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Role of Aldosterone in Cardiac Remodeling Post-Myocardial Infarction and After High Salt Diet

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ROLE OF ALDOSTERONE IN CARDIAC REMODELING POST-MYOCARDIAL INFARCTION AND AFTER HIGH SALT DIET

By

Avtar Lal

This Thesis is Submitted as a Partial Fulfillment of the

Doctor of Philosophy Program

in Cellular and Molecular Medicine,

School of Graduate Studies

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

Your file Votre référence
ISBN: 978-0-494-46514-1
Our file Notre référence
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For my wife

Jyoti

and Children,

Alisha and Karan

AUTHORIZATION

Chapter 1:

Figure 1: Reprinted from Journal of Endocrinology 2005; 186: 1-20. Connell M.C. and E. Davies. 2005. The new biology of aldosterone, with permission from Society for Endocrinology.

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CONTRIBUTION OF COLLABORATORS

All studies were conducted under the supervision of Frans H.H. Leenen at the University of Ottawa Heart Institute. All experimental work presented in Chapter 2 was carried out by Avtar Lal. All experimental work presented in Chapter 3 was carried out by Avtar Lal with the exception of coronary artery ligation, implantation of miniosmotic pump, and measurements of LV dP/dt max and plasma catecholamines. All experimental work presented in Chapter 4 was carried out by Avtar Lal with the exception of coronary artery ligation. The manuscripts presented in these chapters 2, 3, and 4 were written by Avtar Lal.

Studies involving coronary artery ligation were performed by Hao Wang and Xiaohong Hou; studies involving implantation of miniosmotic pump and measurements of LV dP/dt max were performed by Bing Huang; and studies involving measurement of plasma catecholamines were carried out by Roselyn White. Scanning electron microscopy (SEM) was performed with the help of Ann-Fook Yang at the SEM lab, Agriculture Canada, Ottawa.

ABSTRACT

High Salt Diet Induces Cardiac Fibrosis by Activating Cardiac Aldosterone

We hypothesized that enhanced cardiac aldosterone production by high salt intake may increase left ventricle (LV) weight and cardiomyocyte size and induce fibrosis in both ventricles. Regular salt (0.6%) or high salt (8%) diet, either without or with spironolactone (20 or 80 mg/kg/day) were given to Wistar rats for 4 and 8 weeks. Both LV weight and cardiomyocyte cross-sectional diameter were increased significantly by high salt diet after 4 and 8 weeks. Both LV and right ventricle (RV) collagen and fibrosis as well as mean arterial pressure (MAP) remained unchanged after 4 weeks, but increased significantly after 8 weeks on high salt diet. Spironolactone (80 mg/kg) prevented the high salt-induced increases in LV weight and cardiomyocyte cross-sectional diameter as well as LV and RV collagen and fibrosis, and attenuated the increase in MAP. Spironolactone (20 mg/kg) was somewhat less effective. The high salt induced changes in cardiac and cardiomyocyte hypertrophy, and cardiac fibrosis were prevented by spironolactone, which is consistent with the concept that cardiac aldosterone mediates these cardiac effects of high salt diet.

Role of brain renin-angiotensin-aldosterone-system (RAAS) in cardiac remodeling post-MI

To assess the contribution of the brain RAAS in the activation of the cardiac RAAS and remodeling post-MI, Wistar rats with intracerebroventricular (icv) infusion of spironolactone and transgenic (TG) rats deficient in brain angiotensinogen were studied. An MI was induced by acute coronary artery ligation. Spironolactone was administered

by icv infusion (100 ng/h) or orally (80 mg/kg/day) for 6 weeks post-MI in Wistar rats. TG rats and control Sprague-Dawley (SD) rats were followed for 8 weeks. MI decreased LV peak systolic pressure (LVPSP) and LV dP/dt max and increased LV end diastolic pressure (LVEDP) and plasma catecholamines and serum aldosterone, which were prevented or attenuated by both icv and oral spironolactone at 6 weeks post-MI. MI increased internal circumferences, cardiomyocyte diameter, fibrosis, laminin and fibronectin, and aldosterone in the LV or and RV, which were also significantly prevented/ inhibited by both icv and oral spironolactone at 6 weeks post-MI in Wistar rats as well as at 8 weeks post-MI in TG rats. Magnitude of beneficial effects of icv spironolactone at low doses was largely equal to that achieved with its oral administration at much higher doses, indicating that in addition to other sites of actions, aldosterone appears to activate central nervous system (CNS) pathways and thereby influences peripheral mechanisms involved in cardiac remodeling. The findings in TG rats support the pivotal role of locally produced angiotensin II in the brain in cardiac remodeling post-MI. The brain RAAS appears to activate a cascade of events, among others an increase in cardiac aldosterone, which play a major role in cardiac remodeling post-MI.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzymes
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotrophic hormone
AF	Activating factor
Ang	Angiotensin
ANP	Atrial natriuretic peptide
AT_1	Ang II type 1
ATF	Activating transcription factors
BNP	Brain natriuretic peptide
BP	Blood pressure
CaMK	Ca^{2+}/-calmodulin-dependent protein kinases
CHF	Congestive heart failure
CI	Confidence interval
CNS	Central nervous system
CRE	cAMP-response-element
CSF	Cerebrospinal fluid
CYP11A1	Cytochrome P450 side-chain cleavage
CYP11B1	Cytochrome P450 11β-hydroxylase
CYP11B2	Cytochrome P450 aldosterone synthase
CYP17	Cytochrome P450 17α-hydroxylase
CYP21A	Cytochrome P450 21-hydroxylase
Dahl-R	Dahl salt-resistant

Dahl-S	Dahl salt-sensitive
ECM	Extracellular matrix
ENaC	Epithelial sodium channel
EPHESUS	Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study
ERK	Extracellular signal-regulated kinase
HSPs	Heat shock proteins
h	Hour
HR	Heart rate
3β-HSD	3β-hydroxysteroid dehydrogenase
11β-HSD2	11β-hydroxysteroid dehydrogenase type-2
Icv	Intracerebroventricular
IP3	1,4,5 triphosphate
Ki-RasA	Kirsten Ras GTP-binding protein-2A
LV	Left ventricle
LVEDP	Left ventricular end diastolic pressure
LVEDV	Left ventricular end diastolic volume
LVEF	Left ventricular ejection fraction
LVH	Left ventricular hypertrophy
LVPSP	Left ventricle peak systolic pressure
MAP	Mean arterial pressure
MCR C-19	19-amino acid sequence at the C terminus of rat MR
MCR N-17	17-amino acid sequence at the N terminus of rat MR

MEK	Mitogen/extracellular signal-regulated kinase
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MnPO	Median preoptic nucleus
mpkCCD	Mouse kidney cell line of cultured collecting duct cells
MR	Mineralocorticoid receptors
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide phosphate oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced from
NE	Norepinephrine
OLC	Ouabainlike compounds
OVLT	Organum vasculosum lamina terminalis
PI3K	Phosphoinositide 3-kinase
PIAS	Protein inhibitor of activated signal transducer and activator of transcription
PKA	Protein kinase A
PKC	Protein kinase C
PRA	Plasma renin activity
PVN	Paraventricular nucleus
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
RALES	Randomized Aldactone Evaluation Study

RSNA	Renal sympathetic nerve activity
RV	Right ventricle
Sc	Subcutaneous
SD	Sprague-Dawley
SFO	Subfornical organ
SGK	Glucocorticoid-regulated kinase
SHR	Spontaneously hypertensive rats
SON	Supraoptic nucleus
SRC	Steroid receptor coactivators
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TIF	Transcriptional intermediary factor
TG	Transgenic
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
WMD	Weighted mean difference
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticulata

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Chapter 1

GENERAL INTRODUCTION

PREFACE

Heart failure is a major health problem worldwide. Congestive heart failure (CHF) following myocardial infarction (MI) is associated with high mortality rate, readmissions and prolonged hospital stays (Johansen et al 2003). Despite major advancements in its treatment, the morbidity and mortality in CHF remain high (Peacock et al 2006). Cardiac remodeling after MI is important for the development of heart failure and represents a series of changes in ventricular size and structure occurring after MI that affect the infarcted and non-infarcted zone of the left ventricle (LV) as well as the right ventricle (RV) (Pfeffer et al 1991). Processes such as infarct expansion, late-phase ventricular dilation, and fibrosis of viable myocardium are maladaptive and contribute to LV dysfunction and progressive heart failure (Pfeffer et al 1991, Delyani et al 2001, Sun and Weber 2000).

A number of local and systemic mechanisms have been implicated in cardiac remodeling and dysfunction post-MI. Among these, the renin-angiotensin-aldosterone system (RAAS) appears to be a one of the major contributors.

Aldosterone, a physiological mineralocorticoid isolated about 50 years ago (Simpson et al 1953), is an important component of the RAAS. It is the primary mineralocorticoid that is synthesized in the zona glomerulosa of the adrenal cortex and released into the circulation to be carried to target organs, where it binds to mineralocorticoid receptors (MR) to exert its actions. The production of aldosterone has also been demonstrated in extra-adrenal tissues, namely brain (Gomez-Sanchez et al 1997), heart (Silvestre et al 1998, 1999, Xiu et al 2002, Takeda et al 2000), and blood vessels (Hatakeyama et al 1994, Takeda et al 1995, Takeda 2004).

The role of cardiac and brain aldosterone in cardiac remodeling and LV dysfunction is still incompletely understood. Studies from our laboratory have given an important lead in this direction, particularly regarding the role of endogenous ouabainlike compounds (OLC) and the brain renin-angiotensin system (RAS) in increasing sympathetic hyperactivity in rat models of hypertension as well as after MI. The present studies address this important issue.

Role of Cardiac and Brain Renin-Angiotensin-Aldosterone System in Cardiac Remodeling and LV Dysfunction

For this thesis, the role of the cardiac and or brain RAAS in cardiac remodeling and dysfunction was studied in two rat models.

In the first experimental model of 'High salt-diet', high salt intake induces cardiac hypertrophy with fibrosis and cardiomyocyte hypertrophy in Wistar rats. The role of cardiac aldosterone in inducing this cardiac remodeling was investigated by oral treatment with spironolactone.

In the second experimental model of 'post-MI', an MI was induced by acute coronary artery ligation. The role of cardiac and brain aldosterone in cardiac remodeling and LV dysfunction post-MI was investigated by oral administration of spironolactone. The specific action of aldosterone in the brain in cardiac remodeling and dysfunction post-MI was investigated by intracerebroventricular (icv) infusion of spironolactone and comparing its effects with oral spironolactone. The precise role of the brain angiotensins in cardiac remodeling post-MI was investigated by using transgenic (TG) rats deficient in

brain angiotensinogen. MI-induced changes in cardiac remodeling in TG rats were studied by comparing its effects with its parent strain Sprague-Dawley (SD) rats.

The literature review will provide an overview of the mechanisms involved in the production of aldosterone in adrenal, brain, heart and blood vessels; actions of aldosterone in brain, heart, blood vessels, and kidney; role of aldosterone in pathophysiological states; effect of high salt diet on heart and blood pressure; and the rationale of the present studies.

1 LITERATURE REVIEW

1.1 PRODUCTION OF ALDOSTERONE IN DIFFERENT TISSUES

1.1.1 Aldosterone Synthesis in the Adrenal

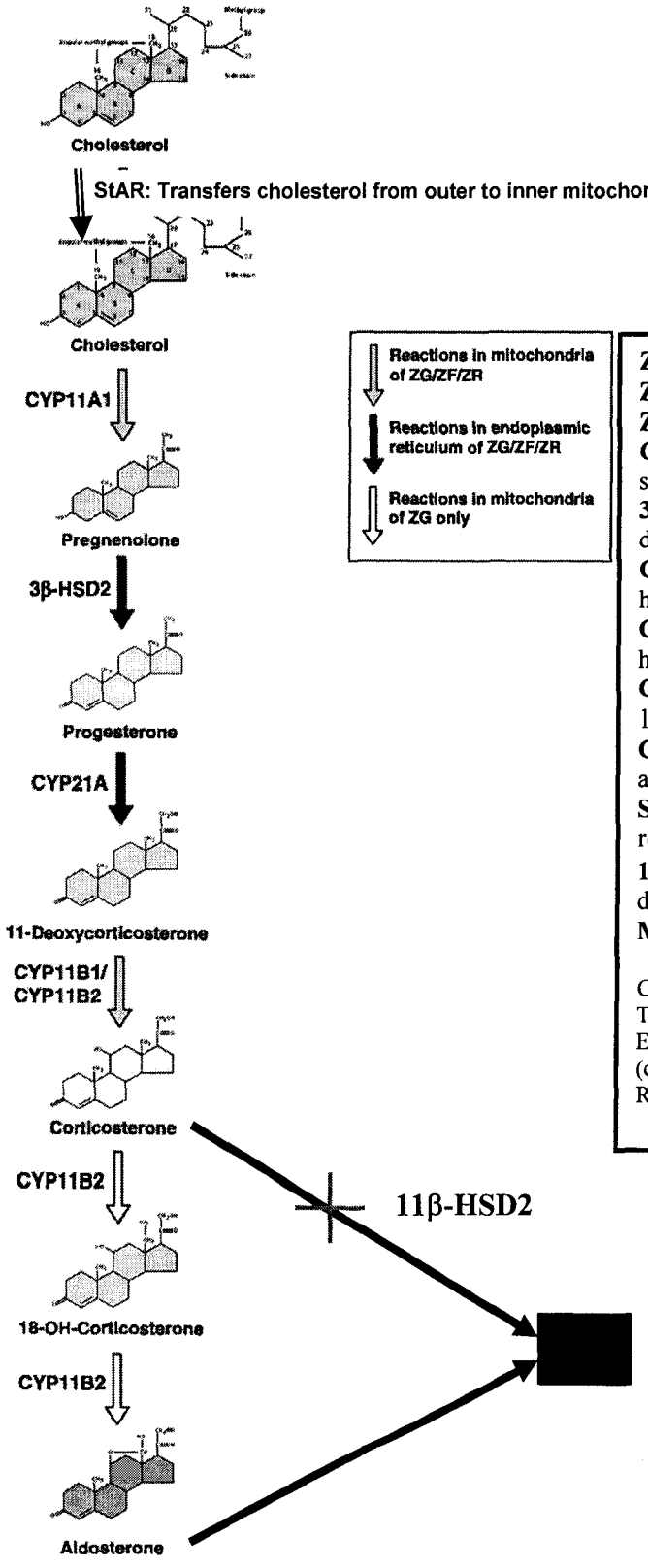
All of the enzymes required for the synthesis of aldosterone are present in the adrenal gland (Figure 1). Cholesterol is required for the synthesis of glucocorticoids and mineralocorticoids. Cholesterol comes from multiple sources, such as circulating cholesterol and cholesterol esters, liberation of cholesterol from endogenous cholesterol stores via activation of cholesterol esterases, and by increased *de novo* biosynthesis. The transfer of cholesterol from the outer to the inner mitochondrial membrane is regulated by a steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). This transfer is considered to be the 'rate-limiting step' in steroidogenesis (Stocco 2001). StAR is present in all steroidogenic tissue and plays a key role in steroidogenesis (Stocco 2001a). In patients with congenital lipoid adrenal hyperplasia, mutations in the StAR

gene result in an inability to make steroids and accumulation of cholesterol in the adrenal gland (Stocco 2001).

Following translocation to the mitochondrion, cholesterol is converted to aldosterone by a series of enzymatic reactions catalyzed by dehydrogenases and mixed function oxidases, many of which belong to the cytochrome P450 (CYP) superfamily of haem-containing enzymes. The next step - conversion of cholesterol to pregnenolone - is mediated by the cytochrome P-450 side-chain cleavage enzyme in the mitochondrial matrix, which is encoded by the CYP11A1 gene on human chromosome 15. This enzyme catalyses three reactions, two hydroxylations and one cleavage of a bond between C-20 and C-22, to produce pregnenolone (Lieberman and Lin 2001). Before the discovery of StAR, this reaction was considered the 'rate-limiting step' in the steroid biosynthesis. Pregnenolone is released into the cytosol and is converted to progesterone by 3β-hydroxysteroid dehydrogenase, which is located on the membrane of the smooth endoplasmic reticulum (Mason 1993). Progesterone undergoes 21-hydroxylation by cytochrome P450 21 hydroxylase (CYP21A) enzyme producing 11-deoxycorticosterone. The enzyme was localized immunocytochemically on the smooth-surfaced endoplasmic reticulum (Shinzawa et al 1988).

The next steps involve the conversion of 11-deoxycorticosterone to corticosterone, 18-hydroxycorticosterone and aldosterone. These reactions are catalyzed by aldosterone synthase, located on the inner mitochondrial membrane and encoded by CYP11B2. The expression of this enzyme is limited to the zona glomerulosa, preventing production of aldosterone in the other cortical areas.

Figure 1: Biosynthesis of Aldosterone in Adrenal Cortex



ZG: Zona glomerulosa
ZF: Zona fasciculata
ZR: Zona reticulata
CYP11A1: Cytochrome P450 side-chain cleavage
3β-HSD2: 3β-hydroxysteroid dehydrogenase type 2
CYP17: Cytochrome P450 17α-hydroxylase
CYP21A: Cytochrome P450 21-hydroxylase
CYP11B1: Cytochrome P450 11β-hydroxylase
CYP11B2: Cytochrome P450 aldosterone synthase
StAR: Steroidogenic acute regulatory protein
11β-HSD2: 11β-hydroxysteroid dehydrogenase type-2
MR: Mineralocorticoid receptor

Connell MC and Davies E. 2005. The new biology of aldosterone. J Endocrinol 186: 1-20.

Aldosterone synthase is homologous (about 93%) to 11β-hydroxylase, encoded by CYP11B1, which catalyses the conversion of 11-deoxycortisol to the glucocorticoid, cortisol in human (Bassett et al 2004) and deoxycorticosterone to corticosterone in rats. The 2 genes are located on chromosome 8q21-22 in humans (Wagner et al 1991) and chromosome 7 in rats (Inglis et al 1995). There are considerable differences in functional and substrate specificity of the two enzymes. The amino acids 147 and 248 differ between these two enzymes. The position of amino acid 147 of aldosterone synthase has an important effect on the efficiency of 11β hydroxylation of deoxycorticosterone and is the key difference between the two enzymes in determining the functional specificity. In contrast, amino acid 248 does not affect enzyme efficiency (Fisher et al 1998).

1.1.1-a Regulation of Synthesis of Aldosterone in Adrenal

Aldosterone synthesis and secretion by the adrenal gland are stimulated by a number of factors including Ang II, adrenocorticotrophic hormone (ACTH) and extracellular K^+ (Quinn and Williams 1988).

Modes of regulation: Aldosterone synthesis is regulated by two phases, acute and chronic regulation.

1) Acute regulation occurs in minutes to hours of stimulus. This acute synthesis of aldosterone may involve rapid synthesis from intermediate compounds in the steroidogenic pathways or de novo synthesis from cholesterol. Aldosterone production is mediated possibly as a consequence of StAR protein activation, leading to increased transport to the inner mitochondrial membrane (Cherradi et al 1998).

2) Chronic regulation occurs in hours to days and the aldosterone production is regulated at the level of expression of aldosterone synthase (Bassett et al 2004).

1.1.1-a-i Renin-Angiotensin System Regulating Adrenal Synthesis

Aldosterone biosynthesis is regulated by the RAS. Renin is synthesized and released by the juxtraglomerular cells in the afferent arteriole of the kidney. Renin catalyzes the hydrolysis of angiotensinogen to angiotensin (Ang) I, which is then converted to Ang II by angiotensin converting enzyme (ACE). Ang II acts on the adrenal zona glomerulosa to stimulate aldosterone production. This adrenal response to Ang II occurs within minutes, indicating that no new protein synthesis is required. This acute, Ang II-mediated release of aldosterone may involve rapid synthesis from intermediate compounds or synthesis from cholesterol possibly through StAR activation. Chronic stimulation by Ang II results in zona glomerulosa hypertrophy and hyperplasia, increased CYP11B2 expression and subsequent enhanced aldosterone synthesis.

The most characterized pathway activated by Ang II is via phospholipase C, though the exact mechanism of aldosterone production is not fully understood. Ang II is known to act on Ang II type 1 (AT_1) receptors (Kakiki et al 1997) which cause phospholipase C to stimulate intracellular production of inositol 1,4,5 triphosphate (IP_3) and 1,2-diacylgylcerol, which activate protein kinase C (PKC). IP_3 also increases the concentration of intracellular free calcium ($[Ca^{2+}]_i$), causing several Ca^{2+}/-calmodulin-dependent protein kinases (CaMK)1 and CaMKIV to phosphorylate and activate activating transcription factors [ATF-1, ATF-2, and NURR-1 and cAMP-response-element (CRE)-binding protein (Spat and Hunyady 2004)]. These bind CRE and other cis-acting elements (e.g., NBRE-1 and AD-5) which are unique to the 5' untranslated

region of the CYP11B2 gene. The ability of ATF-1 and CRE-binding protein to enhance transcription is partially regulated by their state of phosphorylation. Activated CaMK1 or CaMKIV possibly phosphorylate CRE-binding protein or ATF-1 leading to increased CYP11B2 transcription (Bassett et al 2004).

Ang II may have an alternate pathway of stimulating the aldosterone production whereby AT_1 receptors couple with src family of tyrosine kinases, inhibit CYP 17 expression and increase the aldosterone production (Sirianni et al 2001). AT_1 receptors also couple with 12-lipoxygenase pathway (Gu et al 2003). 12-lipoxygenase is expressed in glomerulosa cell and upregulated by Ang II. The overexpression of 12-lipoxygenase in H295R human adrenocortical cells stimulates aldosterone production (Gu et al 2003).

