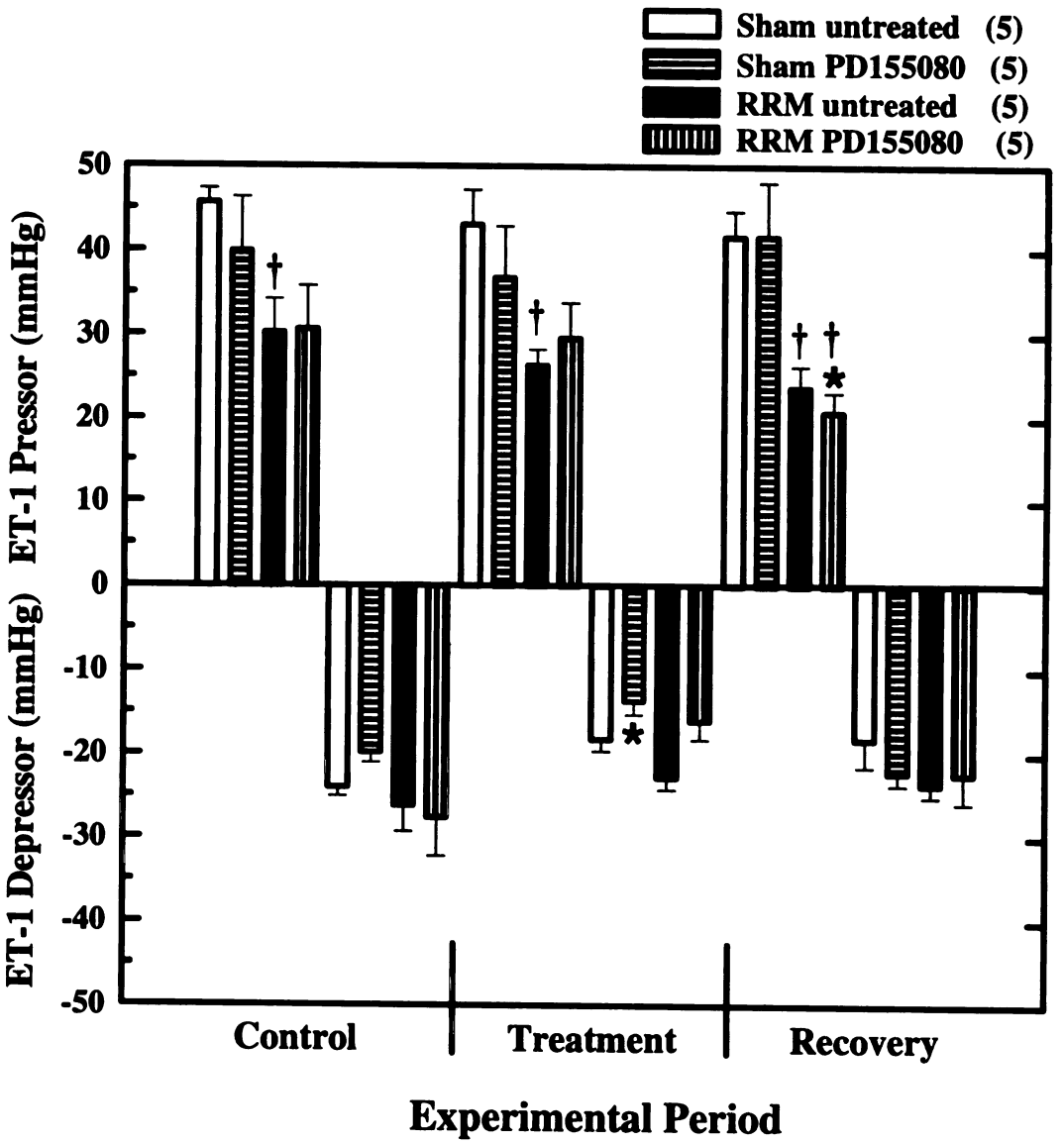

Sham untreated (5)
Sham PD155080 (5)
RRM untreated (5)
RRM PD155080 (5)
ET-1 Depressor (mmHg) ET-1 Pressor (mmHg)
50
40
30
20
10
0
-10
-20
-30
-40
-50
†
*
Control
Treatment
Recovery
Experimental Period

Figure 29

Figure 30: Water intakes, urine outputs and water balances in response to chronic ET_A (PD155080, 25 mg/kg/b.i.d.) receptor antagonist administration in sham and RRM rats on normal salt intakes.

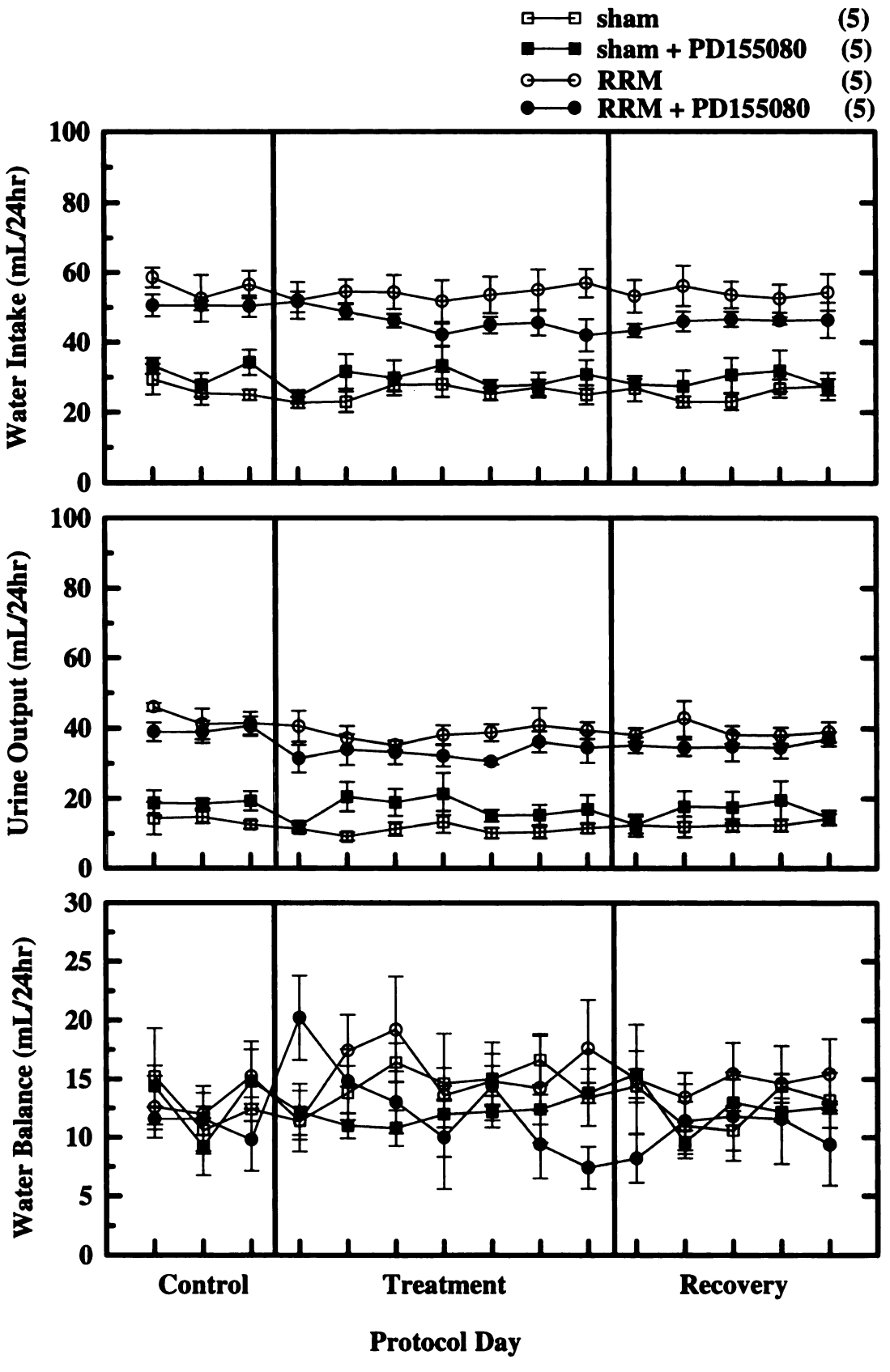

sham (5)
sham + PD155080 (5)
RRM (5)
RRM + PD155080 (5)
Water Intake (mL/24hr)
Urine Output (mL/24hr)
Water Balance (mL/24hr)
Control
Treatment
Recovery
Protocol Day

Figure 30

Figure 31: Urinary sodium excretions during chronic ET_A (PD155080, 25 mg/kg/b.i.d.) receptor antagonist administration in sham and RRM rats on normal salt intakes. All rats were maintained on a fixed sodium intake of 2 mEq/day administered in the i.v. saline solution. *Asterisk indicates decreases in sodium excretion from within group control values. † Dagger denotes increases in sodium excretions from within group control values.