1.1.1-a-ii Potassium ions Regulating Adrenal Synthesis of Aldosterone

Extracellular K^+ concentration is a key determinant of aldosterone secretion. In vivo studies using mice with targeted deletion of genes in the RAS demonstrated that K^+ could substitute for Ang II and increase adrenal CYP11B2 expression and aldosterone production (Chen et al 1997, Okubo et al 1997). The effects of Ang II and extracellular $[K^+]$ are synergistic (Spat 2004). Increased $[K^+]$ causes zona glomerulosa cell membrane depolarization, leading to the opening of voltage-dependent L- and T-Ca^{2+} channels and a rise in $[Ca^{2+}]_i$. This leads to activation of calmodulin and calmodulin-dependent protein kinase which phosphorylate transcription factors to stimulate CYP11B2 gene transcription (Spat and Hunyady 2004). Antagonist of calmodulin (calmidazolium) and calmodulin-dependent protein kinases, KN93 inhibit K^+ and Ang II stimulation of CYP11B2 reporter gene expression (Condon et al 2002). Ang II and K^+ regulate CYP11B2 transcription through common Ca^{2+}-dependent signaling pathways and

transcription factors (Clyne et al 1997). Calcium channel blockers such as nifedipine completely blocked the K^+ and partially blocked Ang II stimulation of CYP11B2 mRNA, providing evidence that intracellular calcium is involved in agonist induction of CYP11B2 gene transcription, mRNA levels and protein expression (Yagci et al 1996, Denner et al 1996).

1.1.1-a-iii ACTH Regulating Adrenal Synthesis of Aldosterone

ACTH interacts with specific receptors in the adrenal cortex to stimulate the production of glucocorticoids. It also contributes to the regulation of aldosterone biosynthesis. Acutely, ACTH stimulates aldosterone production via cAMP-mediated pathways and protein-synthesis-independent mechanisms involving macrophages-derived factor, steroidogenic-inducing protein and calmidazolium (Cozza et al 1990, Cooke 1999). Chronically, ACTH suppresses plasma aldosterone both in humans and rats (Fuchs-Hammoser et al 1980, Holland and Carr 1993, Aguilera et al 1996). The mechanism of chronic inhibition is unclear but cAMP may downregulate the expression of AT_1 receptors in adrenocortical cells (Bird et al 1994), thereby desensitizing the adrenal cells to Ang II. ACTH may transform proliferating zona glomerulosa cells into zona fasciculata cells through induction of CYP11B1 and CYP17 enzymes which divert precursors from the mineralocorticoid to the glucocorticoid pathway (Bird et al 1996).

1.1.2 Aldosterone Synthesis in Brain

A variety of enzymes involved in the synthesis of aldosterone have been demonstrated in the brain. Immunoreactivity of StAR, mediating the transfer of cholesterol from the outer to the inner mitochondrial matrix for further enzymatic

reactions (Stocco and Clark 1996) has been observed in multiple brain regions, including the hypothalamus, cerebellum, pons, and cerebral cortex (King et al 2002). The cytochrome P450 side chain cleavage, first rate-limiting step in steroid biosynthesis involving the conversion of cholesterol to pregnenolone, has been found to occur in oligodendrocytes, glial cell and rat C6 glioma cells (Mellon and Griffin 2002, Mellon and Deschepper 1993). Immunoreactivity of this enzyme has also been demonstrated in pituitary, thalamus, hippocampus, diencephalon and cortex of the brain (Compagnone et al 1995) and has been found to coexist with StAR in various brain regions, including the hypothalamus, cerebellum, pons, and cerebral cortex (King et al 2002). The enzyme, 3β-hydroxysteroid dehydrogenase isomerase involved in synthesis of progesterone from pregnenolone, has been demonstrated in glial cells and Schwann cells (Koenig et al 1995). Cytochrome P450 21-hydroxylase, converting progesterone to 11-deoxycorticosterone has been demonstrated in the neuronal cell, glial cells and in the fibers of the tractus reticulothalamics, by immunohistochemical localization using antibody against bovine adrenocortical cytochrome P450 21-hydroxylase (Iwahashi et al 1993).

The final step in the synthesis of aldosterone from deoxycorticosterone is catalyzed by aldosterone synthase (Mitani et al 1997). The expression of aldosterone synthase mRNA has been demonstrated by RT-PCR in the whole brain and hypothalamic minces and was localized by immunohistochemistry in the hippocampus neurons and the cerebellar cortex particularly within the Purkinje cells (Mackenzie et al 2000a, 2002, Gomez Sanchez et al 1997). Moreover, its enzymatic activity was confirmed by incubating minces of hypothalamus, hippocampus and cerebellum with [1,2 ^{3}H]-

deoxycorticosterone and demonstration of the end products, aldosterone, corticosterone and 18-hydroxy-deoxycorticosterone. The enzymes implicated in the steroidogenesis are found in the astrocytes and oligodendrocytes in the rat brain (Mellon and Deschepper 1993). Incubation of slices with metyrapone, an inhibitor of 11β-hydroxylase, inhibits the synthesis of aldosterone and corticosteroids (Gomez Sanchez et al 1997).

In humans, mRNA encoding CYP11A, CYP17, 3β hydroxysteroid dehydrogenase, 21-hydroxylase, and 11β-hydroxysteroid dehydrogenase type-2 (11β-HSD2, that inactivates glucocorticoids) were expressed in amygdala, caudate nucleus, cerebellum, corpus callosum, hippocampus, thalamus and spinal cord (Yu et al 2002). Recently, Geerling et al (2006, 2006a) reported that the abundance of 11β-HSD2 protein in the nucleus tractus solitarius (a cardiovascular afferent integrative center in the hindbrain) correlated with the nuclear translocation of MR in response to systemically administered aldosterone in adrenalectomized rats. The expression of 11β-HSD2 neurons was found in nucleus tractus, ventrolateral division of the ventromedial hypothalamic nucleus and a few scattered neurons in the medial vestibular nucleus, just rostral to the nucleus tractus solitarius. Zhang et al (2006) reported the expression of 11β-HSD2 mRNA in paraventricular nucleus (PVN) of the hypothalamus of SD rats. Inhibition of 11β-HSD2 activity in PVN by microinjection of carbenoxolone (metabolite of glycyrrhizic acid, an 11β-HSD2 inhibitor) into PVN increased blood pressure (BP), heart rate (HR) and renal sympathetic nerve activity (RSNA). ICV injection of carbenoxolone and glycyrrhizic acid excited PVN neurons and increased the BP, HR and RSNA. Pretreatment with icv spironolactone prevented the increases of these carbenoxolone-induced PVN activity, RSNA, mean arterial pressure (MAP), or HR in these rats.

Aldosterone synthase was detected in caudate nucleus, corpus callosum, spinal cord, and thalamus, but not in amygdala, cerebellum or hippocampus (Yu et al 2002). Gomez Sanchez et al (2005b) measured aldosterone levels in the plasma and brain of adult female Wistar rats given regular, high, and low salt diet as well as in adrenalectomized rats maintained on 0.9% NaCl. As compared to regular salt diet, low salt diet increased whereas high salt decreased both the plasma and the brain aldosterone levels. In the adrenalectomized rats, plasma level of aldosterone was 1 ± 0.8 pg/ml, while the brain aldosterone was 9.9 ± 1.9 pg/g wet tissue. Recently, our lab measured aldosterone contents in the hypothalamus of Wistar rats. Icv infusion of Na^+-rich artificial cerebrospinal fluid (aCSF), increasing cerebrospinal fluid (CSF) [Na^+] by ~ 5 mmol/L increased hypothalamic aldosterone contents by 30% without increasing circulating aldosterone (Huang et al 2006).

Deoxycorticosterone is a substrate for aldosterone synthase. Gomez Sanchez et al (2005b) reported that after administration of deoxycorticosterone (0.083 mg/day, sc by pellet), plasma aldosterone was undetectable, but the brain level was 11.9 ± 3.1 pg/g vs 6.4 ± 2.6 pg/g wet tissue without treatment. The authors concluded that aldosterone is produced in the brain. The amount of mRNA for steroid enzymes are quite low in the hypothalamus, indicating that aldosterone may act in an paracrine fashion directly or indirectly in the areas that appear to be important in BP regulation or other functions (Gomez Sanchez et al 1997)

Aldosterone in the central nervous system (CNS) may either be produced by *de novo* synthesis in the brain from cholesterol (Gomez-Sanchez et al 1997) or from circulating steroid precursors, such as progesterone and 11-deoxycorticosterone (Mellon

and Griffin 2002). In addition, since steroids are lipophilic and able to cross the blood brain barrier, some of the aldosterone detected in the brain may actually be derived from the circulation.

1.1.2-a Regulation of Brain Synthesis of Aldosterone

The regulation of aldosterone synthesis in the brain is incompletely understood. However, recently our lab elucidated a possible mechanism of regulation of aldosterone in the brain by local [Na^+].

1.1.2-a-i CSF [Na^+] Regulating Brain Synthesis of Aldosterone

Huang et al (2006) reported an increase of aldosterone contents in hypothalamus by ~33% with the chronic increase of CSF [Na^+] by 4-5 mmol/L. Icv infusion of Na^+-rich aCSF had no effect on plasma aldosterone, indicating an increase of aldosterone in the hypothalamus may be due to increase of local production in the brain. However which enzyme is activated by Na^+-rich aCSF was not evaluated. Gomez Sanchez et al (2005b) reported that high salt diet decreased in parallel the aldosterone levels in plasma and whole brain in Wistar rats. Ye et al (2003) measured CYP11B2 mRNA in various areas of the brain from Wistar-Kyoto rats that had been fed with low, normal, and high sodium diets for 12 days. Sodium depletion increased CYP11B2 expression in adrenal by 57-fold, in hippocampus by 14-fold, and in cerebellum by 5-fold, but did not alter the expression in the brainstem and hypothalamus. High salt diet on the other hand decreased the expression of CYP11B2 in adrenal (by ~20%), but did not change the expression in hypothalamus, hippocampus, cerebellum and brain stem. They did not measure the content of aldosterone in the brain. Huang et al (2004) reported that high salt increased

CSF [Na^+] in SHR salt but not in Wistar-Kyoto rats. In contrast to the effects of high salt diet and plasma [Na^+] on adrenal regulation of aldosterone production and release, an increase in CSF [Na^+] appears to increase hypothalamic aldosterone.

Blockade of the biosynthesis of steroids in the CNS by icv infusion of the 3β-HSD blocker trilostane prevented hypertension in Dahl S rats on high salt diet (Gomez Sanchez et al 2005a). If this finding is further substantiated by, e.g., use of a specific aldosterone synthase inhibitor such as FAD-286 to block aldosterone synthesis (Fiebeler et al 2005), regulation of aldosterone by sodium appears opposite for the brain versus adrenal cortex (Huang et al 2006).

1.1.2-a-ii Ang II Regulating Brain Synthesis of Aldosterone

Ye et al (2003) measured CYP11B2 mRNA in various areas of the brain and adrenal from rats after infusion of Ang II (200 ng/kg/min, sc) for 7 days. The regulation of aldosterone synthase mRNA by Ang II differed in the brain compared to adrenal. Ang II infusion significantly increased CYP11B2 expression in the adrenal gland by 5-fold, but had no effect on the expression in the hypothalamus, brainstem and cerebral cortex.

1.1.3 Cardiac Synthesis of Aldosterone

For the cardiac synthesis of aldosterone, enzymes involved in the synthesis have been found in the heart. The mRNA for StAR, a crucial factor in the rate limiting-step in aldosterone biosynthesis is expressed in cultured neonatal rat cardiomyocytes and rat heart (Casal et al 2003). The enzyme, 3β-hydroxysteroid dehydrogenase isomerase, involved in synthesis of progesterone from pregnenolone, has been evidenced in cardiac tissue (Delcayre and Silvestre, 1999). Using the quantitative reverse transcriptase-

polymerase chain reaction, 11β-hydroxylase and aldosterone synthase were found in the heart of 2-month-old rats. The total amount of both aldosterone synthase and 11β-hydroxylase mRNA molecules in the whole heart was ~ 100-fold lower than in the adrenal (Silvestre et al 1998). This ratio is comparable to that of ACE mRNA, whose total quantity is about 150-fold lower in the heart than in lungs. Cardiac levels of 11β-hydroxylase mRNA were 7-fold higher than those of aldosterone synthase (Heymes et al 1994).

Aldosterone was determined in both cardiac homogenates (50-90 pg/ mg protein), which represents the quantity of steroids intracellularly as well as in cardiac perfusion (10 pg/h per g of tissue), which represents the quantity of steroids released into the coronary circulation of rats (Silvestre et al 1998, 1999). Cardiac homogenates were also able to convert [^{3}H] deoxycorticosterone to [^{3}H] aldosterone and [^{3}H] corticosterone (Silvestre et al 1998), further confirming the activity of enzymes in the heart. The estimated concentration of aldosterone in heart is about 5.8 ng/mg, a value about 17-fold higher than the mean plasma value (0.3 ng/ml), possibly due to slower degradation in cardiac tissue than in plasma, or it may be segregated intracellularly once produced (Silvestre et al 1998, Delcayre and Silvestre, 1999). Aldosterone probably accumulates in a compartment other than extracellular fluid, e.g., it binds to cell receptors and/ or reaches intracellular sites. This concept is supported by the biphasic pattern of aldosterone in coronary effluent in rat Langendorff hearts when aldosterone was perfused followed by washout. The rapid phase corresponds to disappearance from extracellular fluid and a slow phase corresponds to washout from a secondary compartment (Chai et al 2006).

Takeda et al (2000) also detected the aldosterone synthase mRNA and activity and production of aldosterone in the heart of Wistar-Kyoto rats.

In humans, Kayes-Wandover and White (2000) and Young et al (2001) detected StAR in the normal and the failing heart and the cardiac expression of the mRNA for cytochrome P450 side chain cleavage (CYP 11A), 3β-hydroxysteroid dehydrogenase, cytochrome P450 21-hydroxylase in the left and right atrium and ventricles, apex, intraventricular septum, atrioventricular node and whole adult and fetal hearts. The expression of CYP11B2 mRNA has been detected in fetal heart. Levels of CYP11B2 transcript of fetal heart were 1000 times lower than those in the adrenal.

Strain-differences in the expression of aldosterone synthase were reported by Rudolph et al (2000). Atrium and ventricular expression of aldosterone synthase was seen in Wistar, but not in SD rats under basal conditions. However, following Ang II stimulation, the myocardial expression of aldosterone synthase was seen in both strains.

The exact site where aldosterone is synthesized in the heart remains to be defined, but cardiomyocytes, smooth muscles and vascular endothelial cells may be contributing. Considering the production of aldosterone and expression of StAR mRNA in cultured neonatal rat cardiomyocytes (Casal et al 2003), cardiomyocytes may be the predominant site for aldosterone synthesis in the heart.

1.1.3-a Regulation of Cardiac Synthesis of Aldosterone

1.1.3-a-i Ang II Regulating Cardiac Synthesis of Aldosterone

Ang II stimulated StAR mRNA and the production of aldosterone in cultured neonatal rat cardiomyocytes, whereas aldosterone caused concentration-dependent

decreases in StAR mRNA expression. This inhibitory effect of aldosterone was completely prevented by spironolactone. These findings indicate that aldosterone (by acting through MR) exerts a negative feedback control on StAR (Casal et al 2003). Cardiac perfusion of Ang II for 1 week raises the concentration of aldosterone synthase mRNA by 3.5-fold in the LV of adult Wistar rats. The production of aldosterone increases by 4-fold in the homogenate and by 16-fold in the coronary sinus perfusate of isolated perfused heart. Cardiac perfusion of Ang II also increases corticosterone by 3-fold in the homogenates and by 2-fold in the perfusate. Deoxycorticosterone concentration increases by 3.5-fold in the homogenate and by 4-fold in the perfusate of isolated perfused heart. With gradual increase in the concentration of Ang II from 10^{-9} to 10^{-7} M, the production of aldosterone and corticosterone rises by 2- to 5-fold (Silvestre et al 1998). On incubating cardiac homogenates with [^{3}H] deoxycorticosterone, Ang II increases the conversion to aldosterone by 2-fold, indicating increased activity of aldosterone synthase by Ang II (Silvestre et al 1998).

1.1.3-a-ii ACTH Regulating Cardiac Synthesis of Aldosterone

ACTH increases the synthesis of aldosterone in cardiac homogenates as well as coronary sinus perfusate of isolated perfused heart. Chronic treatment with ACTH for 7 days increases the production of aldosterone in homogenate by 4.5-fold and in the perfusate by 15-fold and decreases plasma aldosterone levels. It also increases corticosterone and deoxycorticosterone in the homogenate and in the perfusate of isolated perfused heart without having any effect on aldosterone synthase mRNA. ACTH increases the synthesis of aldosterone by 4-fold (from 50 pg/mg of protein to 200 pg/mg of protein) in cardiac homogenates and by 6-fold at 1 hour (from 10 pg/h per g of tissue

to 60 pg/h per g of tissue) and by 12-fold at 2 hours of perfusion (from 10 pg/h per g of tissue to 120 pg/h per g of tissue) in the perfusate of isolated perfused heart. The concentration of corticosterone also increases in the homogenate and the perfusate with ACTH. There occurs a dose-dependent rise of aldosterone and corticosterone levels in the coronary sinus perfusate with increasing ACTH concentration in isolated perfused rat heart (Silvestre et al 1998). A marked increase in the early steps of cardiac biosynthetic pathways (corticosterone and deoxycorticosterone) by ACTH may be sufficient to overcome the lack of increase of aldosterone synthase (Silvestre et al 1998).

1.1.3-a-iii Low Na+/high K+ diet Regulating Cardiac Synthesis of Aldosterone

A low Na+/high K+ diet increases the aldosterone synthase mRNA by 4-fold in the LV and RV. It also raises the concentration of aldosterone by 3.5- and 11-fold, corticosterone by 3.5- and 2-fold and deoxycorticosterone by 3.5- and 5-fold both in the homogenate and in the perfusate of isolated perfused rats' heart respectively (Silvestre et al 1998).

1.1.3-a-iv High Sodium Intake Regulating Cardiac Synthesis of Aldosterone

Cardiac aldosterone synthase activity, CYP11B2 mRNA and aldosterone were increased by 2-fold after 8 weeks administration of high salt (0.9% NaCl in drinking water) in Wistar-Kyoto rats (Takeda et al 2000).

1.1.4 Vascular Synthesis of Aldosterone

Aldosterone has been shown to be produced in the mesenteric artery of Wistar rats (Takeda 2004). Endothelial and smooth muscle cells cultivated from human pulmonary artery and cultured rat aortic endothelial cells also produces aldosterone

(Hatakeyama et al 1994, Takeda et al 1995). The mRNA for P450 side chain cleavage gene CYP11A has been detected in the mesenteric artery of Wistar rats (Takeda et al 1994) as well as aorta of humans (Kayes-Wandover and White 2000). In humans, expression of mRNA for 3β-hydroxysteroid dehydrogenase, cytochrome P450 21 hydroxylase have been detected in aorta (Kayes-Wandover and White 2000) and of CYP11B2 in umbilical endothelial cells (Takeda et al 1996). The mRNA for aldosterone synthase is expressed in the mesenteric arteries and cultured vascular endothelial cells in rats. The amount of CYP11B2 mRNA cultivated from blood vessels, endothelial cells and smooth muscle cells is 50-100-fold lower than that the adrenal gland (Takeda 2004, Hatakeyama et al 1994). Takeda et al (1996) reported the activity of CYP11B2 on the conversion of [^{14}C] deoxycorticosterone to [^{14}C] aldosterone in human vascular endothelial cells. They also detected the radioactive peak of progesterone, deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone in the perfusate after perfusing the mesenteric artery with Kreb-ringer solution containing [^{14}C] pregnenolone (Takeda et al 1995, Takeda et al 1995a). Moreover, these steroids were also detected in the incubation medium of human vascular endothelial cells after incubation with [^{14}C] pregnenolone (Takeda et al 1996). The results indicate that the precursors of aldosterone exist in the vasculature (Takeda et al 1995a, Takeda et al 1996).

1.1.4-a Regulation of Vascular Synthesis of Aldosterone

1.1.4-a-i Ang II Regulating Vascular Synthesis of Aldosterone

Ang II stimulates the production of aldosterone in mesenteric artery of Wistar rats (Takeda 2004, Takeda et al 1995a) and increases the expression of aldosterone synthase

mRNA in endothelial cell and smooth muscle cell from human pulmonary artery (Takeda et al 1996). In cultured human vascular endothelial cells, aldosterone secretion increases from a basal rate of 30 ± 4 to 50 ± 6, 81 ± 8, and 130 ± 8 fmol/24 h with the addition of 10^{-9} and 10^{-8}, 10^{-7} mol/L Ang II respectively. Ang II also induces a dose dependent increase in CYP11B2 mRNA in smooth muscle cells and endothelial cells (Hatakeyama et al 1994, Takeda et al 1996). In human vascular endothelial cells, the conversion of [^{14}C] deoxycorticosterone to [^{14}C] aldosterone was increased from 4 ± 2% (control) to 7 ± 3, 11 ± 4, and 29 ± 6% with 10^{-9}, 10^{-8}, 10^{-7} mol/L of Ang II respectively, indicating a dose-dependent stimulation of aldosterone synthase activity (Takeda et al 1996). Ang II-mediated aldosterone synthesis was inhibited by an AT_1 receptor antagonist in vascular endothelial cells and by an ACE inhibitor in mesenteric artery of Wistar rats (Takeda 2004, Takeda et al 1995, 1996), further indicating the involvement of Ang II in mediating aldosterone synthesis in blood vessels.

1.1.4-a-ii Potassium ions Regulating Vascular Synthesis of Aldosterone

Potassium increases the production of aldosterone from the mesenteric artery by 50% (Takeda et al 2004). In cultured human endothelial cells, potassium increases the production of aldosterone from 30 ± 4 fmol/24 h (control) to 43 ± 3 and 75 ± 9 fmol/ 24 h with 7-9 mmol/L concentration of potassium. Potassium increases the expression of CYP11B2 mRNA in a dose-dependent manner. The conversion of [^{14}C] deoxycorticosterone to [^{14}C] aldosterone was increased from 4 ± 2% (control) to 6 ± 2 and 10 ± 3% with 7-9 mmol/L concentrations of potassium, indicating dose dependent increase of aldosterone synthase's activity (Takeda et al 1996).

1.1.4-a-iii ACTH Regulating Vascular Synthesis of Aldosterone

ACTH increases the secretion of aldosterone in cultured human vascular endothelial cells to 45 ± 3 and 120 ± 4 fmol/ 24 h with 10^{-10} and 10^{-8} mol/L of ACTH from a basal rate of 30 ± 4 fmol/ 24 h. ACTH does not effect the expression of CYP11B2 mRNA and the conversion of [^{14}C] deoxycorticosterone to [^{14}C] aldosterone in cultured human vascular endothelial cells (Takeda et al 1996).

1.2 ACTIONS OF ALDOSTERONE IN DIFFERENT TISSUES

Aldosterone together with other adrenal steroids, the glucocorticoids (cortisol in humans and corticosterone in rodents) maintains homeostasis in a large number of physiological systems. Aldosterone has a variety of actions and broadly these are divided into two major types, genomic and non-genomic. In genomic action, aldosterone binds to MR, translocates to the nucleus, modulates gene transcription and protein synthesis and has a considerable latency of effects. The non-genomic effects on the other hand, have a rapid time-course and these actions are transmitted by specific membrane receptors, unlike that of the classical genomic actions.

1.2.1 Mineralocorticoid Receptors (MR)

MR are found in both Na^+ transporting epithelia (kidney and colon) and nonepithelial tissues (e.g. brain and heart) (Funder 2005).

1.2.1-a MR in Brain

In the brain of adult Wistar rats, MR mRNA was found in the hippocampus, subfornical organ (SFO), organum vasculosum lamina terminalis (OVLT), supraoptic

nucleus (SON), hypothalamus, median preoptic nucleus (MnPO), PVN, and choroid plexus. The levels at most of these sites were similar except hippocampus and SFO. In the hippocampus, the MR mRNA levels were ~1.5-fold higher than in the kidney, which is considered a positive control. In the SFO, the MR mRNA levels were similar to that in kidney (Amin et al 2005). Sutanto and de Kloet (1991) reported the presence of MR mRNA in the neurons of the hippocampal formation, lateral septum, medial and central amygdala by in situ hybridization techniques using specific probe for MR.

Amin et al (2005) performed immunoreactivity of MR in Wistar rats by using antibodies for MR directed against a 17-amino acid sequence at the N terminus (MCR N-17) or a 19-amino acid sequence at the C terminus of rat MR (MCR C-19). The authors did not find differences in the immunopositive signal distributions in the area of interest between the two antibodies and they used MCR N-17 for expressing MR in Wistar rats. High levels of MR were found in the pyramidal subfields of the hippocampus. Compared to this, a similar or greater immunodensity was observed in the hypothalamic nuclei such as SON, magnocellular PVN, suprachiasmatic nucleus, periventricular, arcuate and medial preoptic nucleus, choroid plexus, ventricular ependyma, endothelial and smooth muscles of blood vessels and the pia-arachnoid. Immunoreactivity was also present in the circumventricular organs, OVLT and SFO. Pietranera et al (2001) also found immunoreactive expression of MR using MCR N-17 antibody in the OVLT, MnPO, amygdala and bed nucleus of stria terminalis, but not in the preoptic area, SON and PVN in SD rats.

Recently, Gomez-Sanchez et al (2006), produced monoclonal antibodies against 10 different peptide conjugates, 6 from the N-terminal (A/B domain) and 4 from the C-

terminal (steroid binding domain), with the anticipation that their individual affinities for the MR would differ depending on its conformation, which in turn is dependent upon the location of the receptor within the cell and the protein associated with it. Five monoclonal antibodies against the rat MR 1-18 peptides were studied; 6G1 and 1D5 were evaluated and seemed to be similar. Immunoreactivity against rat MR 1-18 6G1 was seen in hippocampus, choroid plexus, cerebellum and the staining was primarily nuclear with lighter cytoplasmic staining in different tissues. Antibodies raised against rat MR 64-82 stained the hippocampus (primarily nuclear) and choroid plexus (cytosolic and nuclear). Monoclonal antibody elicited by rat MR 79-97 peptides stained hippocampus and choroid plexus, both cytosolic and nuclear. Antibodies from the rat MR 365-381 recognized nuclei of hippocampus and cerebellum. Monoclonal antibodies against the peptide 832-846 produced weak immunohistochemistry staining that was similar to the above antibodies.

Sutanto and de Kloet (1991) also visualized MR by in vivo autoradiography in adrenalectomized rats with a tracer dose of [^{3}H] corticosterone or [^{3}H] aldosterone. [^{3}H] aldosterone was bound to MR in anterior hypothalamic nuclei, amygdaloid nuclei, circumventricular organ, and layer of cortex.

1.2.1-b MR in Heart

Immunohistochemical methods using monoclonal anti-idiotypic antibody H10E, which interacts with the steroid binding domain of MR, revealed the presence of immunoreactive material in the heart and large blood vessels of rabbits (Lombes et al 1992). In the heart, a positive staining was observed in cardiomyocytes, endothelial cells

and fibroblasts (Lombes et al 1992). In humans, the expression of MR was examined at the mRNA and protein level by in situ hybridization with cRNA probes specific for human mRNA and immunodetection of anti-MR antibody. In situ hybridization signal equivalent to that of whole kidney was present on cardiomyocytes. Specific immunolabelling of cardiomyocytes with anti-MR antibodies demonstrated the presence of MR protein (Lombes et al 1995). Gomez-Sanchez et al (2006) also reported the immunohistochemical visualization of MR in the heart using antibodies raised against the peptides comprising amino acids 1-18, 79-97, and 365-381.

1.2.1-c MR in Vessels

Immunostaining of MR was higher in aorta and pulmonary artery than in the carotid, renal, and mesenteric artery. In the large artery, staining was localized to endothelial and vascular smooth muscle cells (Lombes et al 1992). Hatakeyama et al (1994) examined the presence of MR in endothelial cells and smooth muscle cells using quantitative RT-PCR method. Lombes et al (1992) did not find any MR staining in the smaller arterioles and capillaries. However, our lab reported a moderate immunoreactivity of MR in the endothelia and vascular smooth muscle cells of small and medium sized vessels and capillaries (Amin et al 2005). Takeda et al (1997) reported the expression of MR mRNA in the mesenteric artery of 2-9 week-old Wistar-Kyoto rats as well as spontaneously hypertensive rats (SHR). MR present on the vascular smooth muscle of coronary vessels was expressed using antibodies raised against rat MR 1-18 and 365-381 (Gomez-Sanchez et al 2006).

1.2.1-d MR in Kidney

In the kidney, immunoreactivity of MR was localized in distal convoluted tubules, collecting ducts, loops of Henle, with no staining in the glomeruli or proximal tubules of adult Wistar rats (Amin et al 2005). Rundle et al (1989) also identified MR immunoreactivity in the superficial nephron segments, including distal tubule and collecting duct by using polyclonal antiserum against the hinge region of human MR and indirect peroxidase immunohistochemistry. Farman et al (1991) performed immunohistochemistry of MR in rabbits' kidney, using monoclonal anti-idiotypic, anti-MR antibody and Lombes et al (1990) by using a monoclonal antibody (H10 E), generated by auto-anti-idiotypic procedure and directed at the aldosterone-binding-site of MR. They found that MR were localized in all parts of the distal nephron and absent in the glomerulus and proximal tubule. MR predominated in the distal and all along the connecting tubules in its cortical, medullary and papillary portions and in the papillary interstitial cells and the epithelial cells lining the papilla. Gomez-Sanchez et al (2006) visualized MR in collecting tubules, distal convoluted tubules and cortical collecting tubules by performing immunohistochemistry using antibodies against rat MR 1-18, 64-82, 365-381 and 832-846.

1.2.2 MR Selectivity for Aldosterone

MR is open to binding both glucocorticoids and mineralocorticoids. The circulatory levels of glucocorticoids are much higher than mineralocorticoid, aldosterone, hence the MR could practically be bound only to glucocorticoids. However, this does not

happen and in a situation of excess amount of glucocorticoids, aldosterone still exerts a specific action on MR at the pre-receptor, receptor and post-receptor levels.

1.2.2-a Pre-Receptor-Level Selectivity

This selectivity is conferred by 11β-HSD2 and glucocorticoid binding globulin.

1.2.2-a-i 11β-HSD2

The enzyme 11β-HSD2, a nicotinamide adenine dinucleotide (NAD)-dependent enzyme has a high affinity for glucocorticoids and inactivates them. Aldosterone is not a substrate for 11β-HSD2 enzyme and hence is protected from metabolism (Figure 1). NAD has ~600-fold intracellular abundance over NADH and is an obligate co-substance for the dehydrogenation of cortisol to cortisone. When 11β-HSD2 is blocked, intracellular glucocorticoids levels rise to occupy MR. Depending upon the intracellular redox state, glucocorticoids can be agonist or antagonist in MR. The mRNA and activity of 11β-HSD2 enzyme has been detected in various tissues such as kidney, brain (Funder 2005, 2005a, Geerling et al 2006, Zhang et al 2006) and mesenteric artery of rats and human aorta (Kayes-Wandover and White 2000, Takeda 2003).

1.2.2-a-i-A 11β-HSD2 in the Kidney

The enzyme 11β-HSD2 is expressed at very high abundance in kidney. With this, a high ratio of plasma free cortisol to aldosterone (100:1) could be reduced in terms of the intracellular concentration (1:10 or 1:100). Hence the enzyme operates to prevent glucocorticoid occupancy on the MR (Funder 2005, 2005a).

1.2.2-a-i-B 11β-HSD2 in the Heart and Blood Vessels

The activity of 11β-HSD2 enzyme is about 100-fold lower in the heart than in the renal collecting duct (Lombes et al 1995), but its coexpression with MR in the heart and the cultured vascular smooth muscle cells (Lombes et al 1995, Kornel 1994) may facilitate its action. Mihailidou et al (2004) demonstrated the role of intracellular redox state in determining the activity of cortisol. When rabbit cardiomyocytes were treated with 10 nM of aldosterone, a 10-fold increase in patch current (to measure Na^+-K^+ pump current, arising from the $3Na^+$:$2K^+$ exchange) was seen in 15 min. Cortisol at 100 nM had no effect when given alone and blocked the agonist effects of aldosterone down to ~10%. When intracellular redox state was altered by infusion of oxidised glutathione directly into the cardiomyocytes, no change in basal current was seen, however when cortisol was added it became MR agonist, mimicking the effect of aldosterone.

The vascular smooth muscle cells also express MR and 11β-HSD2. When 11β-HSD2 is blocked, increased intracellular cortisol could activate MR (Alzamora et al 2000). In a study on Na^+/H^+ exchanger activity in human vascular smooth muscle cells, aldosterone raises intracellular pH. Cortisol does not activate vascular smooth muscle cells at a concentration of 100 nm when given alone, until carbenoxolone is added (to inhibit 11β-HSD2) through MR activation. When this is done cortisol becomes MR agonist elevating intracellular pH similar to aldosterone (Alzamora et al 2000).

1.2.2-a-i-C 11β-HSD2 in the Brain

The 11β-HSD2 mRNA is expressed in the embryo of mouse in the hippocampus, rhinecephalon and hypothalamus. Postnatally, it was expressed in the thalamus and

cerebellum. The mRNA expression peaked at the end of first postnatal week and declined thereafter. Postnatal brain showed considerable activity of high affinity 11β-HSD2, which is parallel to its mRNA expression (Robson et al 1998). In adult brain, autoradiograms showed specific labeling over a few discrete regions of the brain, from diencephalons to brain stem (Roland et al 1995). The distribution of 11β-HSD2 mRNA was found in ventrolateral ventromedial hypothalamus, PVN, subcommissural organ, and commissural portion of the nucleus tractus solitarius.

A high mRNA expression of 11β-HSD2 limited to the subcommissural organ and lower expression in the ventromedial nucleus of hypothalamus, amygdala, locus coeruleus, nucleus tractus solitarius, and medial vestibular nucleus was also found. These areas are compatible with the proposed selective central control of BP (subcommissural organ, nucleus tractus solitarius) and salt appetite (ventromedial nucleus, amygdala) (Robson et al 1998). Roland et al (1995) found marked expression of 11β-HSD2 mRNA in the commissural portion of the nucleus tractus solitarius and subcommissural organ, a circumventricular organ and moderate levels in ventrolateral portion of ventromedial hypothalamus. Geerling et al (2006, 2006a) found immunoreactivity of 11β-HSD2 in the nucleus of solitary tract, ventrolateral division of ventromedial hypothalamic nucleus, medial vestibular nucleus, ependymal cells that form the subcommissural organ. Zhang et al (2006) also reported the expression of 11β-HSD2 mRNA in the PVN of the hypothalamus and the cortex of SD rats and the levels were highest in the PVN.

Since MR expression alone is insufficient to define aldosterone target cells, the 11β-HSD2 and MR were found to clearly co-localize not only in the kidney, colon and

lungs, but also in neuroepithelia near the hippocampus of the mouse embryo (Brown et al 1996). Recently, Geerling et al (2006, 2006a) surveyed the entire brain for MR immunoreactivity and discovered clusters of dense nuclear and perinuclear MR in a restricted distribution within the nucleus of the solitary tract. The cells with dense nuclear MR also expressed the 11β-HSD2. Immunoreactivity of 11β-HSD2 was found throughout the cytoplasm of a small group of neurons in the medial nucleus of solitary tract in the same restricted distribution as the MR cluster. Zhang et al (2006) reported the activity of 11β-HSD2 in the PVN. Inhibition of this activity in the PVN by microinjection of carbenoxolone into the PVN or ICV injection of carbenoxolone and glycyrrhizic acid increased BP, HR and RSNA. This could be due to aldosterone-like responses to corticosterone (or cortisol) by binding to MR (Mihailidou et al 2005, Fuller et al 2005), the precise mechanism of which is not clear. It was suggested that another effect of 11ß-HSD2 - the conversion of NAD to NADH - may be important in determining the outcome of glucocorticoids binding to MR. By mechanisms not fully understood, an altered redox state induced by inhibition of 11ß-HSD2 activity may enable corticosterone to function as a MR agonist (Mihailidou et al 2005, Funder 2004).

Pretreatment with icv spironolactone prevented the increases of carbenoxolone-induced PVN activity, RSNA, BP, or HR in these rats. The findings suggest that MR in PVN contribute to sympathetic regulation, which may be activated by aldosterone or corticosterone depending on the state of 11β-HSD2 activity.

1.2.2-a-ii Glucocorticoid Binding Globulin

The specificity of MR for aldosterone can also be conferred by corticosterone / cortisol binding globulin, which binds to glucocorticoids and reduces their free circulating levels. Transcortin and albumin bind 95% of circulating glucocorticoids. On the other hand, only 50% of the aldosterone is bound to albumin (De Kloet et al 1977, Delcayre and Silvestre, 1999). The differential plasma binding allows aldosterone an order of magnitude advantage over corticosterone (Funder 1998).

1.2.2-b Receptor-Level Selectivity

At the receptor level, mineralocorticoid selectivity is conferred by MR binding affinity and ligand-induced conformational changes.

1.2.2-b-i MR Binding Affinity

MR bind both mineralocorticoids and glucocorticoids with high affinity. Funder (2005) reported their affinity to MR as deoxycorticosterone = corticosterone $\geq$ aldosterone = cortisol. Corticosterone has approximately 3-fold higher affinity for MR than aldosterone. The relative potency of aldosterone and corticosterone reflects the algebraic sum of the differences, regarding their affinity and the binding in plasma. In the heart, aldosterone is approximately 3-fold better competitor than corticosterone to MR in vivo. In the hippocampus, the aldosterone displacement curve was markedly shifted to the right and corticosterone has a better in vivo binding capacity than aldosterone (Funder 1994, 2005). Sutanto and de Kloet (1991) reported the relative binding affinities of deoxycorticosterone, aldosterone, cortisol, and corticosterone in the hippocampal cytosol and these were 3.9, 1.5, 2.1 and 1.2 IC 50 (nM) respectively.

In in-vitro kidney cytosol, obtained from adrenalectomized rats, corticosterone binds to MR equally as do aldosterone and deoxycorticosterone (Sutanto and de Kloet 1991). Kidney and colon express 11 β-HSD2, at high levels, the enzyme does not operate to exclude glucocorticoids, but to reduce their ability to bind for receptor occupancy by approximately an order of magnitude. In such a circumstance, there is still an order of 10-fold higher intracellular glucocorticoid than aldosterone levels, but somehow not activated (Funder 1994, 2005).

1.2.2-b-ii Ligand-Induced Conformational Changes

The mineralocorticoid selectivity is also conferred by ligand-induced conformational changes, which differ between glucocorticoid and mineralocorticoids and lead to differential transactivation capabilities. Even though aldosterone and cortisol bind to MR with a same order of affinity, the dissociation constants Kd, corresponding to the 'off' to 'on' ratio, are different. The dissociation of glucocorticoids from the MR is 4-times more rapid than that of aldosterone, despite similar-affinity constants (Lombes et al 1994). The half life ($t_{1/2}$) of aldosterone-MR compexes was 872 min, whereas the corresponding $t_{1/2}$ for cortisol-, corticosterone-MR complexes was 232 min and 202 min respectively. This indicates that aldosterone-receptor complexes are more stable and more efficient. Probably, MR has as an intrinsic property, whereby it discriminates aldosterone from glucocorticoids, independent of 11β-HSD2 effect (Lombes et al 1994). This constitutes an additional molecular mechanism that ensures selectivity of aldosterone action in a kinetic point of view. Another important step towards mineralocorticoid specificity is the characterization of N/C-terminal interaction. Cortisol produces much weaker N/C-interaction than aldosterone, and it is possible that N/C-

interaction may contribute to the observed functional difference in MR bound to the two ligands (Rogerson and Fuller 2003).

1.2.2-c Post Receptor-Level Specificity

A few coregulators have been described; some are general modulators, pleiotropic in their action and cellular expression, whereas others seem to be highly restricted to specific steroid receptor or with limited tissue distribution. The N-terminal domains of steroid receptors are highly specific with composite activating and repressing domains.

1.2.2-c-i Coactivators of MR

MR has been shown to interact with and is potentiated by steroid receptor coactivators (SRC)-1, mainly through its interactions involving the activating factor (AF)2 domain of ligand-binding domain. The ligand-binding domain lies in the C-terminal region and takes part in several functions including nuclear localization. SRC-2/ transcriptional intermediary factor (TIF) 2 and cAMP response element protein-binding protein (CBP) /p300 could also increase MR AF2 function. TIF 1α (21) or CBP/p300 are active through AF1a and TIF2 and CBP/p300 through AF1b domains (reviewed in Pascual-Le Tallec and Lombes 2005). The protein inhibitor of activated signal transducer and activator of transcription (STAT)-3 (PIAS)3 interact with in vivo and in vitro with TIF2. Jimenez-Lara et al (2002) reported a potential role of PIAS3 as transcriptional modulator of TIF2-mediated signaling. In mammalian two-hybrid assays and co-immunoprecipitation experiments in humans neuroblastoma SK-N-MC cells with co-expressed tagged protein, PIAS3 interacted strongly with the MR. The interaction of PIAS3 and MR was enhanced in the presence of aldosterone (Tirard et al 2004).

Peroxisome proliferators-activated receptor gama coactivator 1 is also a strong coactivator (reviewed in Pascual-Le Tallec and Lombes 2005).

1.2.2-c-ii Corepressor of MR

Silencing mediator of retinoic acid, nuclear receptor corepressor and thyroid hormone receptor function as corepressor of nuclear receptor. Death-associated protein and PIAS1 are corepressors of MR (reviewed in Pascual-Le Tallec and Lombes 2005).

1.2.3 Mechanism of Action of Aldosterone

Aldosterone may act through Genomic or Nongenomic actions.

1.2.3-a Genomic Actions

Aldosterone causes genomic effects by binding to MR. MR belongs to the nuclear receptor superfamily and is composed of several functional domains which include an N-terminal domain, a highly-conserved DNA-domain, and a C-terminal ligand-binding domain (Arriza et al 1987). The unbound MR is present in the cell cytosol. Unliganded MR is associated with a complex of chaperone protein, including the heat shock proteins (HSPs) 90, 70 and 56, which maintain the receptor as inactive state. The receptor-associated HSPs are necessary to enable MR to bind steroid with high affinity (Trapp and Holsboer, 1995). Hormone binding results in a conformational change, which causes dissociation of the activated associated protein from the HSPs, dimerization and translocation to the cell nucleus (Rogerson et al 2004). The hormone-activated receptor accumulates in dynamic clusters in the nucleus (Fejes-Toth et al 1998). The activated receptor/hormone complex binds to steroid response elements in the 5'UTR of

aldosterone-response genes that activate or repress gene transcription. The receptor-steroid complex may also act through a process of transcription interference or synergy whereby it interacts with other transcription factors that themselves bind DNA to activate or repress transcriptional activity (Karin 1998).