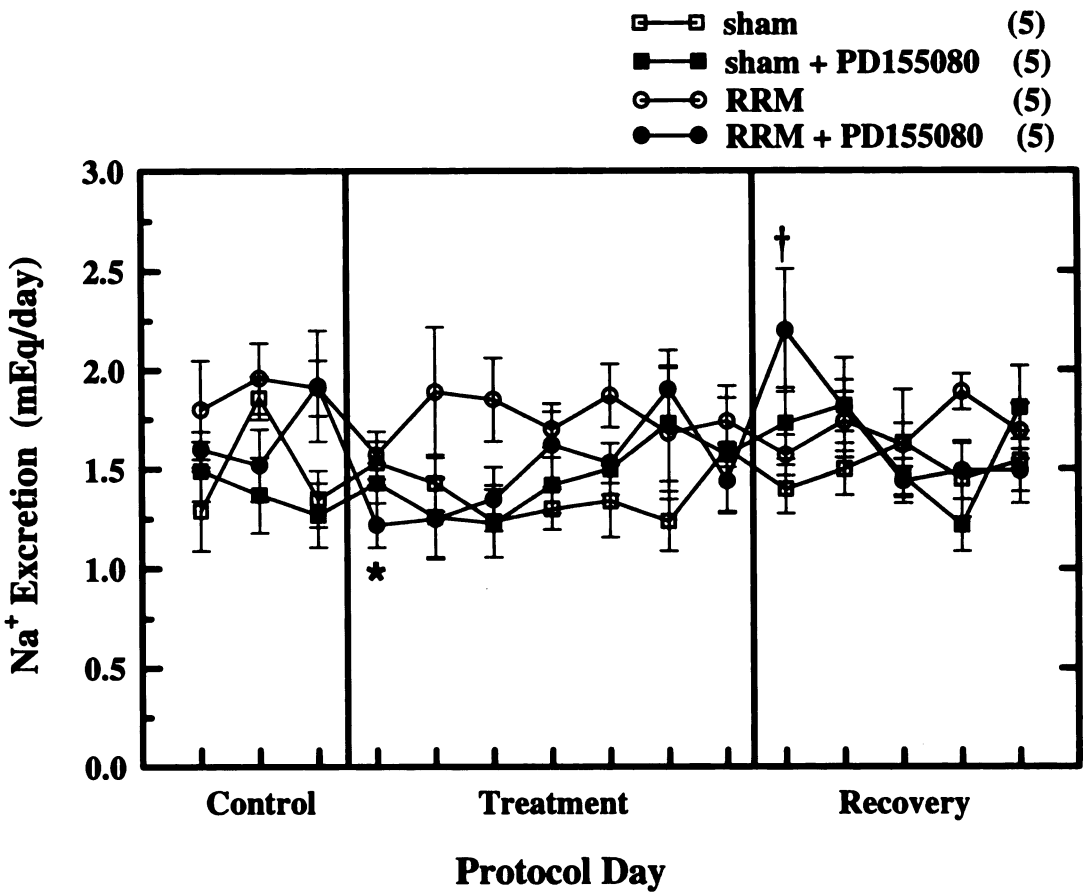

sham (5)
sham + PD155080 (5)
RRM (5)
RRM + PD155080 (5)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Na+ Excretion (mEq/day)
*
†
Control
Treatment
Recovery
Protocol Day

Figure 31

Table 4: Effects of PD155080 on renal parameters, ET-1 plasma concentrations, and PD155080 plasma concentrations in sham and RRM rats maintained on a normal salt intake. All RRM renal parameters (BUN, Scr, Ccr, Upro) are significantly different from the corresponding sham values at each time point but are not annotated for ease of comparison. BUN, blood urea nitrogen, Scr, serum creatinine, Ccr, creatinine clearance, Upro, urinary protein excretion, $[ET\text{-}1]_p$, plasma ET-1 concentration, $[PD155080]_p$, plasma PD155080 concentration. *Asterisks indicate differences from within group control measurements.

Table 4:

	BUN mg/dl	Scr mg/dl	Ccr ul/min/100 g body wt	Upro mg/day/100 g body wt	[ET-1]p pg/ml	[PD155080]p ug/ml
Sham						
Untreated (n=5)						
Control	13.4 ± 1.5	0.625 ± 0.08	340 ± 30	22 ± 2.1	1.4 ± 0.2	
Treatment	14.3 ± 1.8	0.810 ± 0.13	270 ± 66	19 ± 2.2	1.4 ± 0.2	
Recovery	13.5 ± 1.0	0.760 ± 0.14	284 ± 19	25 ± 4.2	1.4 ± 0.3	
PD 155080 (n=5)						
Control	14.4 ± 0.9	0.491 ± 0.06	401 ± 52	15 ± 2.2	1.0 ± 0.1	
Treatment	13.3 ± 1.5	0.683 ± 0.05	321 ± 40	16 ± 1.7	7.5 ± 5.3	7.5 ± 1.9
Recovery	15.4 ± 1.3	0.887 ± 0.23	270 ± 44	22 ± 2.6	14.8 ± 8.7	
RRM						
Untreated (n=5)						
Control	60.2 ± 4.6	1.784 ± 0.31	141 ± 32	48 ± 8.2	1.0 ± 0.1	
Treatment	47.8 ± 5.4 *	1.476 ± 0.22	137 ± 16	59 ± 10.9	1.4 ± 0.1	
Recovery	62.2 ± 6.9	1.686 ± 0.36	141 ± 30	57 ± 5.5	1.7 ± 0.1*	
PD 155080 (n=5)						
Control	52.0 ± 2.9	1.250 ± 0.12	187 ± 18	62 ± 5.5	1.1 ± 0.2	
Treatment	43.5 ± 8.5	1.366 ± 0.12	151 ± 23	50 ± 7.6	3.1 ± 0.7	6.26 ± 4.1
Recovery	54.5 ± 8.0	1.410 ± 0.11	148 ± 12	64 ± 6.5	2.1 ± 0.7	

DISCUSSION

A close relationship exists between hypertension and CRF. Hypertension, the second leading cause of renal disease, is commonly observed in renal failure patients that have progressed to ESRD. Long-term multicenter studies of renal disease patients have concluded that elevations of BP are a strong independent risk factor for ESRD (Klag *et al.*, 1996). Many recent studies have shown that control of BP is associated with a slower rate of decline in renal function (Brazy *et al.*, 1989; Pettinger *et al.*, 1989) and reduced risk of hypertension-related diseases.

The primary purpose of the experiments described here was to investigate the role of humoral factors, specifically, AngII and ET, in the pathogenesis of hypertension in the RRM model of CRF. I hypothesized that the relative contribution of these two hormones to both short-term and long-term BP regulation in CRF differs depending on the level of salt intake. The work is highly relevant to the treatment of human CRF, which currently entails both regulation of dietary salt, and aggressive drug therapy aimed at controlling BP.

My experimental approach was designed to study the mechanism(s) of hypertension associated with CRF using the RRM animal model. The mechanisms by which BP is regulated in the short-term are not identical to those involved in long-term BP control and hypertension may result from a disorder of any of these. I decided that a

complete evaluation of the possible causes of hypertension in CRF required investigation of physiological systems contributing to both short-term and long-term BP regulation. This was achieved by examining the effects of acute and chronic therapy with specific pharmacological inhibitors of the renin-angiotensin and endothelin systems.

Previous investigation led me to hypothesize that the relative importance of each of these systems in BP control differs depending on the dietary intake of salt. It is known decreasing salt intake causes activation of the RAS, whereas changes in salt intake do not appear to affect ET production (Schiffrin *et al.*, 1996). Since salt intake is so relevant to the pathophysiology of human CRF, I decided to fully investigate the influence of each on BP under different levels of salt intake.

I. Comparison of responses to alterations in salt intake in RRM and sham rats prior to administration of inhibitors of the RAS and ET.

A. Blood pressure

1. Reduced renal mass: HS>NS>LS

The development of hypertension during the 3 levels of salt intake was very diverse and directly related to the amount of salt consumed (Figure 32). Even though the HS groups were studied after the least amount of time following partial ablation (2 weeks), they exhibited the highest BP. On the other end of the salt intake spectrum, the LS groups never became hypertensive even 12 weeks following RRM.

Some investigators have proposed that all hypertension is due primarily to a renal defect restricting the excretion of sodium (de Wardener, 1990b). During increases in salt intake in individuals where renal function is compromised, the kidneys experience an inability to excrete the excess salt leading to sodium and water retention. This volume

expanded state leads to a temporary elevation of cardiac output (CO). Later in the disease progression sodium and water balance are re-established through pressure diuresis/natriuresis and the hypertension maintained by a chronically elevated peripheral vascular resistance (PVR)(Lombard *et al.*, 1989). An elevated PVR is the basic hemodynamic abnormality underlying the maintenance of most forms of hypertension. In RRM hypertension this elevated PVR has been associated with an increased SNA, elevated levels of natriuretic hormones, and an altered activity of the RAS.

During sodium restriction RRM rats have diminished serum sodium concentrations, reduced intravascular volume, and an activated RAS, but BP remains in the normal range (Ylitalo *et al.*, 1976). The progression of BP is attenuated when excision method RRM rats are maintained on sodium restricted diets (Ylitalo *et al.*, 1976; Terzi *et al.*, 1992) and exacerbated by sodium loading (Koletsky, 1959; Ylitalo *et al.*, 1976; Douglas *et al.*, 1964). The general consensus is that BP elevation observed in the ligation method of RRM is not salt sensitive.

2. Sham: HS=NS=LS

Wide variations in salt intake had no effect on BP when renal function was unaltered, as exhibited in sham rats in these experiments. All groups of sham rats remained with normal BP ranges (100-110 mmHg). Intrarenal and hormonal compensations apparently were sufficient to maintain sodium and water balance without the recruitment of alterations in systemic BP. My results confirm earlier reports by Ylitalo and colleagues who demonstrated that alterations in salt intake ranging from highs of 10-15 mEq/day to lows of 0 mEq/day did not alter BP over a period of 4-6 weeks from levels observed in rats maintained on NS (Ylitalo *et al.*, 1976). Elevations in BP

associated with drinking 1.0% saline have been reported in normal rats but these increases were not observed in all rats (66%) and only after 6-12 months on the increased salt intake (Koletsky, 1959).

B. Blood urea nitrogen: NS=LS>>HS

Changes in BUN levels were utilized throughout my thesis as an estimate of a decline in GFR. A declining GFR is the hallmark of diagnosis in patients with CRF. For the most part, BUN levels increased as the time following renal ablation increased. In the HS rats, slightly elevated to high normal BUN levels were measured, suggesting that little overall renal deterioration had yet occurred.

This finding is surprising given the approximately 50% mortality in the HS rats. It is likely that HS rats did not die due to renal dysfunction per se but may have experienced some other lethal cardiovascular event (i.e. myocardial infarction or stroke) due to the increased pressure and excessive salt intake. High salt itself is probably responsible in part for the low BUN values observed in these rats. Guyton and co-workers measured a significant increase in GFR when isotonic saline was substituted for tap water in partially nephrectomized dogs (Langston *et al.*, 1963). During the switch to isotonic saline BUN values that had more than doubled following partial nephrectomy were reduced almost back to normal levels (tap water: 53.1 $\pm$ 2.1 vs. saline: 27.0 $\pm$ 1.0 mg/dl). Similar findings were reported by Koletsky who showed that renal excretory function was less compromised in RRM rats drinking 1% saline than in RRM rats drinking tap water (Koletsky, 1959).

The BUN levels measured in the NS and LS experiments were similar in magnitude even though BUN in LS rats was measured twice as long after nephrectomy.

This may be due to a protective effect of LS on glomerular injury. BP was in the normotensive range in LS, therefore renal deterioration due to an elevated BP exerted little or no role in the progression of renal disease under these conditions. Also Dworkin and colleagues have reported in RRM that salt restriction lessens renal deterioration in the presence and absence of BP reduction (Lax *et al.*, 1992; Dworkin *et al.*, 1996). The renoprotective effect of salt restriction in the absence of reductions in BP has been observed in other models of renal disease such as the uninephrectomized SHR (Benstein *et al.*, 1990). It has been suggested that the beneficial effects of salt restriction are related to inhibition of compensatory renal growth. Sodium restriction has been reported to slow the growth of the kidney as well as other organs and inhibit tubular cell hyperplasia (Solomon *et al.*, 1972; Gallaher *et al.*, 1990; Ostlund *et al.*, 1991). These studies support my experiments in RRM on LS in that salt restriction was sufficient to slow the progression of renal deterioration.

C. Water intake and urine output

1. Reduced renal mass: HS>NS>LS

Increased WI is commonly observed in CRF and RRM due to water loss caused by osmotic diuresis and impaired renal concentrating ability. Thirst can be stimulated by the retention of osmotically active substances or by high AngII levels under certain conditions (Mitch and Wilcox, 1982). A diminished ability to concentrate the urine in RRM leads to increases in UO and WI.

All groups of RRM rats in my experiments had elevated WI and UO when compared to sham animals maintained on the same level of salt intake. Increases in UO paralleled WI so that WB was not increased in RRM. This was to be expected because

fluid intake and output must be precisely balanced under steady-state conditions or continuous expansion would lead to circulatory collapse within days.

WI and UO were greatest in RRM rats on HS and least under LS conditions. Similar results have been reported in rats undergoing excision of renal mass and varying salt intake (Ylitalo *et al.,* 1976; Ylitalo and Gross, 1979). A centrally mediated increase in circulating AVP has been implicated in the increased fluid intake during HS. Adding salt to the drinking water increases the osmolarity of the blood which is detected by osmoreceptors in the hypothalamus. Activation of hypothalamic osmoreceptors initiates increases in AVP release and mechanisms that involve somatic responses leading to the consumption of water. Increased plasma levels of AVP have been found in RRM rats in addition to elevated WI (Gavras, 1982).

2. Sham: HS>NS=LS

The addition of salt to the drinking water elicited an increase in WI in sham rats when compared to rats maintained on NS or LS. No differences in WI were observed between sham rats kept on NS or LS. Others have reported increased WI in normal rats on elevated salt intakes (Ylitalo and Gross, 1979; Ylitalo *et al.*, 1976). The mechanisms are linked to activation of osmoreceptors in the hypothalamus and a sodium appetite in the rat.

D. Mortality

One interpretation from these data is that the best therapy for CRF is a dietary intake very low in NaCl. I did not specifically monitor pathological factors associated with low NaCl intake, but upon general observation, the groups of rats maintained on this regimen looked the healthiest (*i.e.* gained weight) and survived the longest as compared

to any other level of salt intake. Mortality estimates were proportional to the level of salt intake and inversely proportional to the length of time following partial renal ablation. Roughly 50% of RRM rats maintained on HS, 20% on NS and < 10% on LS died before completing the study. The degree of hypertension associated with each of these levels of salt intake surely played an important role in these mortality estimates. Others have shown that partial nephrectomy plus salt reduces life span by almost 25% over rates observed with partial nephrectomy alone (Koletsky, 1959). The LS experimental data strongly suggest that patients with renal disease be kept on a restricted salt intake.

II. Influence of ACEI under varying levels of salt intake

A. Acute

The acute experiments described above were designed to evaluate the role of the RAS short-term BP regulation under varying salt intakes. Acute results following ACE inhibition in RRM demonstrated that BP was not altered at any level of salt intake (Figure 2). On the contrary in sham rats, BP was lowered during all levels of salt intake while only reaching significance during LS. Bolus administration of enalaprilat was designed to acutely inhibit the formation of AngII over a period of minutes to hours. From this type of approach I could evaluate the contribution of AngII's fast pressor effects on the maintenance of BP. High AngII levels are required to cause direct contraction of the vasculature through the fast pressor effect. When circulating levels of AngII are high, it would be expected that administration of ACEI would reduce AngII production thereby lowering BP. It is evident from these acute results that direct vasoconstriction by AngII operating through the fast pressor mechanism plays little role in short-term BP regulation

BP control under LS conditions when the RAS is known to be highly activated. Figure 33 summarizes the relative theoretical influence on BP due to the fast pressor effect in normal and RRM rats under varying salt intakes.

B. Chronic

1. Support for enalapril dose used in experiments

Other investigators have shown hemodynamic effects with ACEI in RRM rats with lower doses of enalapril than were used throughout these studies. Enalapril administration of 25 mg/L (Lafayette *et al.*, 1992) and 50 mg/L (Anderson *et al.*, 1985) in the drinking water has been reported to slow the rise in BP in RRM rats NS. On the contrary, pilot studies in our lab showed that enalapril in the drinking water at 50 mg/L did not significantly attenuate the rise in BP observed in vehicle treated RRM rats over 7 days (Figure 34). The differences in efficacy of enalapril may due to the different experimental approaches used. First of all, Lafayette and Anderson measured BP by tail plethysmography, which is not as reliable a measurement of BP obtained from an arterial catheter. Secondly, they achieved the reduction in renal mass by the ligation method, whereas I utilized the excision method. Lastly, enalapril treatment in their studies began immediately following the completion of the ablation. In my experiment, enalapril treatment was initiated 4 weeks after ablation. This period of time allows for compensatory renal changes to occur, and hypertension to develop. This approach is better suited for the evaluation of the inhibition of the RAS in established RRM hypertension. The inability of enalapril administration at 50 mg/L to significantly lower BP in my experiment when RRM rats were maintained on NS may also be attributed to a

shorter treatment time (1 week) compared to the other studies (3-5 weeks). This duration of administration may not permit enalapril's full antihypertensive effects to be realized.

The difference in antihypertensive effectiveness in my normal salt studies between the 50 mg/L and 250 mg/L dosages can not be attributed to an inadequate inhibition of ACE in the 50 mg/L group because both groups had similar inhibition of AngI pressor responses during treatment days (enalapril 50 mg/L control: 34.5 $\pm$ 3.7 vs. treatment: 3.4 $\pm$ 1.2 mmHg; enalapril 250 mg/L control: 32.7 $\pm$ 4.6 vs. treatment: 5.2 $\pm$ 1.1 mmHg). Similar ACE inhibition was achieved in spite of large differences in the average daily dose of enalapril between 50 mg/L (2.6 mg/day) and 250 mg/L (13.5 mg/day) administration. As mentioned above, both Anderson and Lafayette have shown dosages of 25-50 mg/L to significantly block AngI pressor responses and decrease BP in hypertensive RRM rats albeit in the ligation method. These factors suggest that enalapril did reach the systemic circulation in sufficient quantities to inhibit ACE in each of my treatment groups.

Another justification for the dose of enalapril used throughout my thesis work was the report of additional renal benefit with doses higher of enalapril than those needed to control systemic BP (Ikoma *et al.*, 1991). These investigators suggest that ACEI in dosages in excess of those required for antihypertensive effects have the potential to preserve glomeruli not yet exhibiting sclerotic lesions and to reverse early glomerular lesions. Most of my studies were designed to initiate ACEI therapy well after partial nephrectomy which would be similar to human CRF. The potential to reverse glomerular lesions with high dose enalapril seemed especially attractive.

2. Reduced renal mass

This work was aimed at characterizing how salt intake effects the actions of ACEI in RRM hypertension. The hypothesis was that the relative importance of the RAS in the elevated BP associated with renal failure was largest under conditions of a dietary intake devoid of sodium. As mentioned earlier, this is a critical point given that dietary salt restriction is an important part of the therapy of CRF in humans.

My experimental data support the original hypothesis that the activity of the RAS is inversely proportional to the level of salt intake in RRM. Under conditions of HS in RRM rats, enalapril did not change the progressive increase in BP observed in non-treated rats (Figure 4). My results are in agreement with reports from Terzi who showed that RRM rats on a 0.50% sodium diet (normal-high) developed progressive hypertension over a 12 week period (Terzi *et al.*, 1992). Enalapril by gavage at 3 mg/kg/day starting one week after ablation failed to cause any significant change in the progression of hypertension.

Elevations in BP during HS are thought to result from intravascular fluid volume expansion. This is secondary to excessive retention of sodium and water, and is associated with suppression of the RAS. Since the role of the RAS is minimal under these conditions, it was not surprising that enalapril was unable to lower BP in my experiment. Other mechanisms involved in the progression of hypertension in RRM during high salt intakes have been proposed. Vasoconstrictors other than AngII may play more of a role in BP regulation under high salt conditions. Much of my experimental results have focused on the role of ET in RRM on HS and this will be discussed in detail later in this paper. Dipette and co-workers have suggested a neurogenic mechanism for

salt-induced hypertension in RRM and have measured increased plasma norepinephrine concentrations (Dipette *et al.*, 1982). Salt is known to stimulate growth of many cell types (*i.e.* mesangial cells) and these actions may effect renal function and/or vascular hypertrophy.

When i.v saline administration was fixed at levels known to approximate salt intakes observed in normal rats, enalapril treatment attenuated the progressive rise in BP that was recorded in non-treated RRM rats (Figure 7). Other investigators have reported similar beneficial results using ACEI in RRM rats maintained of NS over longer periods of time. All of these studies that utilized the excision method of RRM report prevention or slowing of renal deterioration and hypertension with prophylactic administration of the ACEI. Enalapril administration for 8 weeks (Amann *et al.*, 1993) or captopril for 12 weeks (Ashab *et al.*, 1995) immediately following partial renal ablation slowed the development of hypertension and renal deterioration observed in non-treated RRM rats. Results from my experiment using RRM rats maintained on NS for 4 weeks prior to enalapril treatment also show a prevention of the progression of hypertension over 1 week administration of the ACEI. I hypothesized further that 1 week of enalapril administration would reverse established RRM hypertension when rats were kept on NS. This hypothesis was incorrect. Perhaps longer administration of enalapril might have lowered established hypertension in my RRM rats but that is only speculation at this time and is not supported by the literature. My results from this experiment suggest that AngII plays a role in long-term BP regulation in RRM under NS conditions. These data support the work of others who have demonstrated that AngII is a necessary component of the progressive elevations in BP observed in RRM rats.