In peripheral tissues such as kidney, the genomic mechanisms of aldosterone following binding to MR enhances Na^+ transport and includes the synthesis and insertion of epithelial sodium channel (ENaC) subunits into the cell membrane to increase of ENaC expression and activity in cell membrane and the activation of existing Na^+ channels by regulatory protein so-called "aldosterone-induced protein" (Garty 1994, 2000). In the brain, the functional studies are suggestive of the same pattern, because the sympathoexcitatory and pressor responses to icv infusion of aldosterone can be prevented by blockade of either MR or Na^+ channels (Gomez-Sanchez 1986; Wang et al 2003). In rats post-MI, central responses to endogenous MR agonist also follow this pattern, because chronic icv infusion of benzamil (Na^+ channel blocker), and spironolactone equally prevent sympathetic hyperactivity and impairment of baroreflex function (Huang and Leenen 2005).

In endothelial cells derived from human blood vessels (which contain MR and ENaC), the ENaC promoter in endothelial cell vascular cell lines responds to aldosterone by transcriptional upregulation of αENaC (Golestaneh et al 2001). Aldosterone exposure for 72 h leads to swelling of adherent human umbilical venous endothelial cells. Aldosterone–induced swelling was prevented by the addition of spironolactone to the culture medium. Aldosterone-treated cells dramatically shrink when amiloride, a direct

inhibitor of ENaC (Saha et al 2005) was added to the primary endothelial cell culture (Oberleithner et al 2004).

1.2.3-a-i Genes Expression in Genomic Action

The actions of aldosterone are mediated by changes in gene expression. The search for MR-regulated genes involved in the control of ion transport has been a major goal in the aldosterone field. Of the induced genes and protein, glucocorticoid-regulated kinase-1 (SGK1) is most firmly established as a mediator of aldosterone action. Others include Nedd4-2, Kirsten Ras GTP-binding protein-2A (Ki-RasA) and phosphoinositide 3-kinase (PI3K).

1.2.3-a-i-A SGK1

SGK1 is an immediate early aldosterone-induced protein, a serine threonine kinase (Stockand 2002). SGK1 stimulates ENaC-mediated Na^+ transport in Xenopus oocyte coexpression assays (Chen et al 1999, Naray-Fejes-Toth et al 1999). In the kidney, SGK1 is selectively induced by aldosterone in the distal nephron, and appears primarily to regulate the plasma membrane abundance of ENaC and control the trafficking of ion transporters (Wang et al 2001). SGK1 gene transcription is rapidly increased by either MR or glucocorticoid receptor in most cell types and its induction is transient in some cell types but not others (Webster et al 1993, Chen et al 1999, Bhargava et al 2001). In Xenopus A6 cells, SGK1 mRNA begins to increase within 15 min following dexamethasone addition, reaches a maximum by 1 h and returns to basal level by 24 h, despite constant hormone levels and receptor activity. This indicates a decrease in SGK1 expression during late phase of mineralocorticoid action while Na^+ current is still increasing. This phenomenon of SGK1 deinduction is also found in a mouse kidney cell

line of cultured collecting duct cells (mpkCCD) on exposure to a constant level of dexamethasone (Chen et al 1999).

SGK1 is rapidly induced in rat nephrons in response to a single subcutaneous injection of aldosterone and then falls rapidly towards baseline (Bhargava et al 2001). In adrenalectomized SD rats, when aldosterone levels were kept constant by sc infusion of aldosterone with miniosmotic pump, the cortical and medullary nephron SGK1 expression rose rapidly and then began to drop between 6 and 24 h. After 24 h of constant aldosterone infusion, SGK1 was still higher than in vehicle-treated adrenalectomized rats though less than the peak value. This indicates a strong induction followed by deinduction of SGK1 and a possible role of this deinduction is to prevent excessive Na^+ reabsorption in the late phase of aldosterone action (Bhargava et al 2004).

1.2.3-a-i-B Nedd4-2

Nedd4-2 is an ubiquitin-ligase that associates with ENaC. Nedd4-2 activity is negatively regulated by SGK1. SGK1 binds to and phosphorylates Nedd4-2, reducing its binding to ENaC (Snyder et al 2004; Connell and Davies 2005). Nedd4-2 binds to and suppresses ENaC activity in a Xenopus oocyte's coexpression assay. Nedd4-2 suppresses ENaC activity by altering its trafficking and stimulating its degradation through ubiquitination. SGK1 interacts with and phosphorylates Nedd4-2 in a PY-motif-dependent manner, leading to reduced interaction between ENaC and Nedd4-2 and hence elevated ENaC cell surface expression (Snyder et al 2002). A subsequent reduction in ENaC ubiquitination by Nedd4-2 increases ENaC density and stability at the apical membrane resulting in increased ENaC-dependent action (Bhargava et al 2004). In oocytes, it was shown that SGK1 induces phosphorylation of Nedd4-2 on ser444 and

ser338. Mutation of these sites interfered with SGK1-dependent phosphorylation of Nedd4-2 and stimulation of ENaC (Debonneville et al 2001). However, in the phosphorylated state, Nedd4-2 catalyzes SGK ubiquitination and degradation, reducing SGK protein levels (Zhou and Snyder 2005).

However, SGK1 can stimulate Na^+ transport even expressed with ENaC subunits with C terminal deletion predicted to abolish its interaction with Nedd4-2. In a mouse model of Liddle's syndrome where one ENaC unit no longer has a PY motif, aldosterone still increased Na^+ transport in freshly isolated collecting tubules and primary culture of CCD. SGK1 appears to increase the function of a number of other ion transport pathways including the collecting duct protein ROMP 1 and $Na^+ K^+$ ATPase, none of which have a PY motif and are not known to be target of Nedd4-2. Nedd4-2 expression does not always parallel ENaC expression, such as colon, where ENaC is expressed in the surface lining cells, while Nedd4-2 is found in the crypt cells that do not express ENaC. Thus part of the aldosterone-SGK1 mediated increase of Na^+ transport is through inhibiting the removal of ENaC from cell surface (Kamynina and Staub 2002).

Loffing-Cueni et al (2006) reported that by immunochemistry Nedd4-2 was found to be highly expressed in the aldosterone-sensitive distal nephron, with low staining intensity in the late distal convoluted tubule (where apical ENaC is high) and early collecting tubule and gradually increasing detection levels towards the collecting duct (where apical ENaC is low). Compared with high salt diet (5%), 2 weeks of low salt diet (0.01%) drastically reduced Nedd4-2 immunostaining and increased apical ENaC abundance in aldosterone sensitive distal nephron. Administration of aldosterone to cultured collecting duct cells mpkCCD-c14 for 5 days led to reduction of Nedd4-2

protein expression. This indicates that Nedd4-2 abundance is regulated by sodium in the diet, by a mechanism probably involving aldosterone. Aldosterone stimulates phosphorylation of Nedd4-2 on ser444 in mpkCCD-c14 cells and in adrenalectomized rats (Flores et al 2005). 14-3-3 proteins constitute a family of highly conserved regulatory molecules that generally bind to phosphorylated residues within their protein targets. 14-3-3 represents a novel class of Nedd4-2-binding protein that affect how Nedd4-2 interacts with ENaC. When phosphorylated, Nedd4-2-ser444 is part of a consensus binding site for 14-3-3 proteins, suggesting that it is the binding of 14-3-3 proteins to Nedd4-2 that sterically interferes with the interaction of Nedd4-2 with ENaC and prevents ENaC ubiquitination (Ichimura et al 2005). Umemura et al (2006) examined the expression of Nedd4L in the kidney, brain, heart and other tissues in Dahl salt-sensitive (S) and Dahl salt-resistant (R) rats. On high (8%) or low (0.3%) salt diet, total Nedd4L expression in kidney was lower in Dahl S than Dahl R rats. High salt diet increased Nedd4L (a ubiquitin ligase having 97% amino acid sequence identity to Nedd4-2) expression in kidney by ~2-fold in Dahl R rats while it was not affected in Dahl S rats.

Nedd4-2 is a substrate for phosphorylation by protein kinase A (PKA) both in vitro and in cells. Ser-327 was critical and mutation of this residue abolished Nedd4-2 inhibition of cAMP. Ser-327 is also phosphorylated by SGK and this residue is required for SGK inhibition of Nedd4-2. Thus Ser-327 is a molecular convergence point for PKA and SGK. Ser-221 contributed to Nedd4-2 regulation by cAMP (but not SGK), whereas Thr-246 contributed to regulation by SGK (but not cAMP). Both vasopressin (via cAMP and PKA) and aldosterone and glucocorticoid (by SGK) increase Na^+ transport by increasing ENaC expression of cell surface. This model is supported by the finding that

over-expression of SGK blunted ENaC stimulation of cAMP, whereas inhibition of SGK increased stimulation. Conversely, cAMP agonists decreased ENaC stimulation by SGK (Snyder et al 2004).

1.2.3-a-i-C Ki-RasA

The expression of Ki-RasA is induced during the early phase of aldosterone action and appears to be necessary for the action of aldosterone on sodium transport in renal epithelial cells. Aldosterone also stimulates the proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 cascade in a MR dependent manner. Activation of the MAPK cascade may represent a key signaling convergence point that stimulates cardiac fibroblast growth by aldosterone (Stockand and Meszaros 2003). Ki-RasA and PI3K are necessary for the action of aldosterone on ENaC and to increase Na^+ reabsorption on the renal epithelia. The ENaC activity reflects the combined sum of all signaling inputs to include the linear Ki-RasA-PI3K-ENaC cascade (Staruschenko et al 2004). However, Ki-RasA appears to have dual action on the ENaC. Overexpression of Ki-RasA in Xenopus laevis oocytes shows that it not only keeps the channel open but also decreases the number the number of the channels in the plasma membrane (Stockand 2002, Stockand and Meszaros 2003).

1.2.3-a-i-D PI3K

The lipid kinase, PI3K is thought to play a role in the action of aldosterone, insulin and vasopressin. Its activity is increased by aldosterone and insulin in the kidney. Inhibition of PI3K reduces the action of aldosterone as well as the stimulation of sodium transport in the kidney by antidiuretic hormone. PI3K plays an important role in insulin signaling and insulin-dependent stimulation of transepithelial Na^+ transport can be

inhibited by inhibitors of PI3K (Record et al 1998). Activation of SGK requires phosphorylation via a PI3K mediated pathways. PI3K dependent phosphorylation plays a role in the mechanism of nuclear import in serum/growth factor treated cells (Maiyar et al 2003). Inhibitors of PI3K inhibits the early action of aldosterone and phosphorylation of SGK1, suggesting that SGK1 may be an integrator of both the aldosterone and insulin pathways with respect to ENaC stimulation (Stockand 2002, Blazer-Yost et al 1999).

1.2.3-b Nongenomic Actions and Receptors

The classical genomic actions of aldosterone have long been accepted. These effects can be inhibited by actinomycin and cycloheximide that block the transcription and translation. However, a number of actions of aldosterone occur through non-genomic mechanism (Wehling 1997). These take place within minutes and have been identified in non-epithelial cells such as vascular smooth muscle cells, lymphocytes, endothelial cells, cardiomyocytes and kidney cells. These rapid-actions are believed to be independent of gene transcription and translation and are insensitive to actinomycin and cycloheximide. In addition, these were identified in erythrocytes which lack nuclei (Losel et al 2004, Falkenstein et al 2000, Falkenstein et al 2000a).

These actions include changes in intracellular cAMP levels, intracellular calcium and increased intracellular pH, sodium current and activation of protein kinase C. The nongenomic actions are the unique characteristic of steroid actions, where aldosterone has 10,000-fold selectivity over cortisol (Wehling 1995). The receptors mediating the nongenomic actions may be the classical intracellular receptor located at different sites and binding to these receptors may initiate messenger cascades, different from those of

classical genomic receptors. Recent research into nongenomic effects has focused on identifying a specific nongenomic receptor, distinct from the MR, but attempts at its isolation and characterization have proven unsuccessful so far.

Non-genomic mediated mechanisms may involve activation of second messenger pathways for cross talk between nongenomic and classical steroid receptors, including MR (Le Moellic et al 2004). A two-step model has been proposed where the non-genomic effects are mediated by classical intracellular MR (Mihailidou 2005, Falkenstein et al 2000, 2000a). In rat arterial smooth muscle cells, the rapid sodium efflux mediated by aldosterone was insensitive to actinomycin, therefore independent of gene transcription. This action was blocked by the MR blockers spironolactone and RU-28318, indicating the involvement of classical MR (Connell and Davies 2005). Aldosterone-induced rapid increase of intracellular pH through the stimulation of Na^+/H^+ antiporter was blocked by RU 28318 (Alzomora et al 2000). The vasoconstriction of superior mesenteric artery and nongenomic effects of aldosterone on Na^+/H^+ exchanger activity, intracellular Ca^{2+} were blocked by eplerenone (Michea et al 2005). The rapid actions of aldosterone on calcium efflux have been demonstrated in cells lines lacking the classical MR and effects on calcium and cAMP have been shown in skin fibroblasts from MR-knockout mice (Haseroth et al 1999).

11β-hydroxysteroid dehydrogenase, which confers mineralocorticoid activity to MR, was involved in nongenomic effects of aldosterone in the human radial branches of the uterine artery. Cortisol was ineffective in activating Na^+/H^+ exchanger activity, but, in the presence of carbenexolone, an inhibitor of 11β-hydroxysteroid dehydrogenase, cortisol rapidly raised the intracellular pH, similar to aldosterone. This rapid effect of

cortisol was suppressed by RU 28318, further indicating the role of combined genomic and nongenomic action on the MR or the combined genomic and nongenomic actions.

Romagni et al (2003) documented a direct nongenomic effect of aldosterone on the resistance arteries in man by a fast reduction of forearm blood flow in man within 4 min during intra-brachial infusion of aldosterone. No constriction was observed in the contralateral forearm. The vasoconstrictor effect was not sustained and the flow returned to baseline after approximately 30 min.

Many nongenomic actions of aldosterone have been demonstrated in the heart. Aldosterone (EC_{50} <1 $_{nM}$) rapidly reduces PKC activity in cultured neonatal rat cardiomyocytes. This effect is mimicked by fludrocortisone (a synthetic corticosteroid with moderate glucocorticoid potency and high mineralocorticoid potency) and was not blocked by spironolactone, which at higher concentrations (0.1 – 1.0 $_{uM}$) shows partial agonistic activity (Sato et al 1997). Mihailidou et al (2005) reported that aldosterone increases Na^+ /K^+ /2Cl^- cotransporter activity and the resultant increase in intracellular [Na^+] results in increase of Na^+ pump activity within 15 min. Effects on both Na^+ /K^+ /2Cl^- cotransporter and Na^+ /K^+ ATPase activity involves phosphorylation directly or indirectly via PKCε. The rapid effects of aldosterone are unaffected by actinomycin D, canrenone or spironolactone.

In the brain, aldosterone acts in the amygdala to influence sodium intake through the classical MR as well as non-genomic mechanism. The nongenomic action on sodium intake was investigated by implantation into the amygdala of DOCA, aldosterone or their A-rink-reduced tetrahydro derivatives 15 min prior to excess to saline. Antisense oligodeoxynucleotide against the MR in the amygdala inhibited DOCA-induced salt

intake. DOCA and aldosterone increased saline intake within 15 min after steroid application. Application of A-ring reduced 3β, 5β tetrahydroaldosterone and 5α-tetrahydrodeoxycorticosterone produced the same increase in sodium intake (Sakia et al 2000). Recently, Karst et al (2005) reported a fast-onset nongenomic enhancement by corticosterone of glutamate transmission in the CA1 hippocampal area. This rapid effect depends on classical MR.

1.2.4 Actions of Aldosterone

Aldosterone acts on different body organs, such as brain, heart, blood vessels and kidney.

1.2.4-a Actions of Aldosterone in Brain

Aldosterone has been shown to exert a variety of important actions in the brain. This is facilitated by its lipophilic properties by which it can cross the blood brain barrier relatively easily (Karssen et al 2002). MR has been identified in hippocampus, hypothalamus, SFO, OVLT, SON, MnPO, PVN, lateral septum, amygdala, choroid plexus, pia-arachnoid, ventricular ependyma, and blood vessels, brain stem sensory and motor neurons (Amin et al 2005, Sutanto and de Kloet 1991, de Kloet et al 2000, Funder 1997). 11β-HSD2 was expressed in various areas in the brain compatible with control of BP (subcommissural organ, nucleus tractus solitarius) and salt appetite (ventromedial nucleus, amygdala) (Robson et al 1998). The effects of mineralocorticoids in the brain include induction of salt appetite, interaction with vasopressin control of cardiovascular functions, and development of hypertension.

1.2.4-a-i Mineralocorticoids Increase Salt Appetite

The MR in amygdala has been implicated in the control of salt appetite (Sakai et al 1996). The salt appetite induced by deoxycorticosterone is prevented by lesions of the amygdala (Schulkin et al 1989), by administration of MR antagonist, ZK 91587 (Vallee et al 1995), or by MR antisense oligodeoxynucleotide through icv infusion (Ma et al 1997) or direct injection into the amygdala (Sakai et al 1996), indicating that mineralocorticoids induce salt appetite by acting on amygdala. The mechanism underlying the increase of salt appetite with aldosterone is not completely known. Recently, a specialized subpopulation of neurons was identified within the nucleus tractus solitarius that express both 11β-HSD2 and MR (Geerling et al 2006). Geerling et al (2006a) proposed that the 11β-HSD2 and aldosterone - expressing target neurons of the nucleus tractus solitarius drive sodium appetite. In adrenalectomized rats, the infusion of aldosterone caused the appearance of dense nuclear MR immunoreactivity in the 11β-HSD2 neurons. Corticosterone had only a small effect on MR nuclear translocation in these neurons. Administration of aldosterone in combination with corticosterone elevated the percentage of the 11β-HSD2 neurons, but not significantly higher than aldosterone infusion alone (Geerling et al 2006a).

1.2.4-a-ii Increase of Vasopressin Release

Treatment with deoxycorticosterone (sc) induced a rise in the number of arginine vasopressin mRNA expressing cells in the PVN of Wistar-Kyoto rats and SHR. The effect was more pronounced in SHR. Deoxycorticosterone also increased the density (number of positive cells/mm^2) of V1aR-immunorective cells in the PVN and the Fos-immunoreactive cells in the PVN, OVLT of the SHR (Pietranera et al 2004) and the

mRNA and immunoreactivity for vasopressin in magnocellular and parvocellular divisions of PVN and SON in SD rats (Grillo et al 1998, Saravia et al 1999).

Saravia et al (1999) reported a decrease in the size of the immunoreactive arginine vasopressin area in the PVN and SON after 9 and 21 days of 3% salt intake. Deoxycorticosterone treatment increased the immunoreactive areas in PVN and SON. Salt intake increases plasma vasopressin, but the addition of deoxycorticosterone further increases it by 2-fold, indicating the role of mineralocorticoids in inducing the production of vasopressin concentration. The higher arginine vasopressin levels in the PVN in deoxycorticosterone-salt treated rats compared to untreated rats drinking salt indicates a role of deoxycorticosterone in sensitizing the PVN cells to increase arginine vasopressin production (Saravia et al 1999).

1.2.4-a-iii Increase of Sympathetic Activity

Sympathetic hyperactivity has been proposed as one of the central mechanisms mediating icv aldosterone-induced hypertension. Icv infusion of aldosterone (300 or 900 ng/h) in Wistar rats with physiological CSF [Na^+] concentration for 4 hours did not change baseline MAP, RSNA and HR but enhanced Na^+-rich aCSF - induced sympathetic hyperactivity and increases in BP and HR. This suggests that activation of brain sodium channels alone may not be sufficient to induce hypertension and that some increase CSF [Na^+] is needed for sympathoexcitation and the development of hypertension (Wang et al 2003). Icv pretreatment with benzamil prevented the RSNA, MAP, and HR responses to icv infusion of aCSF 0.16 M [Na^+] after icv infusion of aldosterone, indicting that short-term icv infusion of aldosterone along with Na^+-rich artificial CSF activates brain benzamil-blockable sodium channels, presumably ENaC,

thereby causing enhanced Na^+ entry and subsequent sympathoexcitation (Wang et al 2003). The enhanced responses to icv infusion of Na^+-rich aCSF after pretreatment with aldosterone were blocked by blockade of OLC by icv Fab fragments, which bind OLC with high affinity, indicating that brain OLC mediates the responses to icv aldosterone and CSF [Na^+].

Chronic icv infusion of aldosterone (25 ng/h) for 2 weeks with a small increase (0.15 M Na^+) in CSF [Na^+] causes hypertension associated with an increase in brain OLC. Benzamil blocked the increases in brain OLC and BP induced by central infusion of aldosterone (Wang et al 2003). Icv benzamil also blocked the increases in brain OLC in Wistar rats by chronic icv infusion of Na^+-rich aCSF (Wang and Leenen 2002). This indicates that in brain benzamil-blockable sodium channels are essential for OLC production and release and thereby leading to sympathetic hyperactivity and hypertension in Wistar rats with chronic icv infusion of Na^+-rich aCSF.