Experimental data from LS studies also support the original hypothesis that the activity of the RAS is inversely proportional to the level of salt intake in RRM. Normotensive RRM rats maintained on LS exhibited the largest decrease in BP in response to enalapril administration. Enalapril's hypotensive effect in RRM on LS was quite remarkable considering BP fell 25-30 mmHg below what is considered the normal range for rats.

Others have investigated the role of the RAS in RRM (excision method) during salt restriction. Work by Brenner has shown that enalapril was more efficacious in reducing proteinuria and preventing the progression of hypertension when RRM rats were maintained on a five fold reduction from normal salt intake (Brenner *et al.*, 1989). Additional results from Terzi in RRM rats demonstrate that moderate sodium restriction retards the progression of hypertension and causes an antihypertensive effect of enalapril that was not observed when RRM rats were maintained on a moderately elevated salt intake (Terzi *et al.*, 1992). Once again these studies incorporated prophylactic administration of enalapril to prevent progressive renal deterioration and hypertension.

These experiments were designed to demonstrate reversal of established hypertension in RRM rats. The lack of even a moderately elevated BP in rats maintained on LS for 8 weeks was unexpected. This might have been due to the severity of salt restriction and/or the lack of sufficient renal mass reduction. The dramatic fall in BP following enalapril administration in RRM rats on LS suggests that the RAS plays an important role in long-term BP regulation under these conditions and that activation of the RAS is enhanced by salt restriction. Overall my experiments show that the relative

influence of the RAS in long-term BP regulation in RRM is largest under conditions of lower dietary salt intakes.

3. Sham

Long-term enalapril administration in sham rats maintained on HS was not addressed in my thesis work because of the lack of an effect on BP with enalapril in RRM rats under these conditions. Enalapril lowered resting BP in sham rats maintained on NS and LS with the largest reduction recorded in the latter group. It is not surprising that ACEI in LS sham rats (where the activation of the RAS is highest) lowered BP. It was unexpected that enalapril would lower BP to such an extent in sham rats on NS. Not all reports in the literature (Amann *et al.*, 1993) confirm my results in NS rats but others in our lab have published similar results (Melaragno and Fink, 1995). These data suggest that AngII is an important contributor to basal levels of BP in normal rats maintained on NS and LS. They also support the role of the RAS in long-term regulation of BP under these conditions.

III. Mechanism of action of ACEI

One of my main objectives in this thesis was to determine the mechanism of action of the BP lowering effect of ACEI in RRM rats. The mechanism of action of ACEI has been debated for years. ACEI were rationally designed to inhibit the formation of circulating AngII. Most evidence supports the hypothesis that ACEI exert their antihypertensive effect through inhibition of AngII formation. The experimental data supporting this hypothesis is extensive although agreement is not universal. Some of the most convincing evidence comes from studies comparing the effects of ACEI to AT_1 receptor antagonists in RRM. Losartan alone produced the same magnitude of

antihypertensive effect as enalapril, and combination of these two agents produced no additional benefit on BP (Lafayette *et al.*, 1992). Some investigators have suggested that the antihypertensive effectiveness of these inhibitors involves a variety of other pathways such as: decreased degradation of BK, increased production of vasodilatory prostaglandin's, inhibition of local tissue RAS, and increased NO production. Studies designed to investigate these other pathways have not produced convincing evidence that ACEI lower BP by mechanisms other than by a reduction in AngII production.

I hypothesized that ACEI affect BP in RRM by inhibiting the production of physiological amounts of AngII. Infusion at physiological rates of AngII into RRM rats given enalapril during NS conditions restored the progressive rise in BP observed in untreated rats. A necessary prerequisite for this type of experimental approach was to establish that ACEI completely inhibit endogenously produced AngII. Work done in our lab has shown that when an AT_1 receptor antagonist (losartan) was given to rats chronically receiving ACEI (enalapril, 250 mg/L), BP did not further decrease over the next 24 hours (Figure 35). In untreated normal rats this dose of losartan caused a significant decrease in BP and blocked pressor responses to exogenous AngII (Sacerdote *et al.*, 1995). This additional evidence supports the assertion that enalapril treatment at 250 mg/L successfully blocks ACE, and suggests that enalapril reduces endogenous AngII formation to a functionally insignificant level.

In the normal salt scenario, exogenous AngII replacement at a rate of 2 ng/min restored the progressive rise in BP normally observed in RRM rats. Likewise in sham rats on a normal salt intake, AngII at 2 ng/min restored the basal level of BP. This low replacement rate of AngII is considered to produce systemic AngII concentrations within

the physiologic range. This work supports the hypothesis that enalapril prevented rises in BP only through inhibition of AngII formation. If enalapril was working through mechanisms other than the inhibition of AngII formation, then I should have not been able to reverse the ACEI's full effect on BP by replacing AngII systemically.

IV. Mechanism of action of AngII in RRM hypertension

A. Role of sodium excretion

AngII exerts multiple actions to control body fluid volume, sodium excretion and blood pressure. Extrarenal (i.e. stimulation of thirst, activation of the sympathetic nervous system, secretion of aldosterone and AVP) and intrarenal (i.e. efferent arteriole constriction, proximal tubule sodium reabsorption) effects make AngII one of the body's most powerful controllers of sodium and fluid homeostasis and long-term BP regulation. Most evidence suggests that direct intrarenal actions of AngII play the major role in the excretion of sodium under physiological conditions (Hall and Brands, 1993). Sodium retention is achieved mainly through AngII mediated increases in proximal tubular reabsorption. Blockade of AngII formation (i.e. ACEI) reduces sodium reabsorption whereas high AngII levels (i.e. AngII infusions) elevate sodium reabsorption.

High circulating AngII concentrations cause increases in BP, and the resulting increase in renal perfusion pressures initiates a transition from sodium retention to sodium excretion via pressure-natriuresis. Thus, the natriuresis associated with high rates of AngII infusion is not caused by decreases in proximal tubular reabsorption (Hall and Brands, 1993). The net effect of AngII on sodium excretion depends on the balance of direct antinatriuretic actions of AngII and the natriuresis resulting from increasing renal perfusion pressure.

In my first experiments in RRM rats maintained on HS, the addition of NaCl to the drinking water resulted in increased sodium excretions (Figure 6). Inhibition of AngII formation by enalapril did not affect $U_{Na}V$, sodium balance, or BP during HS or NS experimental periods. These results were not unexpected because of the anticipated lack of RAS involvement during HS conditions. Therefore it is doubtful that and AngII mediated antinatriuretic effect played a role in RRM hypertension under these HS conditions.

In RRM rats maintained on NS, no changes in $U_{Na}V$ were measured even though significant changes in BP occurred during some treatments (Figure 12). These results do not support increases in sodium reabsorption as the cause of progressive elevations in BP observed in RRM rats on NS. Enalapril administration alone, which successfully inhibited AngII formation, was expected to elicit an increase in sodium excretion. But BP was lower in enalapril treated than untreated RRM groups. The decreased BP, which lessens sodium excretion may have opposed any inhibition of sodium tubule reabsorption during ACEI administration. Another consideration is that the activity of the RAS is low to normal in RRM and the reduction of an AngII mediated effect due to inhibition of already low plasma AngII concentrations may not exert an effect readily observable using our methods. It was expected that the highest rate of AngII replacement (4 ng/min) would increased sodium excretion due to the elevation in BP overwhelming directly mediated AngII proximal tubule reabsorption. This was not observed. My data suggest that the hemodynamic effects of AngII on natriuresis counteract the intrarenal effects on the proximal tubule resulting in no net change in sodium excretion.

In sham rats on NS, enalapril treatment alone was associated with a significant natriuresis that developed only after several days on enalapril and was reversed by two days after enalapril discontinuation. In fact, the majority of the recovery days were associated with a significant sodium retention. The natriuretic response observed in enalapril treated sham rats during the treatment period was most likely due to decreased AngII stimulated tubular reabsorption. This natriuretic effect may even have been greater if it were not for the BP lowering influence limiting natriuresis. The substantial hypotensive effect of enalapril suggests that activity of the RAS is greater in sham than in RRM rats on NS. This explains why the natriuretic response to ACE inhibition was only measurable in sham rats. The decrease in sodium excretion after withdrawal of enalapril supports the role of AngII in mediating sodium reabsorption.

The natriuresis observed after enalapril did not contribute to the hypotensive effect of ACEI. My data show than very low rates of AngII infusion (1 ng/min) were enough to reverse the natriuretic effect of ACEI (Figure 13). This reversal occurred without a full restoration in BP. In fact the hypotensive effect due to enalapril alone or enalapril plus 1 ng/min AngII was not different. My data suggest that in sham rats the actions of AngII in the proximal tubule on sodium reabsorption are more sensitive than the actions of AngII on the systemic vasculature. Work by Hall and colleagues has shown that proximal tubule transport is approximately 1000 fold more sensitive to AngII than contraction of aortic vascular smooth muscle (Hall and Brands, 1993). Upon infusion of higher rates of AngII, no additional changes in sodium excretion or BP were observed. This is the case because the normalization of BP elicited an increase in sodium excretion that offset the AngII stimulated increase in tubule reabsorption of sodium. I concluded

from this experiment that AngII mediated effects on sodium reabsorption do not play a significant role in the maintenance of BP in normal rats on NS.

Urinary sodium excretion was not affected by enalapril administration alone or in combination with AngII replacement in either RRM or sham rats under sodium deplete conditions (Figure 18). The substantial BP lowering effect of ACEI predicts that a decrease in sodium excretion would occur, whereas loss of intrarenal AngII would be expected to increase sodium excretion. These effects appeared to cancel one another, but subtle changes in sodium excretion may not have been detected because the sodium excretory rates in both sham and RRM rats were barely measurable (zero to 0.1 mEq/24h). Infusion of exogenous AngII during enalapril treatment also did not measurably affect sodium excretion, probably because proximal reabsorption of sodium was already maximal under LS conditions, even in the absence of AngII. Because BP fell in the absence of any preceding alterations in sodium excretion, I concluded that changes in sodium balance due to inhibition of AngII formation were unlikely to exert an effect on BP under these LS conditions.

B. Increased responsiveness in RRM

I hypothesized that an increased sensitivity of AngII AT_1 receptors in the vasculature could be involved in the hypertension observed in RRM. Activation of AT_1 receptors in the vasculature results in contraction of VSMC leading to vasoconstriction an elevated BP. If RRM or salt increases the sensitivity of these vascular receptors to AngII then bolus challenges of AngI should elicit greater pressor responses in RRM rats. This did not occur in these experiments. This indicates that reduction of renal mass did not cause an increased vascular response to AngII. Thus, there was no change in the fast

pressor response to AngII in RRM. Previous studies support my observations that acute pressor responses to AngII were not different between RRM and normal rats on both HS and NS (Kanagy *et.al.*, 1993).

Another potential mechanism that was addressed in my experiments was that RRM rats exhibited an increased chronic responsiveness to circulating AngII. Days after discontinuation of enalapril treatment, BP in RRM rats maintained on NS did not return to the rate of increase that was recorded in the vehicle group of RRM rats (Figure 7A). This effect persisted throughout the recovery period and was not observed in sham rats on the same dose of enalapril (Figure 8A). A possible explanation for this sustained antihypertensive effect was persistent inhibition of plasma ACE. Yet AngI pressor responses were back to control levels 2-3 days into the recovery period. Another possibility is that in RRM there is a slowly developing pressor effect of AngII that was inhibited during enalapril treatment. The "slow pressor effect" as described in the introduction is the phenomenon observed when low doses of AngII i.v. infusion produce increases in BP over hours to days. This is in contrast to the fast pressor effect of AngII, which is caused by higher doses of AngII via direct vasoconstriction. If AngII was working to raise BP in RRM through fast pressor effects, then restoration of circulating AngII concentrations after discontinuation of enalapril should have increased BP within minutes to hours. This did not occur. Furthermore, if the fast pressor effect of AngII was operative in RRM rats on NS, acute enalaprilat treatment should have lowered BP, an outcome also not supported by the data (Figure 2).

I hypothesized instead that RRM rats exhibit increased responsiveness to the slow pressor effects of AngII, and that this accounted for the ability of "normal" levels of

circulating AngII to restore hypertension development. To test this idea, I examined the difference in pressor responsiveness between RRM and sham rats drinking enalapril and receiving replacement AngII at a rate of 4ng/min (Figure 36). Sham rats drinking only enalapril had a BP drop of 10-20 mmHg during the treatment period from the 3 control days of the experiment. This hypotensive effect was reversed by infusion of AngII at 4ng/min and BP levels returned to normal. Thus, the peak BP change in sham rats while on enalapril and administered AngII at 4ng/min was 20-25 mmHg. RRM rats drinking enalapril had a BP drop of approximately 5-10 mmHg during the treatment period from their respective 3 control days. Yet, in the RRM rats also administered 4ng/min AngII, the peak change in BP was 40-45 mmHg. These results suggest an enhanced responsiveness to the SPE in RRM rats on NS. This enhancement could explain why BP is elevated in RRM rats despite their having plasma AngII concentrations in the normal physiologic range.

Additional support for the hypothesis of increased responsiveness of the SPE in RRM rats comes from previous work in our lab by Dr. Kanagy, who demonstrated that a 10 ng/min continuous i.v. infusion of AngII in untreated RRM rats on NS elevates BP by 30-35 mmHg over a period of 7-10 days (Kanagy, 1991). This was in contrast to only mild elevations of BP (10-15 mmHg) recorded in sham rats receiving the same infusion rate of AngII.

The role of the SPE in RRM was also evaluated in RRM rats during LS administration. In comparing enalapril administration between sham and RRM rats, it is clear that there was a slower developing hypotensive effect in RRM rats. Likewise, days after discontinuation of enalapril treatment, BP in RRM rats maintained on LS did not

exhibit the rate of increase back to pre-treatment levels that was recorded in the sham rats given the same treatment (Figure 14A vs. Figure 15A). This lack of BP restoration in RRM rats persisted throughout most of the recovery period and was not observed in sham rats. Here again, AngI pressor responses were back to control levels 2-3 days into the recovery period. These results suggest the slow pressor effect of AngII plays a major role in the maintenance of BP in RRM rats on LS.

On the contrary it is evident that the mechanism by which ACEI chronically decrease BP under LS conditions in sham rats is by inhibiting the fast pressor effect of AngII. ACEI lowered BP faster in sham rats than in RRM rats on LS (Figure 14 vs. Figure 15) because plasma AngII concentrations are greater in sham than in RRM rats when both are on a low salt diet (Ylitalo *et al.*, 1976).

One question still remains. Why didn't replacement of AngII restore BP to pre-treatment levels in rats on LS even with higher doses of the peptide? I did not observe a complete reversal of enalapril's hypotensive effect in RRM or sham rats. Administration of AngII i.v. at rates of 10 ng/min partially blunted enalapril's hypotensive effect in sham and did not restore BP levels measured prior to enalapril treatment in RRM. In support of my results, Cowley and coworkers in 1976 reported that sodium-depleted dogs did not show an increase in BP when given AngII at an i.v. rate of 23 ng/kg/min for 9 days (Cowley and DeClue, 1976). It is likely that slightly higher rates of infusion would have been sufficient to restore normal BP. These date are consistent with existing evidence that the SPE of AngII is inhibited by LS (Cowley and McCaa, 1976). Figure 37 summarizes the relative theoretical influence on BP due to the SPE in normal and RRM rats under varying salt intakes.

V. Influence of endothelin under varying levels of salt intake

A. Acute role of endothelin

The direction of my early studies investigating the role of ET in RRM evolved from previous work done in our lab demonstrating the salt dependency of ET-1-induced hypertension (Mortensen and Fink, 1991). At the time I only had access to peptide ETRA's that were restricted to parenteral administration. I hypothesized that ET may be involved in RRM hypertension when rats were maintained on an elevated salt intake. By this time my experiments using ACEI in RRM on HS had demonstrated that the RAS is not involved in BP regulation under HS conditions. I decided to examine the hemodynamic response to both selective ET_A (PD147953) and non-selective ET_A/ET_B (PD145065) receptor antagonism in RRM rats on HS. Since these antagonists were relatively new and little was published about their specificity and potency, I conducted a series of experiments to validate their specificity in ET-1-induced hypertension and determine potential dosing regimens (Figure 19). After I was satisfied with these experiments I tested the specific hypothesis that ET exerts short-term control of BP in RRM rats by immediate and direct contraction of the vasculature. A corollary question that I examined was: does the level of salt intake influence the hemodynamic effects of ET receptor blockade? The results from acute administration of each antagonist in RRM on the 3 levels of salt intake showed that an antihypertensive effect was only observed in RRM rats on HS. The results from these experiments suggest that ET contributes to short-term BP regulation in RRM mainly through activation of ET_A receptors, and preferentially during high salt intakes. The mechanism of this effect is uncertain, since ET formation is not generally responsive to differences in salt intake.

B. Chronic role of endothelin

1. Support for PD155080 dose used in experiments

Since PD155080 was a newly developed non-peptide ET_ARA, dosing regimens were not in place when my experiments started. I developed the dosing regimen of 25 mg/kg b.i.d. from a series of pilot studies involving RRM and sham rats. Since my original observations other evidence has been generated supporting the appropriateness of this dose. *In vivo* evaluations suggest that plasma PD155080 concentrations ranging from 5 to 60 μg/ml exert selective antagonism of ET_A receptors in the rat. Plasma concentrations of PD155080 in RRM and sham rats given PD155080 chronically were within that range (Table 4). The most convincing evidence supporting this dose however, comes from its proven efficacy at reversal of ET-1 induced hypertension (Figure 25).

2. Reduced renal mass

The lack of information regarding the involvement of ET in the excision method of RRM provided an opportunity to be the first to characterize the contribution of ET in this model. I was specifically interested in what role ET plays in long-term BP regulation in RRM and how salt intake may influence its actions. My acute studies suggested that ET's involvement in RRM may be greater under HS conditions. Pilot experiments using PD155080 demonstrated an antihypertensive effect at each level of salt intake, with the largest fall in BP observed under NS conditions (Figure 26). Because of these encouraging results and a variety of other reasons already mentioned, I decided to concentrate on the involvement of ET in long-term BP regulation in RRM rats on NS.

One week treatment with PD155080 caused a significant and sustained decrease in BP to normotensive levels in RRM on NS (Figure 28). This antihypertensive effect

was produced in RRM rats four week following partial nephrectomy and exhibiting a sustained hypertension. The effect of PD155080 on BP was observed within 1 day after administration and reversed by 1 day following discontinuation. These novel findings suggest that ET, acting through ET_A receptors, has an integral part in the maintenance of hypertension in RRM rats on NS.

The overall implication of my chronic experiments utilizing ETRA's in RRM was that ET may play some role in the long-term control of BP at all salt intakes while it exerts a predominant role under NS conditions.

3. Sham

In sham rats PD155080 was given chronically to test the hypothesis that ET was involved in the maintenance of basal levels of BP. After getting preliminary results that suggested ET's involvement in RRM hypertension was greater under HS conditions, I evaluated the influence of salt intake on ET_A receptor antagonism in normal rats. In sham rats maintained on a saline infusion calibrated to deliver 6.0 mEq Na^+/day (HS), PD155080 resulted in a slight hypotensive effect during the first 2 days of administration (Figure 25). In sham rats maintained on a saline infusion calibrated to deliver 2.0 mEq Na^+/day (NS), PD155080 resulted in a slight, inconsistent hypotensive effect during the treatment period when compared control days (Figure 28). My data demonstrated that HS conditions did not alter the BP response to ET_ARA administration in normal rats. Schriffrin has reported that elevations in salt per se did not increase tissue ET-1 content nor elevate circulating levels of the peptide, suggesting that salt did not stimulate ET production or release by itself (Schiffrin *et al.*, 1996). Given that the hypotensive effects due to PD155080 in my experiments were relatively modest and inconsistent, these data

do not support a role of ET in the maintenance of normal levels of BP. My observations are consistent with most reports that do not support a role of ET in the maintenance of basal vascular tone in normal rats (Ohlstein *et al.*, 1993; Teerlink *et al.*, 1995). Figure 38 summarizes the relative theoretical influence on BP due to ET in normal and RRM rats under varying salt intakes.