Similarly, Huang et al (2005a) reported that icv infusion of aldosterone in aCSF containing [Na^+] slightly higher than physiological CSF[Na^+] also increased RSNA, BP and HR in Dahl S (salt-sensitive) rats. The extent of the pressor effect of icv aldosterone appears to depend on the Na^+ in the artificial vehicle. The CSF sampled through the cisterna magna showed a minor (1 mM) increase in [Na^+] by icv infusion of aCSF with 0.16 vs 0.14 M Na^+. However, the CSF [Na^+] levels near the infusion site in the forebrain may be higher to explain the different responses in brain OLC, sympathetic hyperactivity and BP to icv infusion aCSF with 0.16 vs 0.14 M Na^+, as increase in CSF [Na^+] by 2-3 mM has been reported to increase the firing rate of neurons in the PVN and SON (Honda et al 1990). Leon et al (2002), found no increase of BP with icv infusion of aldosterone in

sheep. However, they used regular aCSF and a small increase in CSF [Na^+] may increase the BP on icv infusion of aldosterone. Icv aldosterone may activate MR and ENaC located in the ventricular ependyma (Sanchez et al 2000, Vigne et al 1989, Amin et al 2005) and thereby increases the entry of CSF [Na^+] into ouabain producing cells, such as astrocytes (Kala et al 2000) and/ or neurosecretory neurons (Takahashi et al 1988) in the PVN, leading to increase of OLC synthesis and or release. The increased brain OLC and subsequent increase in activity of brain RAS may also increase the synthesis/release of vasopressin (May 1996) and the later may also contribute to increase of BP.

1.2.4-a-iv Increase of Blood Pressure

The icv infusion of aldosterone produces a dose dependent increase of BP in rats having one kidney removed and given 1% NaCl to drink. Significant increases of BP occurred at day 7 in rats receiving 15 ng/h, day 11 in rats receiving 5 ng/h, and day 18 in rats receiving 1.5 ng/h of aldosterone by icv infusions. At day 20, the BP values were 119 ± 1 mmHg in control, 125 ± 1 mmHg for 0.5 ng/h, 131 ± 1 mmHg for 1.5 ng/h, 140 ± 2 mmHg for 5 ng/h, and 182 ± 5 for 15 ng/h of icv infusions of aldosterone (Gomez-Sanchez 1988). The chronic icv infusion of aldosterone (5 ng/h, 30 days) induced a rise of BP in unilaterally nephrectomized SD rats and this dose was ineffective when used systemically. Subcutaneous infusion of a large dose of aldosterone (500 ng/h) caused a rise in BP, which was almost similar to that produced by 5 ng/h of its icv infusion. Polydipsia and polyuria occur with the systemic infusion of aldosterone (500 ng/h), but these were not seen with icv infusion of 5 ng/h. (Gomez-Sanchez 1986). Kageyama and Bravo (1988) also reported an increase of BP with icv infusion of 50 ng/kg/h of aldosterone for 12 days in dogs, but the same dose was ineffective when infused

subcutaneously. The increase of BP with icv infusion of aldosterone (5 ng/h) was prevented by icv infusion of mineralocorticoid antagonist, prorenone (5 ng/h) (Gomez-Sanchez 1986). The increase of BP with subcutaneous infusion of aldosterone (1 ng/h) in male SD rats was also inhibited by icv infusion of mineralocorticoid antagonist, RU 28318 (1.1 ug/h) (Gomez-Sanchez et al 1990), indicating that systemically administered aldosterone may be entering the brain (Schmiedek et al 1973) to mediate its central effects of increasing the BP by acting in the MR.

1.2.4-b Actions of Aldosterone in Heart

Aldosterone mediates its effects on the heart through MR present in cardiac myocytes, endothelial cells of atria and ventricle, fibroblasts and vascular smooth muscle cells (Young et al 1994, Lombes et al 1992).

1.2.4-b-i In-vitro Actions

1.2.4-b-i-A Increase of Collagen

Aldosterone stimulates collagen synthesis, measured by ^{3}H-proline incorporation in cultured adult rat cardiac fibroblasts in the concentration of 10^{-9} M. This increase is inhibited by prior incubation with spironolactone, indicating a role of aldosterone on MR in mediating this action.

Recently, Rude et al (2005) reported that adult rat ventricular myocytes when treated with aldosterone for 24 hours increase the activity of matrix metalloproteinase (MMP)-2 and MMP-9. Pretreatment with spironolactone abolished the aldosterone-induced MMP activities. Aldosterone induced increases of mitogen/ extracellular signal-regulated kinase (MEK) activities and extracellular signal-regulated kinase (ERK)1/2 phosphorylation and

intracellular reactive oxygen species. U0126, a MEK1/2 inhibitor, abolished the aldosterone-induced increase in MMP activities. Apocynin, a nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase inhibitor, MnTMPyP and N-acetylcysteine inhibited aldosterone-induced ERK1/2 phosphorylation and the increase in MMP activities. PKC is involved in the nongenomic action of aldosterone as chelerythrine, a PKC inhibitor, abolished the aldosterone-induced increases in ERK1/2 phosphorylation and MMP activities. This indicated that aldosterone directly induces MMP-2 and MMP-9 activities in cardiomyocytes through a rapid, nongenomic effect mediated by MR, PKC and MEK/ERK1/2 pathways. Reactive oxygen species also play a role in regulation of MMP activity in response to aldosterone (Rude et al 2005).

Incubation with Ang II increased the collagen synthesis in a similar experiment in a concentration dependent manner. No change of collagen synthesis was observed with 10^{-1} and 10^{-9} M of Ang II, while collagen synthesis increased with 10^{-7} and 10^{-6} M. The 10^{-7} and 10^{-6} M of Ang II also decreased the collagenase activity, in contrast to aldosterone which does not affect collagenase activity (Brilla et al 1994). Since administration of Ang II increases the production of aldosterone in cultured neonatal cardiomyocytes (Casal et al 2003), the effect of Ang II to increase the fibrosis may be direct or indirect through aldosterone.

1.2.4-b-i-B Cardiomyocyte Hypertrophy

Aldosterone (10 nM) increased cardiomyocyte hypertrophy, measured as [^{3}H] leucine incorporation in neonatal rat cultured cardiomyocytes. Spironolactone (1 uM) alone was without any effect on leucine incorporation, but completely blocked the effect

of aldosterone (10 nM), indicating that the effect of aldosterone on protein synthesis is mediated through MR (Sato et al 1996).

1.2.4-b-ii In-Vivo Actions

1.2.4-b-ii-A Cardiac Hypertrophy and Fibrosis

The cellular mechanisms involved in aldosterone-induced fibrosis remain unclear. Aldosterone stimulates proliferation of cardiac myofibroblasts through MR-dependent activation of the Kristen Ras and mitogen-activated protein kinase cascade (Stockand et al 2003). Robert et al (1995) studied the time of occurrence of biological determinants of cardiac fibrosis after administration of aldosterone (0.75 ug/h, sc) and salt to rats. At 3 and 7 days of treatment, no histological and biochemical changes were seen. At 2 weeks, type III pro-collagen mRNA increased by 83% and type I pro-collagen tended to increase in the RV, both types I and III pro-collagen tended to increase in the LV. At 4 and 8 weeks, type I and III pro-collagen mRNA were increased, cardiomyocyte necrosis detected and progressive cardiac fibrosis was seen in both the ventricles. At 4 weeks of aldosterone salt treatment, a marked accumulation of collagen transcripts were seen in the RV as compared to LV, e.g., type I and III pro-collagen increased by 184% and 230% respectively in the RV, whereas these increased by 78% and 162% in the LV. These differences in the RV and LV pro-collagen persisted at 8 weeks.

Systemic infusion of aldosterone along with high salt diet induced LV hypertrophy and interstitial and perivascular fibrosis in the LV and RV. A low dose of spironolactone (20 mg/kg/day), which did not affect BP and cardiac hypertrophy prevented aldosterone-induced myocardial fibrosis (Brilla and Weber 1992). A large dose

of spironolactone (200 mg/kg/day) however prevented the increase of BP, cardiac hypertrophy and fibrosis. This indicates that in the heart, the myocyte and nonmyocyte compartments are under separate control, the myocyte hypertrophy is mostly related to ventricular loading and increase of collagen is due to aldosterone acting on the fibroblasts, in concert with other humoral factors (Brilla et al 1990). In old normotensive rats, an 8-week treatment with spironolactone prevented the age-induced increase in cardiac and arterial fibrosis and improved arterial stiffness despite the lack of hypotensive effect (Lacolley et al 2001). This indicates a role of aldosterone in accumulation of extracellular matrix in heart and artery during aging.

Aldosterone upregulates ACE mRNA expression in rat neonatal cardiomyocytes (Harda et al 2001, Wang et al 2002). Aldosterone also increases cardiac AT_1 receptor density and mRNA and this effect is dose-dependent (Sun and Weber 1993, Roberts et al 1999). This indicates that positive feedback mechanisms, which are induced by aldosterone, might exist in the heart at the level of ACE and AT_1 receptors. MR blockade by spironolactone blocks both feedback mechanisms. The increase of AT_1 receptor density was also prevented by losartan. Ang II may be involved in inducing cardiac hypertrophy and myofibroblast proliferation at the site of fibrosis, probably through the increase of intracellular Ca^{+2}, since calcium channel blocker, mibefradil prevents aldosterone and Ang II-induced cardiac hypertrophy and fibrosis (Ramires et al 1998). Mibefradil also attenuated hydroxyproline concentration and collagen volume fraction and ACE binding density in rats receiving aldosterone. Calcium may thus modulate fibrous tissue formation in rat model of hyperaldosteronism by altering myofibroblasts collagen turnover and cell growth.

Endothelin-1 gene is over-expressed in the endothelium of epicardial and intramyocardial coronary artery and endocardium in DOCA-salt hypertensive rats, indicating the involvement of endothelin in aldosterone-induced cardiac fibrosis (Lariviere et al 1996). This is further confirmed by decrease of cardiac interstitial and perivascular fibrosis with endothelin antagonist, bosentan in DOCA-salt treated rats (Karam et al 1996).

1.2.4-c Actions of Aldosterone in Blood Vessels

Aldosterone is involved in vascular smooth muscle hypertrophy and increase of vascular matrix.

1.2.4-c-i Vascular Smooth Muscle Hypertrophy

Aldosterone increased the incorporation of [^{3}H] leucine into vascular smooth muscle cells and this incorporation is inhibited by an MR antagonist (Hatakeyama et al 1994), suggesting that aldosterone directly induces hypertrophic changes in vascular smooth muscle cells.

1.2.4-c-ii Vascular Fibrosis

The administration of aldosterone to uninephrectomized SD rats receiving high salt diet increased the elastic modulus wall stress, measured as medial cross-sectional area of carotid artery and thoracic aorta and the structure of arterial wall, analyzed by immunohistochemistry for elastin and fibronectin. Eplerenone normalized the aldosterone-induced increases of elastic modulus wall stress, elastin and fibronectin (Lacolley et al 2002).

1.2.4-d Action of Aldosterone in Kidney

Aldosterone actions in the kidney to regulate electrolytes excretion and intravascular volume have been studied most thoroughly. Western analysis with MR-specific antibody detected MR protein expression in rat mesangial cells and renal fibroblasts (Nishiyama et al 2005). RT-PCR analysis revealed significant gene expression in both these cells (Nishiyama et al 2005). Terda et al (2005) showed that MR translocation from the cytoplasm to the nucleus was induced in rat mesangial cells by treatment with aldosterone. Aldosterone diffuses directly across the plasma membrane, binds to MR in the cytoplasm, and the complex is internalized into the nucleus. In the nucleus, the activated receptor acts as a positive transcription element for multiple genes, including ENaC and SGK-1. In addition, aldosterone increases the synthesis of Na^+/K^+ ATPase located in the basolateral cell membrane, which creates electrochemical gradients that drive the diffusion of Na^+ through the sodium channel (Horisberger and Rossier 1992).

Aldosterone increased [^{3}H]-thymidine uptake in renal mesangial cells, suggesting that aldosterone directly induces cell proliferation (Nishiyama et al 2005). Aldosterone induces collagen synthesis in glomerular mesangial cells (Wakisaka et al 1994). Aldosterone stimulates collagen gene expression and synthesis in renal fibroblasts and these effects were prevented by pretreatment with eplerenone (Nishiyama and Abe 2006). Chronic administration of aldosterone and salt (1% in drinking water) to SD rats induces severe glomerular mesangial injury and interstitial fibrosis in the kidney. This was associated with activation of MAPKs, including ERK1/2, c-Jun-NH_2-terminal kinases and big-MAPK-1. Aldosterone-induced renal injury and MAPKs activation were

prevented by concurrent treatment with eplerenone (Nishiyama et al 2004). Eplerenone and inhibition of the ERK1/2 cascade with PD98059 abolished aldosterone-induced cell proliferation and deformity in renal mesangial cells as well as the collagen synthesis in renal fibroblasts (Nagai et al 2005).

1.3 HEART AND EXTRACELLULAR MATRIX

The heart consists of cellular and extracellular compartments. In the cellular compartment, nearly 25% of the cells in the healthy heart are cardiomyocytes and because of their dimensions, these constitute about 75% of the total cell volume. The non-cardiomyocytes are fibroblasts, endothelial cells, smooth muscles, macrophages and plasma cells. Of these different cardiac cells, fibroblasts predominate. Myocardial cells are supported by an ECM that not only stabilizes the physical structure of the tissue, but also plays an active role in regulating cell development, migration, proliferation, shape and function (Cleutjens 1996, Jugdutt 2003). The ECM consists mainly of fibrous proteins (collagen and elastin that provides support and resists deformation) and adhesion molecules (fibronectin and laminin that binds to collagen).

1.3.1 Collagen

Collagen accounts for about 25% of the total protein mass and provides tensile strength and resists stretch, whereas elastin promotes resilience. Collagen has a central core of a long, stiff, triple helical conformation, in which the sequences of glycine-proline-hydroxyproline wind around each other into a superhelix (Jugdutt 2003). Five molecular isoforms of collagen, collagen types I, III, IV, V, VI are present in the heart (Bishop and Laurent, 1995). The major fibrillar collagen types I and III constitute the

bulk of cardiac ECM. Fibrillar collagen type I having the tensile strength approximate to steel and accounts for approximately 75% of total collagen. It is associated with thick fibers and confers tensile strength and resistance to stretch and deformation (Weber et al 1989, Weber and Brilla, 1991). Collagen type III is associated with thin fibers that confer resilience (Weber 1989). Collagen types I and III form aggregate struts of varying thickness and length that are distributed between myocytes and muscle fibers (Pelouch et al 1993). Fibrillar collagen contributes to the transduction of force generated by myocyte and to prevention of muscle fiber slippage (Ju and Dixon, 1996). Basement membrane contains a structural backbone of the collagen type IV to which other protein such as laminin and fibronectin can attach. Type V collagen is localized in basement membrane and interspersed in the interstitium and type VI between cardiomyocytes, linking basement membrane of the individual cardiomyocytes to the surrounding ECM and interstitial cells. Fibroblasts are primarily responsible for the synthesis of collagen in the myocardium (Zak 1973).

The abundance of collagen at any site depends on the balance of its synthesis or degradation. The collagen synthesis involves intracellular synthesis of pro-α chains, hydroxylation of selected prolines and lysines, glycosylation, formation of procollagen triple helixes, secretion into extracellular space, converting into less soluble molecules, assembly into collagen fibrils and aggregation to form fibers. The enzyme propyl-4-hydroxylase catalyzes the formation of 4-hydroxyproline by hydroxylation of proline in the triplet amino acid sequences. The factors stimulating collagen synthesis include, transforming growth factor (TGF)-β1, connective tissue growth factor, cytokines such as

tumor necrosis factor (TNF)-α and interleukin-1 (Jugdutt 2003). The degradation of cardiac ECM is mediated by MMPs (Tyagi 1997).

1.3.2 Fibronectin

Fibronectin is an adhesion molecule, another protein of ECM and is distributed homogenously in the extracellular spaces, in which the cardiomyocytes and collagen are located. Fibronectin has the binding sites for collagen, heparin, fibrin and proteoglycan molecules. Cardiomyocytes adhere to ECM via matrix specific receptor, including those specific for fibronectin and may act a bridge between cardiomyocytes and collagen (Pelouch et al 1993, Jugdutt 2003). It plays a crucial role in guiding cell migration and proliferation in embryonic development. Fibronectin is synthesized by fibroblasts and is degraded by MMP-2, -7, -10, and -14 (reviews, Jugdutt 2003, Ju and Dixon 1996).

1.3.3 Laminin

Laminin is another adhesion molecule that is located in the basement of the cardiomyocytes, fibroblast and endothelial cells where it may bind to collagen type I, III, IV. Laminin is present along the entire length of the basement membrane and is abundant near sarcomeres Z band where collagen bundles are in contact with the sarcolemmal membrane (Price et al 1992). Laminin mediates cell adhesion, migration, growth and differentiation and also plays a role in normal organ function. Laminin is also important for the survival of cardiomyocytes in culture (Lundgren et al 1988). Fibroblasts are responsible for synthesis of laminin under controlled conditions.

1.3.4 MMPs

MMPs degrade matrix protein such as collagen, laminin and fibronectin. Collagenases (MMP-1, -8, -13) are highly specific for destroying fibrillar collagen. Gelatinases (MMP-2, -9) denature fibrillar collagen and collagen type IV and V, fibronectin, laminin and elastin. Stromelysins (MMP-3, -10) degrade fibronectin, laminin and collagen type III and IV. Other members such as MMP-7, -14, and membrane type (MT)-MMP degrade collagen type IV, fibronectin and elastin (Cleutjens 1996). The net activity of MMPs depends on their transcription, activation and inhibition. The transcription of MMP-gene to form pro-MMP is stimulated by factors such as IL-1, IL-6, epidermal growth factor, platelet-derived growth factor, TNF-α, and basic fibroblast growth factor. Other factors such as TGF-β, IL-4, and corticosteroid inhibit the gene expression. The activation of pro-MMP to active MMPs is stimulated by urokinase plasminogen activator/ plasmin system and inhibited by tissue inhibitor of metalloproteinase (TIMP) (reviewed in Creemers et al 2001). The degradation of collagen type I is catalyzed by interstitial collagenase and the telopeptide resulting from degradation are resistant to further degradation since their helical configuration remains intact (Diez and Laviades, 1997). The large telopeptide undergo spontaneous denaturation into nonhelical derivatives that are completely degraded into inactive fragments by interstitial gelatinases. The small telopeptide found in intact form in blood is cleared from the circulation via glomerular filtration and these may be considered as an index of the intensity of degradation of collagen I (Diez and Laviades, 1997, Risteli et al 1993). The activated MMPs are inhibited by interaction with naturally occurring, specific TIMPs (Tyagi 1997).

1.3.5 TIMPs

TIMPs are expressed by a variety of cell type and consist of four structurally related members TIMP-1, -2, -3, and -4. TIMPs bind to the active site of MMPs and inhibit their activity. TIMP-1 is a potent inhibitor of the activity of most of MMPs except MMP-2 and MT-MMP. TIMP-2 potently inhibits the activity of most MMPs, except MMP-9. TIMP-3 binds to MMP-1,-2,-3,-9, and –13. TIMP-4 inhibits MMP-1,-3,-7,-9 (Creemers et al 2001, Cleutjens 1996). A decrease in TIMPs levels can be expected to initiate degradation of ECM through the increase of MMPs levels.

Under normal physiological conditions the matrix components and other connective components are continuously synthesized and degraded. An uncontrolled metabolism of ECM does occur and is a common feature in most forms of acute and chronic heart diseases. The rapid degradation of ECM is seen early after acute MI, when the collagen degradation exceeds synthesis. MMPs are normally in latent phase. When activated these degrade collagen into fragments. The decreased levels of collagen can lead to cardiac dilation or even rupture. The inhibition of MMPs activity can reduce LV dilation and preserve function. The rate of collagen synthesis is very slow, about 0.56% per day in the canine ventricles compared to 7.2% per day for non-collagen protein (Bonnin et al 1981). The half life of collagen is 10 times longer than for non-collagen protein. In addition, fibroblasts which produce most of the collagen, though representing 70% of the cardiac cells remain in G_0 phase of cardiac cell cycle, until they are stimulated to proliferate (Weber et al 1992). This indicates that the replacement of collagen after degradation is very slow, providing a window of potential vulnerability for adverse remodeling in conditions associated with increased ECM degradation such as acute MI.

1.3.6 Fibrosis

A disproportionate accumulation of collagen called fibrosis occurs due to three possibilities, (1) more synthesis of collagen, (2) suppression of collagen degradation by MMPs, (3) overpowering of inhibitors of MMPs. Fibrosis alters myocardial structure and results in increased systolic and diastolic stiffness. The accumulation of collagen in interstitial spaces is called 'interstitial fibrosis' and around intramyocardial artery is called 'perivascular fibrosis'. Interstitial fibrosis leads to myocardial stiffness, whereas perivascular fibrosis affects the dilatory capacity of the vessel.

Fibrosis could be quantified by quantitative morphometry or biochemical assay for hydroxyproline. Morphometric or morphological technique quantifies both interstitial and perivascular fibrosis separately. Biochemical technique on the other hand, quantifies the total collagen in the myocardium and can not be used to identify the amount of collagen in the interstitial and around intramyocardial artery separately. Caspari et al (1975) reported that the collagen contents at birth is same in each ventricle and increases to a similar degree independent of mass of muscle. However, due to greater quantity of muscle developed and myocyte size in the LV than the RV, the concentration of collagen in the RV is 30% greater than the LV. The cardiac fibrosis occurring in response to myocyte necrosis that accompanies impairment of nutrient blood flow is called 'replacement fibrosis' and fibrosis occurring as a result of inflammatory cell mediated events in the absence of myocyte loss is called 'reactive fibrosis'. The fibrosis in the scar or infarct in replacement fibrosis, whereas the fibrosis in the non-infarcted LV and the RV is the reactive fibrosis.