VI. Mechanism of action of endothelin in RRM hypertension

A variety of mechanisms that are influenced by ET may be involved in RRM hypertension. I did not attempt to identify these mechanisms or their relative contribution. Yet through my experimental design some direction of future investigation may be determined.

It has been established that release of ET-1 from endothelial cells in the systemic vasculature causes vasoconstriction and smooth muscle cell growth by stimulating ET_A receptors (Rubanyi and Polokoff, 1994). The time course of the antihypertensive response to PD155080 in my chronic experiments was too short for reversal of vascular structural changes. If vascular endothelial cell production of ET is increased in RRM, I might have expected to see increased circulating levels of the peptide. ET plasma levels were not different between sham and RRM rats on NS suggesting that increased ET production did not occur. Because the release of ET from endothelial cells is directed towards VSMC, it is possible that tissue levels of ET increase in the absence of increased plasma levels. This scenario has been reported by Schiffrin and colleges to occur in DOCA-salt rats (Schriffrin *et al.*, 1996). I did not measure vascular tissue content of ET directly so I can not rule out increased vascular ET production and direct vasoconstriction as a contributor

to RRM hypertension. Therefore, inhibition of ET-1 induced vasoconstriction could have accounted for the BP lowering effect of ET_A receptor blockade in the RRM rats.

An alternative explanation is that endogenous ET-1 raises BP in RRM rats by an action on receptors distinct from those affected by acute vasoconstrictor actions of the peptide, perhaps in the brain, adrenal gland or other organs involved in BP regulation. In DOCA-salt rats, i.c.v. administration of an ET_ARA was shown to elicit an acute antihypertensive effect (Mortensen and Haywood, 1995). A centrally mediated hypertensive mechanism involving ET may be involved in RRM but this was not addressed in my study nor has it been in the literature.

As mentioned in the introduction, previous evidence suggests that alterations in renally synthesized ET may be involved in RRM hypertension. Increases in urinary excretion of ET occur as renal disease progresses and appear to be a good marker of renal deterioration (Benigni *et al.*, 1991). Whether increases in renally derived ET are a marker, or a cause, of renal deterioration and elevated BP is not known. In my experiments, blockade of renal ET receptors by PD155080 could have caused a fall in BP by promoting sodium and fluid excretion via the kidney. My results from RRM rats maintained on NS do not support such an explanation in that PD155080 administration was associated with sodium and water retention rather than diuresis and natriuresis. PD155080 may exert a variety of beneficial effects on the remnant kidney, but investigation of these was not the focus of my experiments.

VII. Therapeutic implications

There are a variety of antihypertensive agents currently on the market. To date only ACEI have been shown to exert protective effects on the kidney while lowering BP

in human CRF. Thus, ACEI are currently the drug of choice in CRF. They may be most beneficial when hypertension exists in the compliant patient where salt restriction can be successfully maintained. ACEI inhibitors generally have a good side-effect profile with persistent cough being the most common adverse effect. These drugs have not been shown, however, to decrease mortality or time to transplantation in patients with CRF. Furthermore, there are instances where ACEI are not the best therapy in CRF. ACEI are contraindicated in pregnancy because of their teratogenic potential. ACEI seem to have limited if any beneficial effect when patients do not comply with restriction of salt intake. It also must be kept in mind that the risk of acute renal failure is increased when ACEI are administered during low salt intakes (Hall and Brands, 1993). Low salt intakes increase the dependence of the renal circulation on the RAS and preferential dilatation of the efferent arterioles by ACEI can further decrease GFR. Therefore discovery of novel therapies that lower BP and prevent renal deterioration could be of great benefit clinically.

ET receptor antagonists may be useful in reducing BP and preventing renal deterioration in situations where ACEI are not appropriate. My work suggests that ETRA treatment may be more effective in CRF patients on high salt intakes. Currently this class of drugs is not known to be teratogenic and therefore may be useful in women with renal insufficiency or in pregnancy-associated hypertension (preeclampsia). Preeclampsia is associated with a generalized endothelial cell dysfunction and significant elevations in plasma ET levels have been reported (Rubanyi and Polokoff, 1994).

My findings indicate that ET_A receptor blockade may be an effective therapy for the hypertension associated with CRF. It was noteworthy that the antihypertensive

response to PD155080 in RRM rats was not accompanied by any measurable decrease in renal function. Further studies involving longer periods of administration will be necessary to specifically address the potential renoprotective effects of ET receptor antagonism in RRM rats.

Figure 32: Mean arterial pressure measured in RRM rats on high, normal and low salt intakes.

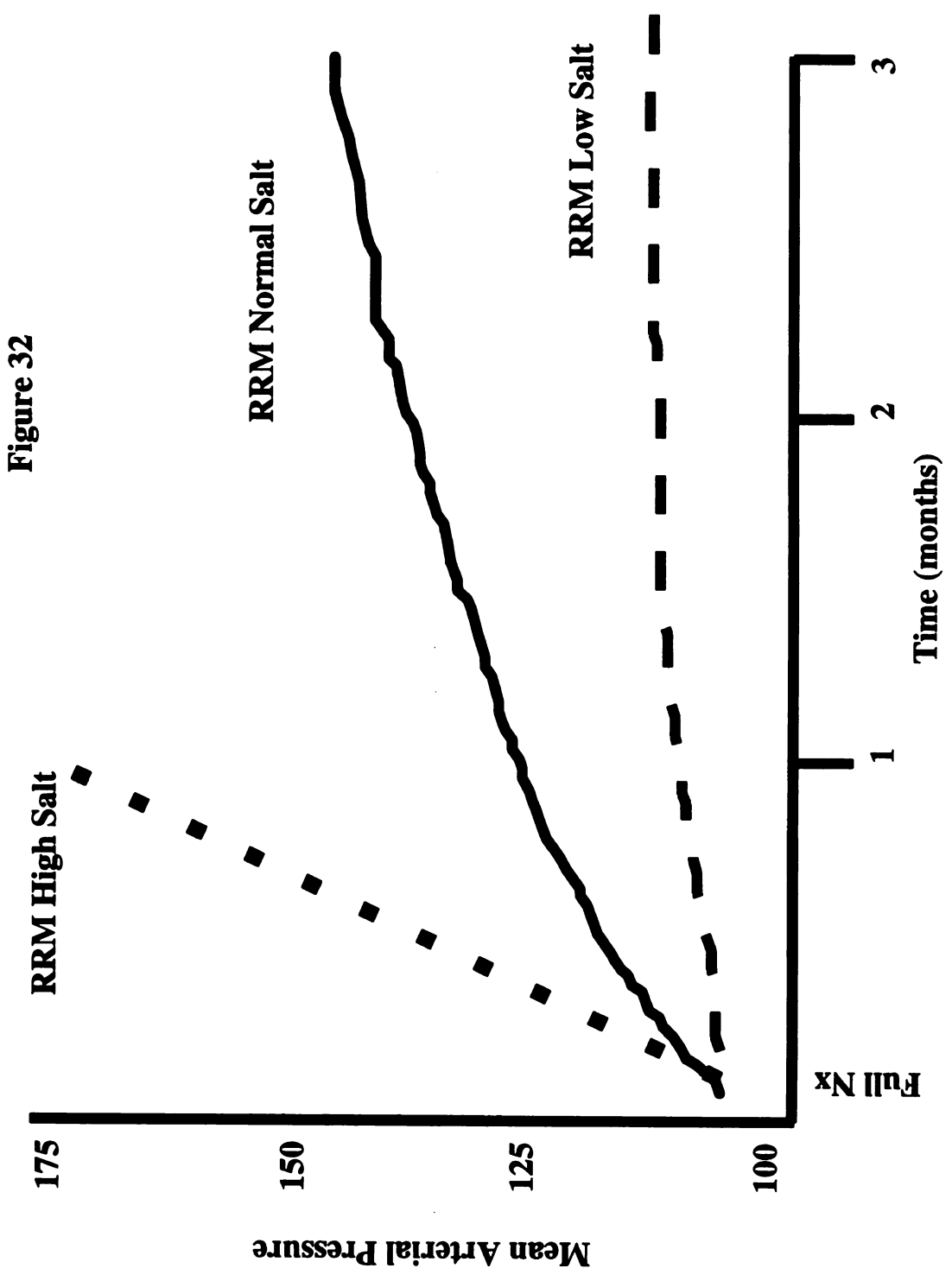
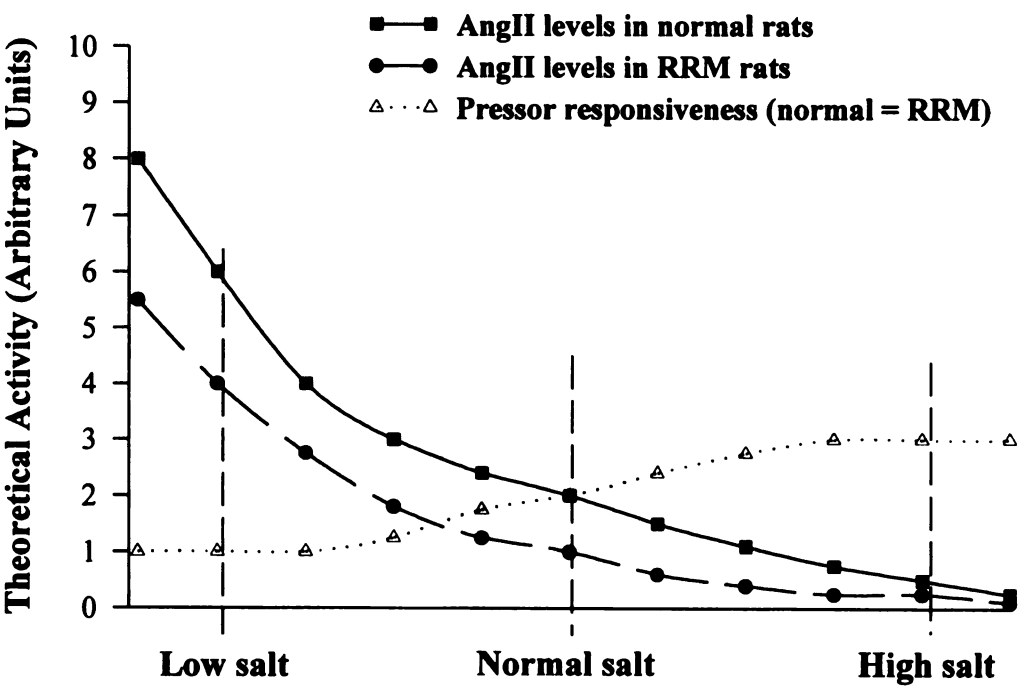


Figure 32
RRM High Salt
RRM Normal Salt
RRM Low Salt
Mean Arterial Pressure
175
150
125
100
Full Nx
1
2
3
Time (months)

Figure 33: Relative influence on blood pressure of the fast pressor effect of AngII in normal and RRM rats under varying salt intakes. Dashed vertical lines represent points on the graph used to estimate the relative influence of blood pressure under differing salt intakes. The theoretical contribution of the fast pressor effect to blood pressure is estimated by the product of the responsiveness of blood vessels to AngII times AngII levels.

Fast pressor effect of AngII



FPE : pressor responsiveness x AngII levels = relative influence on BP

Low	FPE_{normal} : 1 x 6.0 = 6 FPE_{RRM} : 1 x 4.5 = 4.5
Normal	FPE_{normal} : 2 x 2.0 = 4 FPE_{RRM} : 2 x 1.0 = 2
High	FPE_{normal} : 3 x 0.3 = 0.9 FPE_{RRM} : 3 x 0.2 = 0.6

Figure 33

Figure 34: Mean arterial pressure responses to chronic low-dose enalapril administration in RRM rats on normal salt intakes.

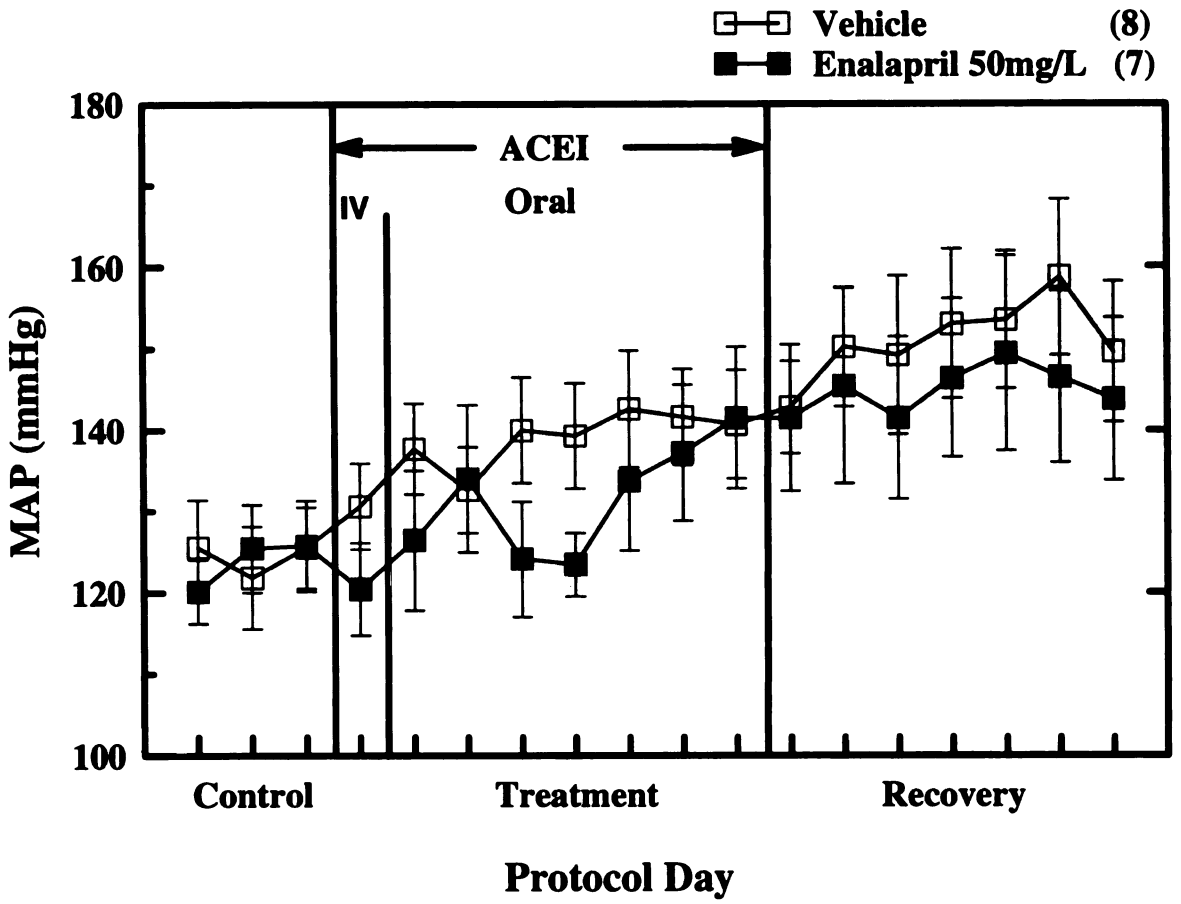

Vehicle (8)
Enalapril 50mg/L (7)
ACEI
IV
Oral
180
160
140
120
100
MAP (mmHg)
Control
Treatment
Recovery
Protocol Day

Figure 34

Figure 35: Mean arterial pressure responses to losartan i.v. bolus during chronic enalapril administration in normal rats on normal salt intakes.

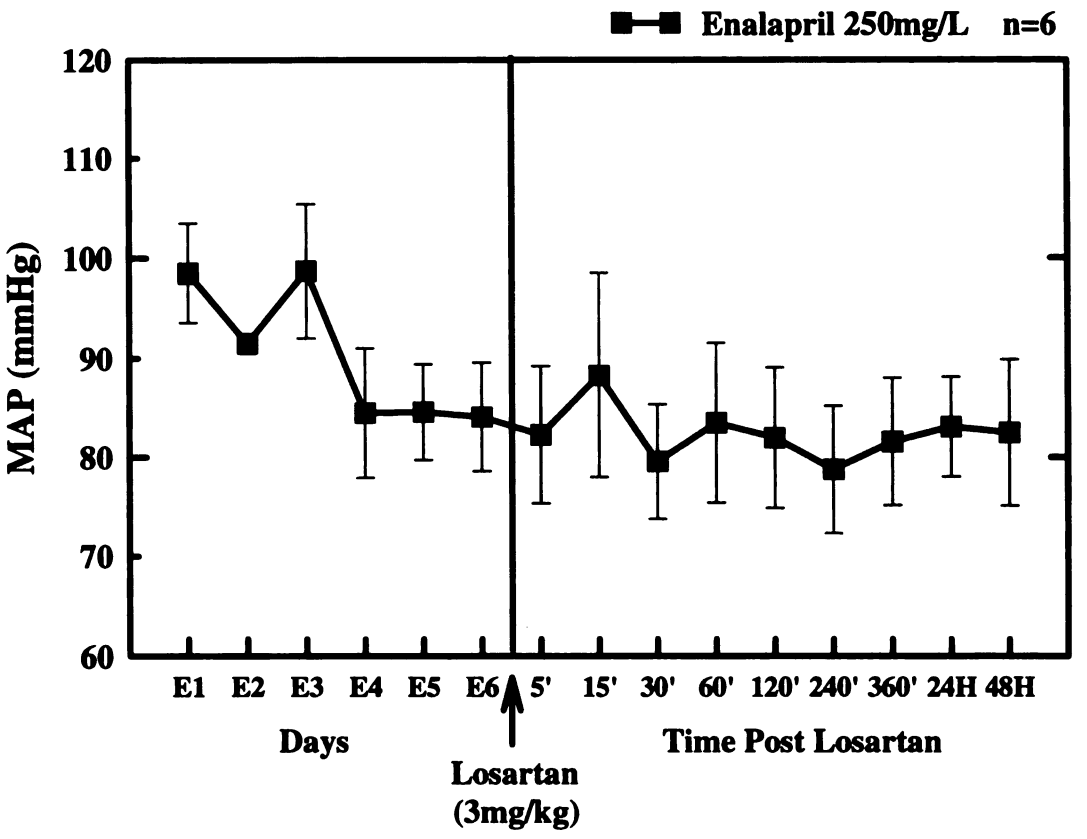

Enalapril 250mg/L n=6
MAP (mmHg)
120
110
100
90
80
70
60
E1 E2 E3 E4 E5 E6 5' 15' 30' 60' 120' 240' 360' 24H 48H
Days
Time Post Losartan
Losartan
(3mg/kg)

Figure 35

Figure 36: Mean arterial pressure responses to chronic enalapril administration in sham and RRM rats on normal salt intakes. Panel A depicts sham rats administered vehicle and enalapril + AngII replacement at 4ng/min. Panel B depicts RRM rats administered vehicle and enalapril + AngII replacement at 4ng/min.