1.4 ROLE OF ALDOSTERONE IN PATHOPHYSIOLOGICAL CONDITIONS

1.4.1 Heart Failure

A major role for aldosterone in the pathogenesis of heart failure was demonstrated from the results of the Randomized Aldactone Evaluation Study (RALES). In patients with moderate to severe heart failure (New York Heart Association, class III-IV), a low dose of spironolactone added to the current best-practice treatment, lead to reduction in mortality and morbidity by 30 and 35% respectively (Pitt et al 1999). In a sub-study of RALES, the blockade of MR significantly lowered circulating procollagen type III, a biological determinant of fibrosis, indicating the role of aldosterone in increasing collagen turnover (Zannad et al 2001). The Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) demonstrated the efficacy of eplerenone in addition to standard therapy in reducing all-cause mortality, sudden cardiac death, cardiovascular mortality/ cardiovascular hospitalization, the incidence of hospitalization for heart failure, and heart failure mortality/heart failure hospitalization in patients with LV ejection fraction $\leq$ 40% and clinical evidence of heart failure post-acute MI when eplerenone or placebo was initiated between 3-14 days (mean 7.3 days) after acute MI (Pitt et al 2003). Treatment with eplerenone in a subgroup of patients with LV ejection fraction $\leq$ 30% resulted in relative risk reduction of 21% vs placebo in both all cause mortality and cardiovascular mortality/ cardiovascular hospitalization, and 23% for cardiovascular mortality. The relative risk for sudden cardiac death was reduced 33% and heart failure mortality/heart failure hospitalization was reduced 25% with eplerenone compared with placebo. Within 30 days of randomization, eplerenone resulted in relative risk reduction of 43% for all-cause mortality, cardiovascular mortality/ cardiovascular

hospitalization and sudden cardiac death (Pitt et al 2006). A number of mechanisms contribute to progressive CHF post-MI, which may be affected by MR blockade.

1.4.2 Sympathetic Hyperactivity in CHF

Increased cardiac sympathetic drive during the initial stage of CHF precedes augmented sympathetic activation (Rundqvist et al 1997). Norepinephrine (NE) spillover in the heart, kidney as well as brain was increased in CHF (Kaye et al 1994, Hasking et al 1986). The degree of plasma NE elevation in patients with CHF correlates with the severity of LV dysfunction and with mortality (Levine et al 1982, Cohn et al 1984). Direct microneurographic recording shows marked increase in sympathetic efferent activity to skeletal muscle that correlates with plasma NE in patients of CHF. Sympathetic nerve activity increases in parallel to the impairment of cardiac performance (Leimbach et al 1986, Ferguson et al 1990).

Heart failure produced by permanent coronary artery ligation in rats mimics the heart failure in humans. LV peak systolic pressure (LVPSP), MAP and LV dP/dt max decrease and LV end diastolic pressure (LVEDP) increases, demonstrating a deterioration of LV function (Leenen et al 1995, Wang et al 2004, Francis et al 2001). Plasma NE and sympathetic activity increase post-MI (Leenen et al 1995, Wang et al 2004, Francis et al 2001). The increase in sympathetic drive in the rat post-MI involves enhanced activity of sympatho-excitatory pathways, as determined by responses to air jet stress. Responses to air jet stress reflect the activity of central sympatho-excitatory pathways (Koepke and Dibona 1985). The peak increase in MAP, RSNA, and HR in response to air stress is 2-3-fold larger in rat post-MI, indicating increased activation of sympatho-excitatory

pathways (Leenen et al 1995, Wang et al 2004). Increase of sympathetic drive post-MI may also be due to decreased activity of sympatho-inhibitory pathways. The peak decrease in MAP, RSNA and HR in response to icv injection of α_2-adrenoceptor agonist, guanabenz is 2-fold greater in rats post-MI compared to sham (Leenen et al 1995), suggesting upregulation of α_2-adrenoceptors in the inhibitory neurons in the anterior hypothalamic area as a result of decreased activity of sympatho-inhibitory pathways. The enhanced responses to icv guanabenz suggest decreased activity of sympatho-inhibitory pathways. Wyss et al (1988) suggested that decreased release of NE leads to decreased activity of sympatho-inhibitory pathways and upregulation of α_2-adrenoceptors on the inhibitory neurons in the anterior hypothalamic area. In animal models of CHF post-MI, FRA-like immunoreactivity (Vahid-Ansari and Leenen 1998) and the number of Fos-positive neurons (Lindley et al 2004) are markedly increased in central cardiovascular regions such as the PVN and SON.

Several mechanisms may contribute to activation of CNS pathways post-MI. Firstly, the cardiac branch of the vagus conveys mechanosensitive and chemosensitive information to the PVN (Lovick and Coote 1988) and cardiac denervation prevents the increase of brain TNF-α production in response to coronary artery ligation (Francis et al 2003) which could activate PVN neurons (Dunn 2000). Cardiac sympathetic afferent fibers are also activated in heart failure to provide excitatory input to the CNS (Ma et al 1997). Secondly, an increase in cardiopulmonary pressures normally has a sympatho-inhibitory effect (Shi et al 1993), but in patients with CHF, these have a positive correlation (Kaye et al 1994, Hasking et al 1986). Increased filling pressures may lead to activation of cardiac sympathetic afferent fibers contributing to sympatho-excitatory state

of chronic heart failure (Wang et al 1999). Thirdly, post-MI the circulatory RAAS becomes activated (Leenen et al 1999a, Hu et al 1996, Xiu et al 2002, Wang et al 2004). Plasma Ang II may activate AT_1 receptors in circumventricular organs such as SFO or OVLT (Lindley et al 2004), which signal to downstream nuclei such as PVN and SON, activating sympathoexcitatory pathways.

The loss of myocardium following coronary artery ligation results in a decrease of BP and increases of LVEDP and LV end diastolic volume (LVEDV) and consequently a decrease in cardiac output and BP (review, Packer 1992). The sympathetic nervous system is activated post-MI to preserve cardiac output and circulatory homeostasis. However, due to impairment of LV function the homeostasis is not achieved (Francis et al 2001). These compensatory mechanisms have deleterious effects on the failing heart and contribute to the progression of LV dysfunction. The increase of sympathetic activity post-MI, by causing arterial and venous constriction and salt and water retention by the kidney increases diastolic and systolic wall stresses on the failing myocardium. The increases in wall stress in response to sympathetic hyperactivity increase myocardial energy expenditure and accelerate the rate of cell death in the failing myocardium which consequently leads to progression of disease (Katz 1990).

1.4.3 Activation of RAAS Post-MI

The RAAS appears to be one of the major contributors to myocardial remodeling. Extensive evidence indicates that increased activity of the circulatory, cardiac and brain RAAS post-MI may contribute to cardiac remodeling and dysfunction post-MI through a variety of direct and indirect effects.

1.4.3-a Circulatory RAAS Post-MI

Different components of the circulatory RAAS are activated post-MI.

1.4.3-a-i Plasma Renin Post-MI

Our lab (Leenen et al 1999a) and Sun et al (2001) reported the time course of activation of plasma renin activity (PRA) in Wistar rats post-MI. PRA showed a significant increase at 6 h and only minor, non-significant increases at 3 days, 4 week, and 2 months post-MI (Leenen et al 1999a). In contrast, Sun et al (2001) found an increase of PRA post-MI to 5-fold at days 3, which further increased to 6-fold at week 1 and 10-fold at week 2, declined to 4-fold at week 3 and normalized at week 4. Other data of PRA post-MI are also variable. At day 1 post-MI, Nogae et al (2000) reported a 4-fold increase of PRA. At day 3 post-MI, Zelis et al (1994) reported non-significant increase of PRA by 43% and 51% with small (15%) and large (35%) MI. At week 1 post-MI, Hirsch et al (1999) and Francis et al (2001) reported a 3-4-fold increases, but Nakamura et al (2004) reported no change of PRA. At week 3 post-MI, Yamagishi et al (1993) and Francis et al (2001) reported a 2-fold increase of plasma renin concentration and activity. At week 4 post-MI, different studies reported an increase by 1.5-4-fold (Francis et al 2001, Nogae et al 2000, Tekeuchi et al 1999, De Resende and Mill 2007), a non-significant increase by 60% (in large MI, Kammerl et al 2000) and ~20% (Burrell et al 2000, Hodsman et al 1988), and no change (Duncan et al 1999, Silvestre et al 1999) of PRA in rats. Bralet et al (1994) reported a 4-fold increase of plasma renin concentration at 4 week post-MI. At week 6 post-MI, plasma renin concentration was increased significantly by 3.5-fold (Schunkert et al 1993). PRA was significantly increased by 2-fold (Francis et al 2001) non-significantly increased by 66% and 20% with large and

small MI (Zelis et al 1994). At week 8 post-MI, Hu et al (1996, 2001) reported 1.5-3-fold increases PRA. Michel et al (1988) reported 3-fold increase but van Veldhuisen et al (1994) reported no change of plasma renin concentration at 8 weeks post-MI. At week 12 post-MI, 1.5-2-fold increases of PRA (Huang et al 1994, Gonzalez et al 1996) and plasma renin concentration (Huang et al 1994) have been reported in normotensive rats.

Several factors could contribute to these variations in PRA levels post-MI.

Firstly, PRA follows circadian variation and in rats and its activity is maximum at 16:00 h and minimum at 9:00 h, a difference of 2-fold (Leenen et al 1975). Watanabe et al (1988) also reported ~2-fold higher PRA at 20:00 h (peak) than at 8:00 h (trough). The timing of blood collection was mentioned in only three (Leenen et al 1999a, Hu et al 2001, van Veldhuisen et al 1994) of the 23 above-mentioned studies and could have affected the PRA.

Secondly, anesthesia affects the PRA. Leenen et al (1981) reported the time course of changes in PRA following induction and maintenance of different anesthetics in rats. PRA increased (2-fold) within 1 min following urethane anesthesia, peaked at 15 min (8-fold) and remained elevated (8-fold) for at least 4 hours following induction of anesthesia. Pentobarbital increased PRA by 5-fold at 15 min. Exposure to ether for 1 min increased PRA by 5-fold at 1 min and by 2-fold at 30 min. Continuous exposure to ether produced more pronounced elevation of PRA for at least 1 h (16-fold). Halothane increases, enflurane decreases (Woodside et al 1984) and ketamine does not affect PRA (Miller et al 1979). Blood was collected 2-3 h after surgery when rats were not under anesthesia (Leenen et al 1999a, Zelis et al 1994, Gonzalez et al 1996, Yamagishi et al 1993), immediately after surgery under halothane (Sun et al 2001), ketamine (Silvestre et

al 1999), pentobarbital (Hu et al 1996, Nakamura et al 2004, De Resende and Mill 2007), and ether (Nogue et al 2000) anesthesia.

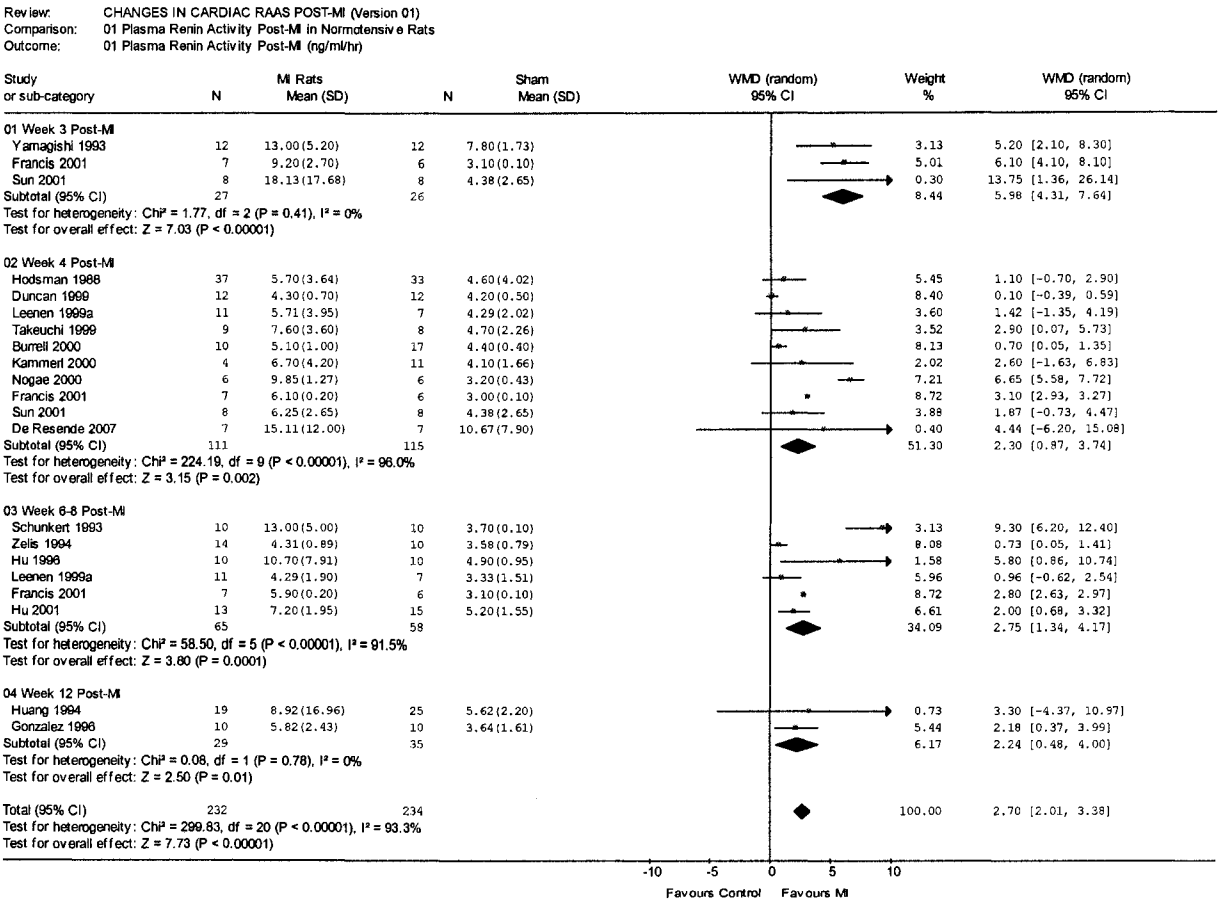
Thirdly, sites/ routes of blood collection could have affected PRA. Blood samples were collected from intra-arterial (Leenen et al 1999a), abdominal aorta (De Resende and Mill 2007, Hu et al 1996, Oie et al 2000), venous (Gonzalez et al 1996, Francis et al 2001), trunk blood after decapitation (Burrell et al 2000, Duncan et al 1999, Hirsch et al 1999, Hodsman et al 1988, Hu et al 2001, Huang et al 1994, Marie et al 1999, Michel et al 1988, Nakamura et al 2004, Nogue et al 2000, Yamagishi et al 1993). The routes of withdrawal were not mentioned by others (Bralet et al 1994, Sun et al 2001, Silvestre et al 1999, Schunkert et al 1993, Takeuchi et al 1999, van Veldhuisen et al 1994, Zelis et al 1994).

Fourthly, the size of the MI may have affected the PRA values (Kammerl et al 2000, Zelis et al 1994). Renal renin gene expression was increased 4-fold in rats with large MI (40-60% of LV), but remained unchanged in rats with small (<20%) and moderate MI (20-40%) at 4 weeks post-MI (Kammerl et al 2000). Among the different studies, PRA showed increases in rats with MI sizes of 30-60% (Burrell et al 2000, De Resende and Mill 2007, Francis et al 2001, Hodsman et al 1988, Gonzalez et al 1996, Hirsch et al 1999, Hu et al 1996, Hu et al 2001, Huang et al 1994, Kammerl et al 2000, Leenen et al 1999, Michel et al 1988, Schunkert et al 1993, Sun et al 2001, Takeuchi et al 1999, Yamagishi et al 1993) and no change in rats with MI sizes of 25-30% (Duncan et al 1999, Marie et al 1999, Silvestre et al 1999).

Cosidering the variations in PRA among different studies, a meta-analysis was performed using the Review Manager 4.2 of the Cochrane Collaboration. Twenty one

studies involving 466 normotensive rats were included in the analysis. An overall significant increase of PRA was found from week 3 to 12 post-MI, weighted mean difference (WMD) random [95% confidence interval (CI)] = 2.70 [2.01, 3.38], ($p < 0.00001$). Sub-group analyses at week 3, 4, 6-8 and 12 weeks also showed significant increase of PRA post-MI ($p < 0.00001$, $p = 0.002$, $p = 0.0001$ and $p = 0.01$ respectively, Figure 2).

Figure 2: Plasma Renin Activity at 3-12 Weeks Post-MI



1.4.3-a-ii Plasma Angiotensin II Post-MI

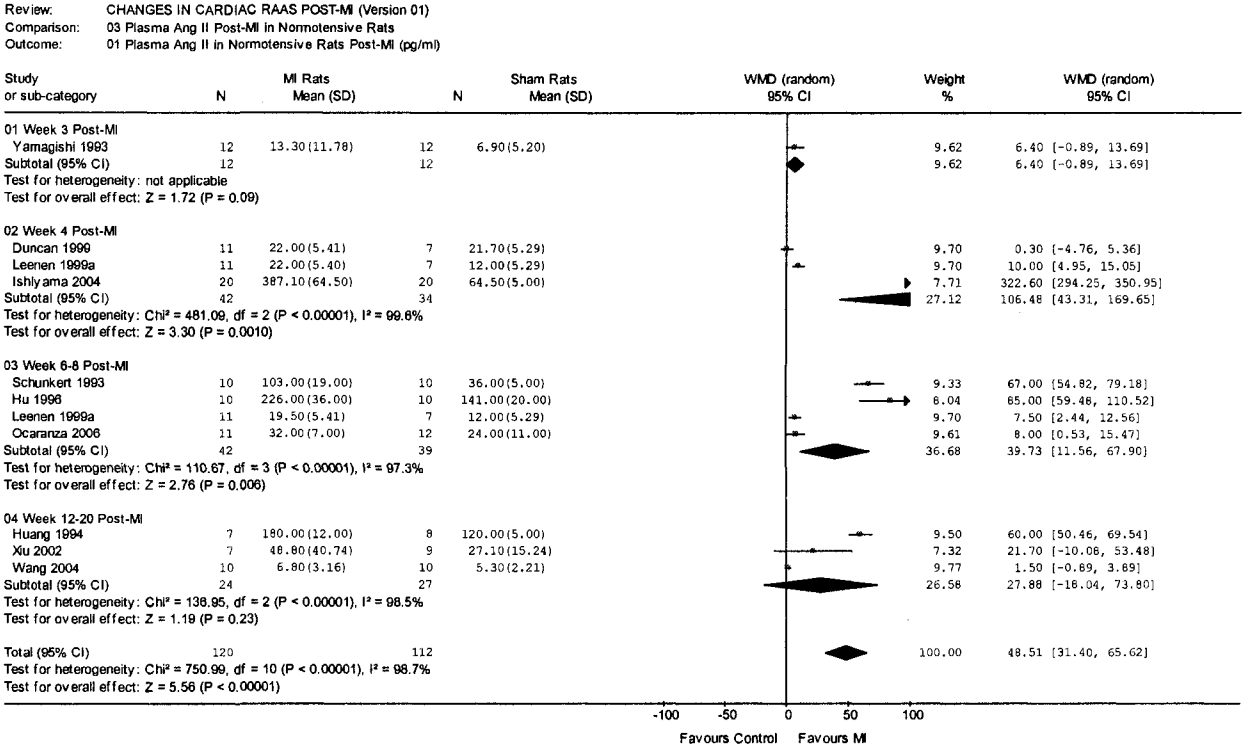
On studying the time course of activation of plasma Ang II, our lab showed a clear increase of plasma Ang II at 6 h post-MI and subsequently modest increases by 50-100%, which were significant at 1 and 4 weeks and 2 months post-MI in Wistar rats (Leenen et al 1999a). At week 1 post-MI, Ocaranza et al (2006) also reported a 2-fold increase of plasma Ang II. At week 2 post-MI, Dedkov et al (2007) reported 1.5-fold increase of plasma Ang II. At week a 3 post-MI, Yamagishi et al (1993) reported 2-fold increase of plasma Ang II. At week 4 post-MI, Ishiyama et al (2004) reported a 6-fold increase of plasma Ang II, but Silvestre et al (1999) and Duncan et al (1999) did not find change in plasma Ang II in normotensive rats. At week 6 post-MI, Schunkert et al (1993) reported a 3-fold increase of plasma Ang II in Wistar rats. At week 8 post-MI, plasma Ang II was increased by 1.5-fold in Wistar rats (Hu et al 1996). In SD rats, Ocaranza et al (2006) reported a 1.5-fold increase of plasma Ang II and Wang et al (2004) reported a tendency of 1.5-fold increase in large MI (>25%) and no change in small MI (<25%). At week 12 post-MI, Huang et al (1994) reported no change of plasma Ang II. At week 20 post-MI, Xiu et al (2002) reported a 1.5-fold increase in plasma Ang II in Wistar rats.

Some variation of plasma Ang II levels could be due to circadian rhythm, as plasma Ang II levels are maximum at 18:00 h and minimum at 6:00 h, with a difference of 3-fold (Schiffer et al 2001). The timing of blood collection, mentioned only in one (Leenen et al 1999a) of the above-mentioned studies could have affected the results of plasma Ang II. The route of blood withdrawal, such as arterial while the rat are freely moving (Leenen et al 1999a, Wang et al 2004), aortic (Hu et al 1996), venous (Ishiyama et al 2004, Ocaranza et al 2006), trunk after decapitation (Duncan et al 1999, Yamagishi

et al 1993), and not mentioned (Silvestre et al 1999, Xiu et al 2002, Schunkert et al 1993) could have affected the study results. The infarct size could have affected the results, as plasma Ang II increased in rats with MI sizes of 25-43% (Hu et al 1996, Ishiyama et al 2004, Schunkert et al 1993, Ocaranza et al 2006, Wang et al 2004, Xiu et al 2002, Yamagishi et al 1993) and remained unchanged in rats with MI sizes of <25-27% (Duncan et al 1999, Silvestre et al 1999, Wang et al 2004).

Due to varations in plasma Ang II among different studies, a meta-analysis of 11 studies involving 232 normotensive rats showed an overall significant increase of plasma Ang II from week 3-20 post-MI, WMD (random) [95% CI] = 48.51 [31.40, 65.62], ($p < 0.00001$). Sub-group analyses also showed significant increase of plasma Ang II at weeks 4 and 6-8 post-MI in normotensive rats ($p < 0.0001$, $p = 0.006$ respectively). No increase of plasma Ang II was found at weeks 3 and 12-20 post-MI (Figure 3).

Figure 3: Plasma Angiotensin II at 3-20 Weeks Post-MI

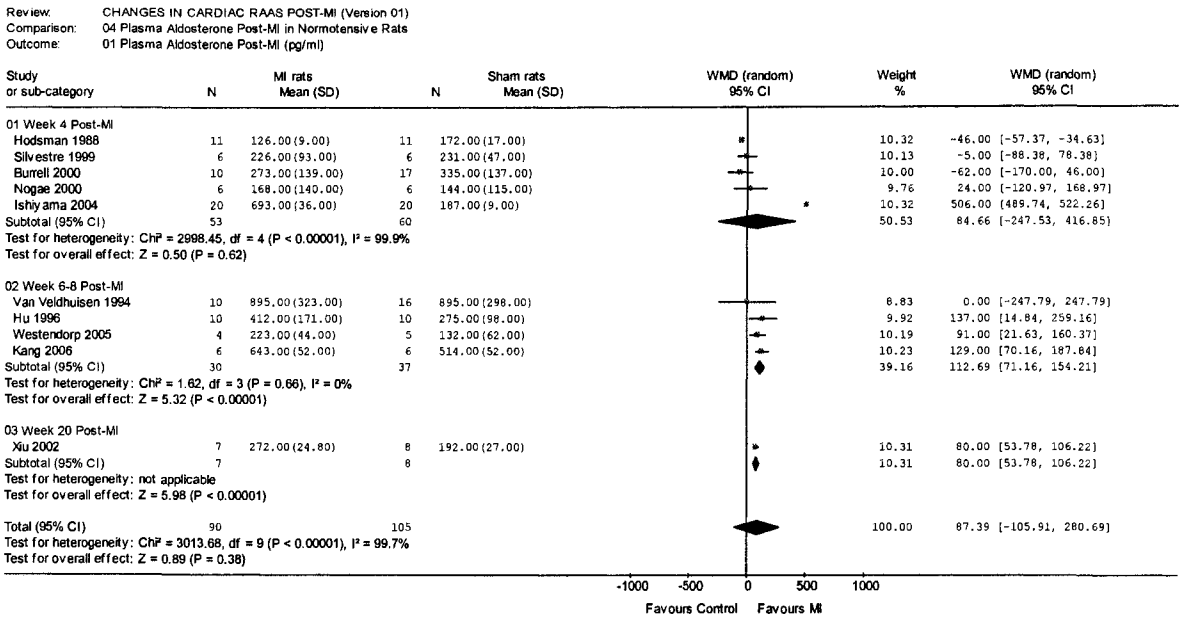


1.4.3-a-iii Plasma Aldosterone Post-MI

The data on plasma aldosterone post-MI are also variable. No change in plasma aldosterone was found at 1-3 weeks post-MI (Nakamura et al 2004, Cittadini et al 2002, Yamagishi et al 1993). At week 4 post-MI, Ishiyama et al (2004) reported a 4-fold increase and Burrell et al (2000) and Nogae et al (2000) reported tendency of increases by 20%, whereas Silvestre et al (1999) and Hodsman et al (1988) reported no change of plasma aldosterone. At week 6 post-MI, Kang et al (2006) reported a 1.5-fold increase of plasma aldosterone. At week 8 post-MI, Hu et al (1996) and Westendorp et al (2005) reported 1.5-fold increases whereas Michel et al (1988) and van Veldhuisen et al (1994) reported no change of plasma aldosterone. At week 20 post-MI, Xiu et al (2002) reported a 1.5-fold increase of plasma aldosterone in Wistar rats.

Several factors could contribute to different levels of plasma aldosterone post-MI. Firstly, plasma aldosterone levels also follow circadian rhythm with 3-fold range and are highest level at ~14:00 h and lowest at 24:00 h in rats (Lemmer et al 2000). The timing of blood collection was 9:00-10:00 h in van Veldhuisen et al (1994) study, but not mentioned in the remaining 13 studies. Secondly, surgery under anesthesia may increase plasma aldosterone by 5-10 times for up to 12 h (Fromm et al 1983). The blood was obtained under anesthesia from arterial and aorta (Hu et al 1996, van Veldhuisen et al 1994, Westendorp et al 2005), venous (Ishiyama et al 2004), and trunk after decapitation (Burrell et al 2000, Hodsman et al 1988, Kang et al 2006, Nogae et al 2000, Michel et al 1988, Silvestre et al 1999, Yamagishi et al 1993).

Figure 4: Plasma Aldosterone at 4-20 Weeks Post-MI



Due to varations in plasma aldosterone, a meta-analysis of 10 studies involving 195 normotensive rats was performed. It showed a significant increase of plasma aldosterone from 6-20 weeks post-MI and WMD (random) [95% CI] were 112.69 [71.16, 154.21], $p < 0.00001$ at week 6-8 and 80.00 [53.67, 106.33], $p < 0.00001$ at week 20 post-MI. No increase of plasma aldosterone was found at 4 weeks post-MI in normotensive rats (Figure 4).

In conclusion, a majority (~70%) of above-mentioned studies on the circulatory RAAS show an increase in plasma renin activity and concentration, Ang II and aldosterone post-MI in rats. The results of the remaining (~30%) of the studies reporting no change of circulatory RAAS post-MI could be related to variations in experimental conditions.

1.4.3-b Cardiac RAAS Post-MI

Different components of the cardiac RAAS are activated post-MI.

1.4.3-b-i Cardiac Renin Post-MI

Expression of renin mRNA was not detected in either the LV or RV of the sham rats, but was found at the site of MI and at other sites of injury involving visceral pericardium and endocardium of the interventricular septum in SD rats (Sun et al 2001). It was first evident on day 3 post-MI, peaked at week 1 and remained elevated over the course of 4 weeks. Renin mRNA expression was not observed in the non-infarcted LV or RV. Cardiac renin activity became evident at the site of MI on day 3, increased over the course of 3 weeks and remained elevated at week 4. Renin activity was undetectable in the non-infarcted LV or RV. Several cells may be responsible for renin synthesis, but macrophages and fibroblasts may primarily be responsible for this (Sun et al 2001). Passier et al (1996) reported a 4-fold increase of renin mRNA at day 2 and 14-fold increase at day 4 in the LV and 2-fold increase in the septum. At 1 week post-MI, renin concentration was increased by 3-8-fold in the LV (Hirsch et al 1999, Passier et al 1996) and by 2-fold in the infarct (Hirsch et al 1999). The increased cardiac renin production following MI could contribute to enhanced local generation of Ang II.

1.4.3-b-ii Cardiac Ang II Post-MI

Our lab reported the time course of changes in cardiac Ang II levels in Wistar rats post-MI (Leenen et al 1999a). In the infarcted area, cardiac Ang II was increased at 6 h post-MI, and this persisted at 1 and 3 days. By week 1, only a modest increase was

present, and by 4 and 8 weeks post-MI, no significant differences were present between the infarcted and sham rats. In the non-infarcted LV, Ang II was markedly increased at 6 h and day 1 post-MI. By day 3, this increase had disappeared relative to sham rats. Cardiac Ang I increased over the first week and remained elevated up to 2 months in the infarct post-MI. In the non-infarcted LV, the increase of Ang I was less and only significant at 1 week post-MI. MI-induced 2-3-fold increases of Ang II content in the non-infarcted LV at 4 weeks (De Resende et al 2006, De Resende and Mill 2007, Silvestre et al 1999, Duncan et al 1999) and 2-fold at 8 weeks (Geng et al 2006). Xiu et al (2002) reported 1.5-fold increase of Ang II production in in-vitro heart perfusion experiment at 20 weeks post-MI. The increased cardiac Ang II post MI could lead on to increased cardiac production of aldosterone.

1.4.3-b-iii Cardiac Aldosterone Post-MI

Silvestre et al (1999) reported a 4-fold increase of aldosterone production in the LV of Wistar rats by in-vitro perfusion experiment at 4 weeks post-MI. Geng et al (2006) reported a 2-fold increase of aldosterone in the non-infarcted LV at 8 weeks post-MI. Xiu et al (2002) reported a 1.5-fold increase of aldosterone production in in-vitro heart perfusion experiment at 20 weeks post-MI in Wistar rats. Mizuno et al (2001) measured the aldosterone levels in samples from the anterior inter-ventricular vein, coronary sinus and aortic root simultaneously in the control people and in patient with LV dysfunction. Plasma levels of aldosterone in these patients were not different from control subjects. However, aldosterone levels were significantly higher in the anterior inter-ventricular vein and coronary sinus than in aortic root, in patient with LV dysfunction, but not in

control subjects. This indicates that cardiac production of aldosterone is activated in patients with CHF and increases in proportion to severity of systolic dysfunction (Mizuno et al 2002).

1.4.3-b-iv Cardiac ACE Post-MI

Cardiac ACE activity is also activated post-MI. Our lab reported that after MI the ACE density was increased at the site of infarct, increased somewhat less in the peri-infarct zone and non-infarct part of the LV, and increased only modestly in the RV at 4 and 8 weeks (Tan et al 2004). Sun et al (1994) reported the time course of increase of ACE density in the LV and RV post-MI. At the site of infarction, ACE binding did not increase at day 3, but was increased 4-fold at week 1, 7-fold at weeks 2, at 9-fold at week 4 and 10-fold at weeks 8 post-MI. In the interventricular septum and the RV, ACE density did not increase till week 1 and then increased 2-fold at week 2, and 2.5-fold at 4 and 8 weeks.

Other workers also reported the activation of cardiac ACE density, mRNA, activity at different time intervals post-MI. Cardiac ACE mRNA was increased by 2-fold in the non-infarcted LV (Burrell et al 2005) and by 3-10-fold in the infarcted LV at day 3-4 post-MI (Burrell et al 2005, Passier et al 1995). At week 1 post-MI, ACE mRNA was increased by 2-fold in the non-infarcted LV (Nakamura et al 2004). ACE activity was increased by 1.5-fold in the non-infarcted LV and interventricular septum (Ocaranza et al 2006, Passier et al 1995), 2.5-fold in the infarcted LV (Passier et al 1995), and 2-fold in the scar (Busatto et al 1997). At week 2 post-MI, ACE activity was increased by 1.5-fold in the non-infarcted LV and by 13-fold in the scar (Busatto et al 1997). At week 3

post-MI, Kobayashi et al (1998) reported increased expression of ACE mRNA in the LV. At week 4 post-MI, ACE mRNA was increased by 2-fold in the non-infarcted LV and by 5-fold in the infarcted LV (Burrell et al 2005). ACE density was increased by 2-fold in the non-infarcted LV and 15-fold in the infarct (Burrell et al 2005). ACE activity was increased by 2-fold in the non-infarcted LV and 8-10-fold in the scar (De Resende et al 2006, De Resende and Mill 2007). At week 6 post-MI, ACE mRNA was increased by 2-fold in the non-infarcted LV (Wollert et al 1994). ACE activity was increased by 2-2.5 in the non-infarcted LV (Schieffer et al 1994, Wollert et al 1994) and the RV (Schieffer et al 1994). At week 8-9 post-MI, ACE mRNA was increased by 5-fold in the non-infarcted LV (Ocaranza et al 2006). ACE activity was increased by 2-3-fold in the non-infarcted LV (Ocaranza et al 2006, van Veldhuisen et al 1994). At week 12-13 post-MI, ACE mRNA was increased by 2-fold in the non-infarcted LV (Hirsch et al 1991) and the scar (Gaertner et al 2002). ACE activity was increased by 2-4-fold in the infarcted LV, interventricular septum (Passier et al 1995, Hirsch et al 1991), RV (Hirsch et al 1991) and in the scar (Gaertner et al 2002).

The increased cardiac ACE activity post-MI could lead on to increased formation of cardiac Ang II. In patients with heart failure, Mizuno et al (2002) reported correlation between cardiac aldosterone formation with ACE activity and suppression of cardiac aldosterone with perindopril. This suggests that cardiac aldosterone synthesis is dependent on ACE, activity probably through the increased synthesis of Ang II.

1.4.3-b-v Cardiac AT_1 Receptors Post-MI

Sun and Weber (1994) reported the time course of increase of Ang II receptor binding in the infarcted LV following MI. Ang II binding was increased by 10-fold at day 3, 12-16-fold from week 1-8 in the infarcted LV (Sun and Weber 1994, Sun et al 1998) and by 2-fold in interventricular septum and the RV at 4 weeks post-MI (Sun et al 1998). These increased binding were markedly attenuated by losartan, but not by AT_2 receptor antagonist, indicating that majority of Ang II receptors are of AT_1 type. Different studies reported increased expression of AT_1 receptors post-MI. At day 1 post-MI, Nio et al (1995) reported 2.5-fold increase of AT_1 receptors expression in the infarcted, but not in the non-infarcted LV. Busche et al (2000) reported a non-significant 1.5-fold increase in the number of cardiomyocytes expressing the AT_1 receptors mRNA at day 1 post-MI. At day 3 post-MI, Lu et al (2001) reported 10-fold increase of AT_1a receptor expression in the non-infarcted LV. At week 1 post MI, Nio et al (1995) reported 4-fold increase in the infarcted and 2-fold increase in the non-infarcted LV and Nakamura et al (2004) reported 1.5-fold (non-significant) increase of AT_1 receptor expression in the non-infarcted LV. At week 2 post-MI, Lu et al (2001) reported 2-fold increase of AT_1a receptor expression in the non-infarcted LV. At week 4 post-MI, De Resende and Mill (2007) reported increased expression of AT_1 receptors in the non-infarcted LV of Wistar rats and Milik et al (2006) reported increased AT_1a receptor mRNA in the non-infarcted LV and the RV of SD rats. On the contrary, Ishiyama et al (2004) reported decrease of AT_1a receptor mRNA in the non-infarcted LV in normotensive male Lewis rats at 4 weeks post-MI, which was reversed with oral administration of losartan or olmesartan. Our lab reported increased binding for AT_1 receptors at the site of infarct, increased somewhat less in the

peri-infarct zone and non-infarct part of the LV, and increased only modestly in the RV at 4 and 8 weeks post-MI in Wistar rats (Tan et al 2004). At week 8-9 post-MI, Geng et al (2006) and Fraccarollo et al (2003) reported increased expression of AT_1 receptor mRNA/protein by 1.5-2-fold in the non-infarcted LV.

AT_2 receptor mRNA did not change in the non-infarcted LV, but was 2-fold increased in the infarcted LV at day 1 post-MI (Nio et al 1995). Busche et al (2000) reported no change in the number of cardiomyocytes expressing the AT_2 receptors mRNA at day 1 post-MI. At day 3 post-MI, AT_2 receptor expression did not change in the non-infarcted LV (Liu et al 2001). At week 1 post-MI, AT_2 receptor mRNA was 2-3-fold increased in the non-infarcted and infarcted LV and these increases were prevented in the non-infarcted and attenuated in the infarcted LV with AT_1 receptor antagonist, but not with AT_2 receptor antagonist (Nio et al 1995), suggesting that a direct action of Ang II mediated through increased receptor expression is involved in the remodeling process of MI. At week 2 post-MI, Liu et al (2001) reported 3-fold increase of AT_2 receptors expression in the non-infarcted LV. At week 4 post-MI, De Resende and Mill (2007) reported no change of AT_2 receptor expression in the non-infarcted LV of Wistar rats. Geng et al (2006) reported no change in the expression of AT_2 receptor mRNA in the non-infarcted LV at 8 week post-MI. On considering these studies, it appears that AT_2 receptor expression increases immediately after MI but not at 8 weeks post-MI. Ang II interacts with AT_1 and AT_2 receptors, but the major cardiovascular effects appears to be mediated through AT_1 receptors. Stimulation of AT_2 receptors antagonizes the effects via AT_1 and may have beneficial effects on ventricular remodeling (Geng et al 2006).

De Resende et al (2006) reported 2.5-fold increase of MR expression and Silvestre et al (1999) reported 23% (non-significant) increase of MR expression in the non-infarcted LV at 4 weeks post-MI. The discrepancy between these studies may be due to difference in infarct size, which is 40% in De Resende et al (2006) vs 25% in Silvestre et al (1999) and the degree of cardiac dysfunction, which was much more severe in De Resende et al (2006) study.

In addition, at 25 days post-MI mRNA levels for the StAR were increased 2-fold in the non-infarcted area of LV (Casal et al 2003). The cardiac levels of 11β hydroxylase and corticosterone are higher than aldosterone synthase and aldosterone. After MI, there is increase of aldosterone synthase mRNA and aldosterone by 2-4-fold and decrease of 11β hydroxylase mRNA and corticosterone by 2-fold, indicating a shift towards production of aldosterone than corticosterone. Despite this, the absolute cardiac levels of corticosterone remain ~ 7 times higher than those of aldosterone post-MI (Silvestre et al 1999). The expression of CYP11B2 mRNA in failing human heart (Young et al 2001) may enhance the production of aldosterone.

Oral treatment with losartan prevented the MI-induced increase of cardiac aldosterone at 4 weeks (Silvestre et al 1999) and 20 weeks (Xiu et al 2002) post-MI. The prevention of cardiac synthesis of aldosterone by losartan indicates that Ang II is mediating this effect through its action on AT_1 receptors. However, this effect of losartan may be peripheral or through its action in the brain.

1.4.3-c Brain RAAS Post-MI

1.4.3-c-i Changes in Brain RAAS Post-MI

Brain levels of aldosterone and Ang II have not yet been reported post-MI. Our lab reported increases of brain AT_1 receptor and ACE densities post-MI (Tan et al 2004). At 4 weeks after MI, AT_1 receptor bindings were increased in OVLT, SFO and PVN in rats with large MI and modestly in the MnPO. At 8 weeks after MI, AT_1 receptor bindings remained elevated in the OVLT, SFO, and PVN and were also increased in MnPO. Rats with small MI showed only minor (5-10%) increases. At 4 and 8 weeks after MI, ACE bindings were significantly increased in the SFO, OVLT and PVN, but only modestly in the MnPO. The ACE bindings were significantly higher in OVLT and tended to be higher in SFO, PVN and MnPO at 4 and 8 weeks post-MI. The finding supports the functional studies that brain RAS through AT_1 stimulation plays an essential role in sympathoexcitation (Zhang et al 1999) and contributes to progression of LV dysfunction (Leenen et al 1999).

Chronic blockade of brain OLC using icv infusion of Fab fragments significantly reduced the MI-induced increases of brain AT_1 receptor densities in SFO, OVLT, PVN and MnPO and ACE densities in SFO, OVLT, and PVN. The findings support the functional studies showing a major role for brain OLC in development of sympathetic hyperactivity after MI involving the brain RAS (Tan et al 2004).

1.4.3-c-ii Blockade of Brain RAAS Affects Sympathetic Activity Post-MI

Ang II and aldosterone locally produced in the brain post-MI may play a major role in CNS pathways leading to sympathetic hyperactivity (Wang et al 2004).

1.4.3-c-ii-A AT_1 Receptors

Icv infusion of losartan at doses devoid of effects when infused peripherally, prevents sympathetic hyperactivity and improves baroreflex function in rats post-MI (Zhang et al 1999, DiBona et al 1995). Blockade of forebrain AT_1 receptors with losartan attenuated the increased neuronal activity in rats with CHF post-MI (Zhang et al 2002). Icv infusion of enalaprilat prevents the MI-induced sympathetic hyperactivity and decreases HR (Francis et al 2004) and injection of AT_1 receptor mRNA antisense into the PVN normalizes the enhanced cardiac sympathetic afferent reflex and decreases the resting RSNA (Zhu et al 2004).

The role of Ang II locally produced in the brain post-MI in increasing sympathetic activity was studied in TG rats lacking angiotensinogen specifically in the brain (Schinke et al 1999). To produce these animals, this group injected angiotensinogen antisense DNA driven by a glial fibrillary acidic protein promotor in the transgene into germ cells of SD rats. Antisense RNA against angiotensinogen mRNA is expressed specifically in the astrocytes, which represent the main source of angiotensinogen in the brain. These RNA do not affect angiotensinogen mRNA synthesis and degradation, but may induce hybrid arrest of translation of the angiotensinogen mRNA, leading to inhibition of angiotensinogen gene expression in the astrocytes (Schinke et al 1999). These animals exhibit markedly reduced brain angiotensinogen (Schinke et al 1999) and angiotensins (Huang et al 2001) and normal plasma angiotensinogen concentration and resting PRA (Schinke et al 1999, Baltatu et al 2000, Wang et al 2004).

SD rats showed a clear impairment of arterial baroreflex control of both RSNA and HR post-MI, with decreases of maximum slopes of RSNA (%/ mmHg) and of HR in

SD-MI vs SD-sham. In TG rats none of these parameters of arterial baroreflex changed significantly. SD rats showed 2-fold increases of HR, MAP and RSNA response to air stress at 8 weeks post-MI. After MI, TG rats did not show any enhanced response to air stress for HR and the responses for MAP and RSNA were significantly less than SD rats. Icv Ang II causes dose-related increases in RSNA, HR, and BP, likely via activation of neurons in the MnPO and juxtaventricular neurons of the SFO and OVLT (Veerasingham and Leenen 1997). In sham rats these responses were significantly enhanced in TG vs SD rats probably due to up-regulation of AT_1 receptors with low levels of Ang II in the brain of TG rats. At 8 weeks post-MI, responses to icv Ang II were significantly decreased in SD rats, but not in TG rats (Wang et al 2004), probably due to down-regulation of AT_1 receptors in the presence of high levels of Ang II only in SD rats.