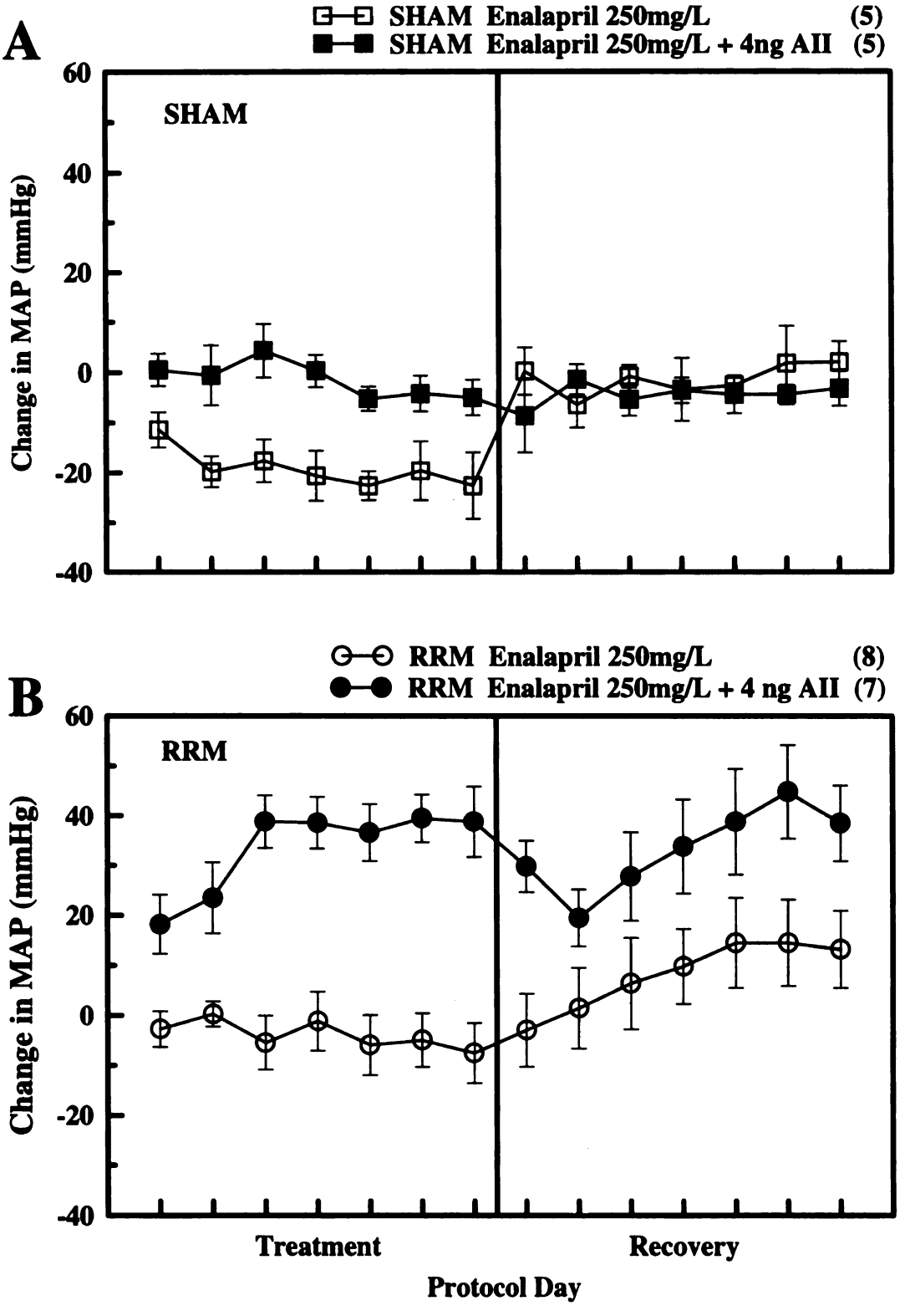
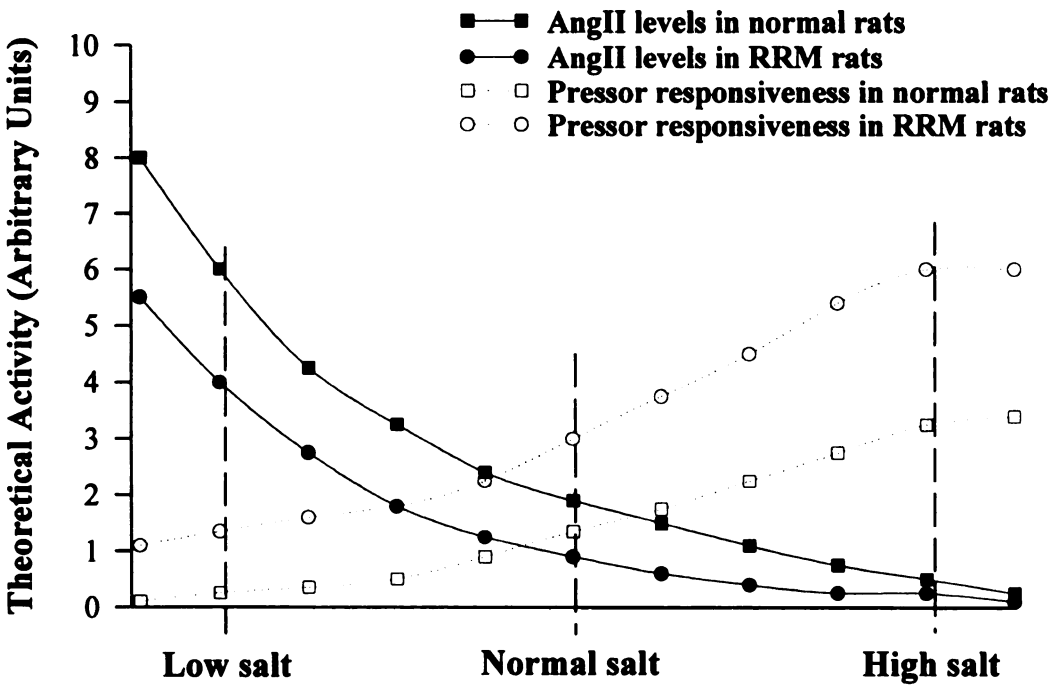

A
SHAM Enalapril 250mg/L (5)
SHAM Enalapril 250mg/L + 4ng AII (5)
SHAM
Change in MAP (mmHg)
60
40
20
0
-20
-40
B
RRM Enalapril 250mg/L (8)
RRM Enalapril 250mg/L + 4 ng AII (7)
RRM
Change in MAP (mmHg)
60
40
20
0
-20
-40
Treatment
Recovery
Protocol Day

Figure 36

Figure 37: Relative influence on blood pressure of the slow pressor effect of AngII in normal and RRM rats under varying salt intakes. Dashed vertical lines represent points on the graph used to estimate the relative influence of blood pressure under differing salt intakes. The theoretical contribution of the slow pressor effect to blood pressure is estimated by the product of the responsiveness of blood vessels to AngII times AngII levels.

Slow pressor effect of AngII

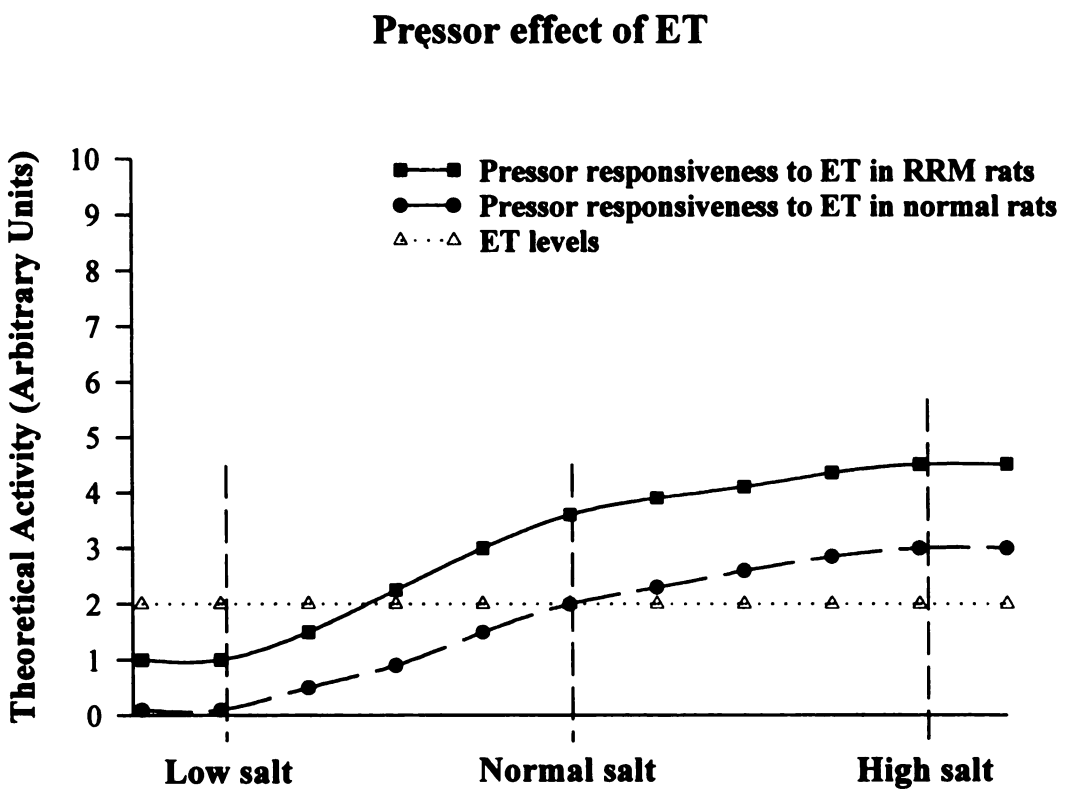


SPE : pressor responsiveness x AngII levels = relative influence on BP

Low	SPE_{normal} : 0.1 x 6 = .6 SPE_{RRM} : 1.5 x 4 = 6
Normal	SPE_{normal} : 1.5 x 1 = 1.5 SPE_{RRM} : 3.0 x 1 = 3
High	SPE_{normal} : 3 x 0.3 = .9 SPE_{RRM} : 6 x 0.1 = .6

Figure 37

Figure 38: Relative influence on blood pressure of endothelin in normal and RRM rats under varying salt intakes. Dashed vertical lines represent points on the graph used to estimate the relative influence of blood pressure under differing salt intakes. The theoretical contribution of ET to blood pressure is estimated by the product of the responsiveness of blood vessels to ET times ET levels.



	pressor responsiveness x ET levels = relative influence on BP
Low	normal : 0.1 x 2 = 0.2 RRM : 1.0 x 2 = 2
Normal	normal : 2.0 x 2 = 4 RRM : 3.5 x 2 = 7
High	normal : 3.0 x 2 = 6 RRM : 4.5 x 2 = 9

Figure 38

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