1.4.3-c-ii-B Aldosterone and MR

MI induces increases of baseline integrated RSNA and HR (Francis et al 2001, Francis et al 2001a). Icv infusion of spironolactone decreased the MI-induced increases of RSNA and HR at 4 weeks (Francis et al 2001a). Huang and Leenen (2005) also reported a tendency of an increase in RSNA at 4 weeks post-MI and this tendency was prevented with icv infusion of spironolactone.

For sympathetic reactivity, air-jet stress-induced increases of MAP, RSNA and HR were increased by 100-120% at 4 weeks post-MI and icv infusion of spironolactone prevented these increases (Huang and Leenen 2005). Icv injection of guanabenz caused decreases in MAP, RSNA and HR in a dose-related manner. The peak inhibitory

responses were increased post-MI by 120-150% and icv infusion of spironolactone prevented these MI-induced changes (Huang and Leenen 2005).

Measuring arterial baroreflex function, intravenous nitoprusside and phenylephrine induce ramp decreases and increases in MAP and ramp increases and decreases in RSNA and HR (Huang and Leenen 2005). Compared with the responses in sham rats, the reflex curve of RSNA or HR plotted against MAP showed a deterioration of baroreflex function in MI rats. Icv infusion of spironolactone improved the MI-induced deterioration of baroreflex (Huang and Leenen 2005, Francis et al 2001a). The prevention of MI-induced increase of RSNA and improvement of impaired baroreflex by icv infusion of spironolactone indicate that MR may be mediating these changes (Francis et al 2001a, Huang and Leenen 2005).

After MI, TNF-α synthesis increases in the hypothalamus, LV and RV, and plasma within minutes to hours and the increase persists for at least 4 weeks (Francis et al 2004). TNF-α activates hypothalamic neurons, particularly those in the PVN (Dunn 2000; mechanism discussed in section 5.2.3), which probably manifests peripherally by increases of plasma norepinephrine (Kang et al 2006). Intraperitoneal administration of etanercept (TNF-α antagonist) prevented the MI-induced activation of PVN neurons and increase of plasma norepinephrine (Kang et al 2006). Increase of plasma TNF-α levels post-MI was attenuated by icv and oral spironolactone (Kang et al 2004, Francis et al 2003) and increases of cardiac and brain TNF-α levels were prevented by oral spironolactone (Kang et al 2004). Oral treatment with eplerenone attenuated the MI-induced increases of plasma TNF-α, norepinephrine, PVN neuronal activation and staining of TNF-α, similar to that produced by etanercept (Kang et al 2006) indicating

that the stimulation of TNF-α post-MI may be mediated through stimulation of MR in the brain, though the exact mechanism is not known.

1.4.3-c-iii Brain RAAS and Cardiac Function Post-MI

Several studies show that activation of brain RAAS contributes to deterioration of cardiac function post-MI. Our lab reported that icv infusion of spironolactone markedly improved the cardiac function at 4 weeks post-MI, significantly attenuated the increase of LVEDP, and decreases in LVPSP, LV dP/dt max and systolic BP to levels that were modestly but still significantly different from control levels (Huang and Leenen 2005). In contrast, Francis et al (2001a) reported a tendency of LVEDP to decrease after icv infusion of spironolactone for 4 weeks and Francis et al (2003) reported no change in LVEDV and LV ejection fraction (LVEF) after icv infusion of spironolactone for 3 weeks. However, Francis et al (2001a) measured LVEDP by using a polyethylene catheter and a regular pressure transducer under pentobarbital anesthesia at 4 weeks post-MI and they (2003) measured LVEDV and LVEF by echocardiography under ketamine anesthesia. In the study of Huang and Leenen (2005), hemodynamics were measured using Millar catheter when rats were not under minimal level of anesthesia. Different infarct sizes could have also produced these variations. The infarct size was ~50% in Francis et al (2001a, 2003) studies vs 30% in Huang and Leenen (2005) study. Central blockade may be less effective in improving cardiac function in case of severe damage of the LV by a large MI.

Icv infusion of benzamil that blocks amiloride-sensitive Na^+ channel including ENaC significantly attenuated the MI-induced increase of LVEDP, and decreases in LVPSP, LV dP/dt max and systolic BP at 4-8 weeks post-MI.

Icv infusion of losartan, from 0.5 to 8 weeks or 4 to 8 weeks modestly improved the MI-induced increase of LVEDP at 8 weeks post-MI (Leenen et al 1999). Francis et al (2004) did not find any improvement of LV end diastolic volume and LV ejection fraction (measured by echocardiography under ketamine anesthesia) with icv infusion of enalapril at 4 weeks post-MI. Again, the large infarct size of 54% (Francis et al 2004), could have prevented the improvement of in MI-induced hemodynamics. In TG rats deficient of brain angiotensinogen, MI-induced decreases in LVPSP, LV dP/dt max and systolic BP and increases in LVEDP and RV weight were largely prevented at 8 weeks post-MI (Wang et al 2004).

Altogether these findings suggest that in rats post-MI, increased binding of aldosterone to brain MR leads to increased expression of ENaC and the latter is essential in the CNS pathways leading to sympathoexcitation and cardiac dysfunction post-MI.

1.5 EFFECTS OF HIGH SALT ON THE HEART AND BLOOD PRESSURE

1.5.1 High Salt Induces Cardiac Hypertrophy

High dietary salt causes cardiac hypertrophy in normotensive rats (Zhu et al 2004, Morgan et al 2001, Hao et al 1997). The cardiac hypertrophy could be due to increase of LV and/or RV weight. Different concentrations (1% to 8%) of NaCl have been used in different studies.

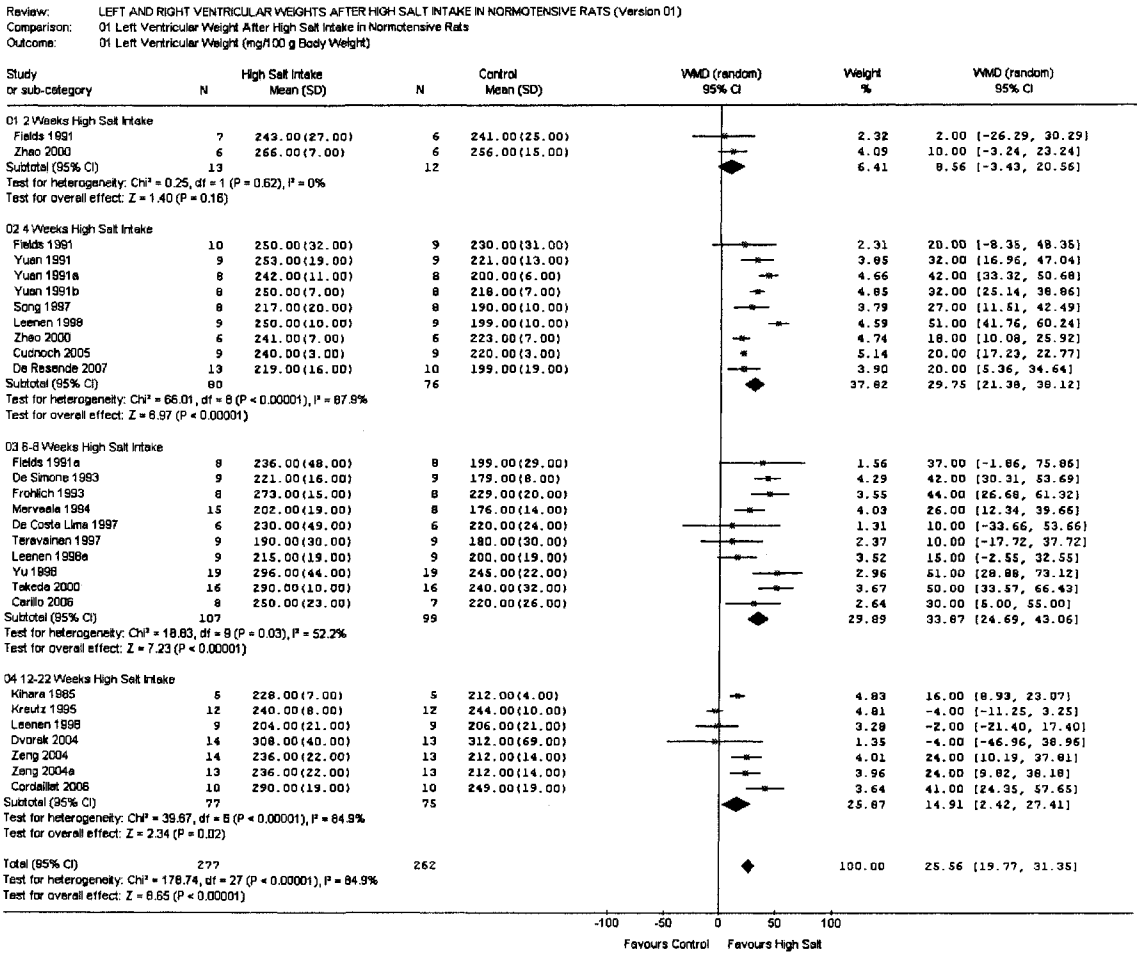
1.5.1-a High Salt Increases LV Weight

Our lab reported no change in the LV weight after two weeks of high salt intake in Wistar (Fields et al 1991) and Dahl salt-resistent (Dahl R) rats (Zhao et al 2000). At four weeks of high salt intake, our lab reported increases of the LV weight in Wistar (by 9-14%, Fields et al 1991, Yuan and Leenen 1991), Wistar-Kyoto (by 11-21%, Leenen and Yuan 1998, Song et al 1997, Yuan and Leenen 1991) and Dahl R (by 8-15%, Yuan and Leenen 1991, Zhao et al 2000). Other labs also reported ~10% increase in the LV weight at 4 weeks of high salt consumption in Wistar (De Resende and Mill 2004) and SD (Cudnoch-Jedrzejewska et al 2005) rats.

Six weeks of high salt intake increased LV weight in Wistar rats (by 6-19%, Fields et al 1991, Teravainen et al 1997, Leenen and Yuan 1998). Eight weeks high salt intake increased LV weight in Wistar (by 5-23%, Carillo et al 2006, da Costa Lima et al 1997, de Simone et al 1993, Fields et al 1991, Teravainen et al 1997) and Wistar-Kyoto (by 15-21%, Frohlich et al 1993, Mervaala et al 1994, Takeda et al 2000, Yu et al 1998) rats. More prolonged high salt intake from 12 to 22 weeks showed variable effects, i.e., increase of the LV weight by 8-16% in Wistar (Zeng et al 2004, Zeng et al 2004a), Wistar-Kyoto (Kihara et al 1985) and SD (Cordaillat et al 2005) rats, to no change of the LV weight in Wistar-Kyoto (Kreutz et al 1995, Leenen and Yuan 1998) and SD (Dvorak et al 2004) rats. Seventy weeks of high salt consumption caused a significant increase in LV weight in Wistar rats (by 30%, Lima et al 2006).

Due to the variation in the LV weight at different time intervals and among the different studies at the same duration of high salt intake, a meta-analysis of 28 studies involving 539 normotensive rats was performed.

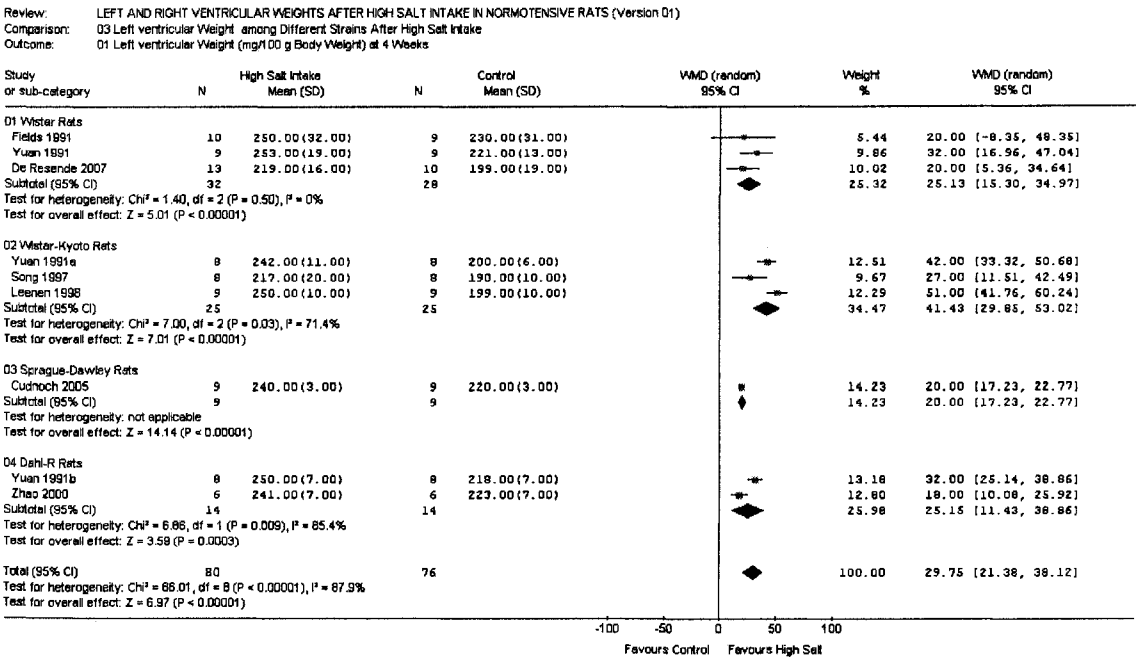
Figure 5: Effect of High Salt Intake on Left Ventricular Weight in Normotensive Rats



It showed an overall significant increase of LV weight after high salt intake in normotensive rats, WMD (random) [95% CI] = 25.6 [19.8, 31.4] ($p<0.00001$ vs control). Except for 2 weeks (WMD, [95% C] = 8.6, [-3.4, 20.6]), all the sub-group analyses at 4, 6-8 and 12-22 weeks also showed significant increases of LV weight after high salt intake, WMD [95% CI] = 29.8 [21.4, 38.1] ($p<0.00001$), 33.9 [24.7, 43.1] ($p<0.00001$), 14.9 [2.4, 27.4] ($p<0.02$) respectively in normotensive rats (Figure 5).

Yuan and Leenen (1991) reported a tendency of a larger increase in LV weight in Wistar-Kyoto rats (21%) compared with Wistar or Dahl R rats (~15%) at 4 weeks of high salt intake. To evaluate if the effect of high salt intake on LV weight is strain-dependent, additional meta-analyses of different strains of rats after 4 and 8 weeks of high salt intake were performed.

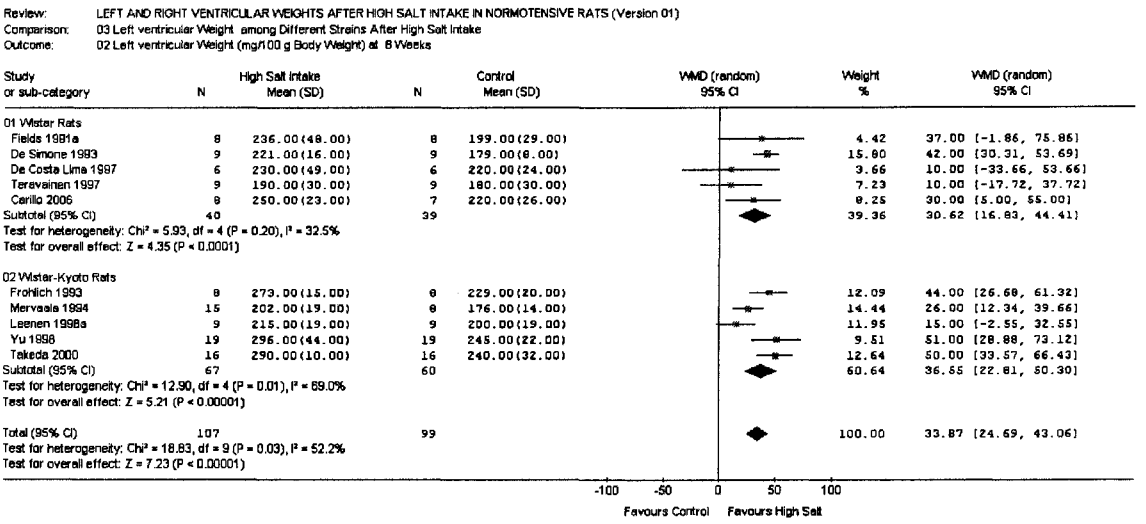
Figure 6: Effect of 4 Weeks of High Salt Intake on LV Weight in Wistar, Wistar-Kyoto, SD and Dahl R Rats



All the strains (Wistar-Kyoto, Wistar, Dahl R and SD rats) showed significant increases of LV weights after 4 weeks of high salt intake, WMD (random) [95% CI] = 41.4 [29.9, 53.0], p<0.0001, 25.1 [15.3, 35.0], p<0.00001, 25.2 [11.4, 38.9], p=0.0003, and 20.0 [17.2, 22.8], p<0.00001, respectively. The meta-analysis shows a tendency of larger effect of high salt diet on LV weight in Wistar-Kyoto rats than other strains (Figure 6). Eight weeks of high salt intake also showed marked increases in LV weight in

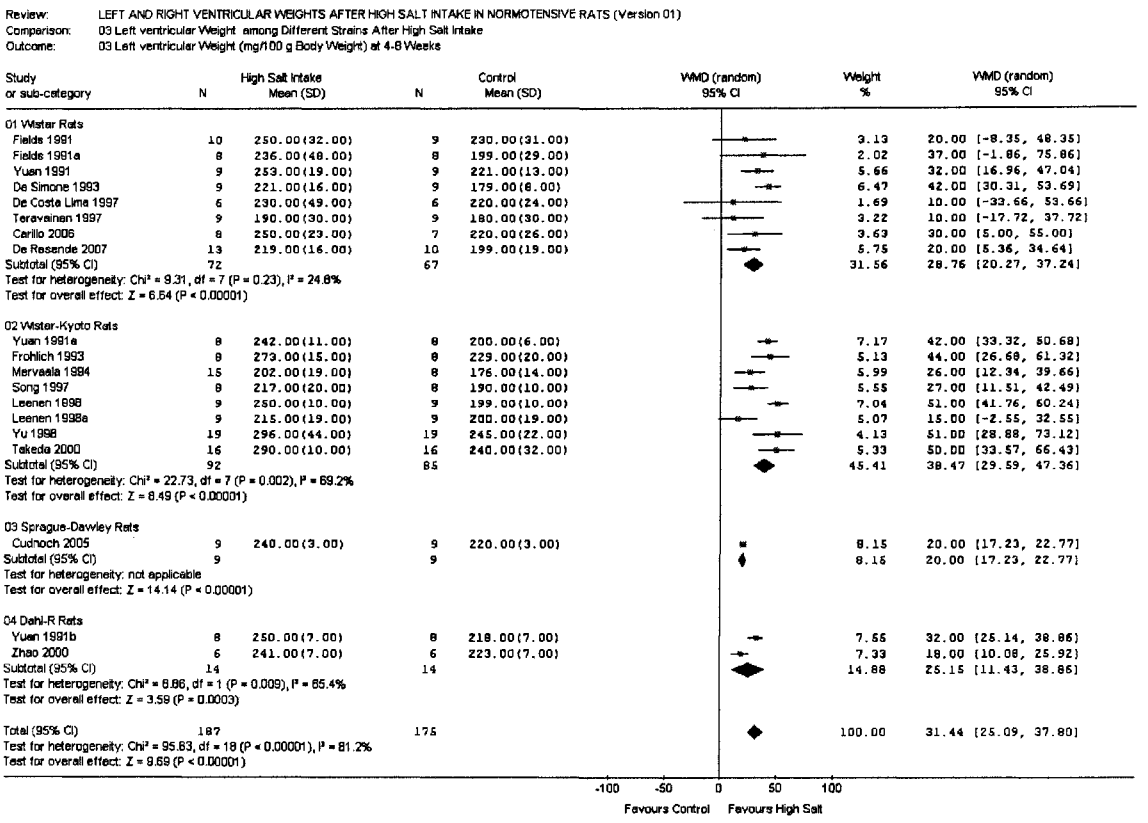
Wistar-Kyoto and Wistar rats; WMD (random) [95% CI] = 36.6 [22.8, 50.3], p<0.0001 and 30.6 [16.83, 44.4], p<0.0001 respectively and no differences in LV weight were found among the strains (Figure 7).

Figure 7: Effect of 8 Weeks of High Salt Intake on LV Weight in Wistar and Wistar-Kyoto Rats



On merging the data of 4 and 8 weeks of high salt intake, a clear increase of LV weight was found among different strains, WMD (random) [95% CI] = 38.5 [29.6, 47.4], p<0.00001 in Wistar-Kyoto, 28.8 [20.3, 37.2], p<0.00001 in Wistar, 25.2 [11.4, 38.9], p=0.0003 in Dahl R, and 20.0 [17.2, 22.8], p<0.00001 in SD rats (Figure 8). Meta-analysis shows a tendency for a larger effect of high salt intake on LV weight in Wistar-Kyoto rats than other strains in the order, Wistar > Dahl R> SD rats.

Figure 8: Effect of 4-8 Weeks of High Salt Intake on LV Weight in Wistar, Wistar-Kyoto, SD and Dahl R Rats



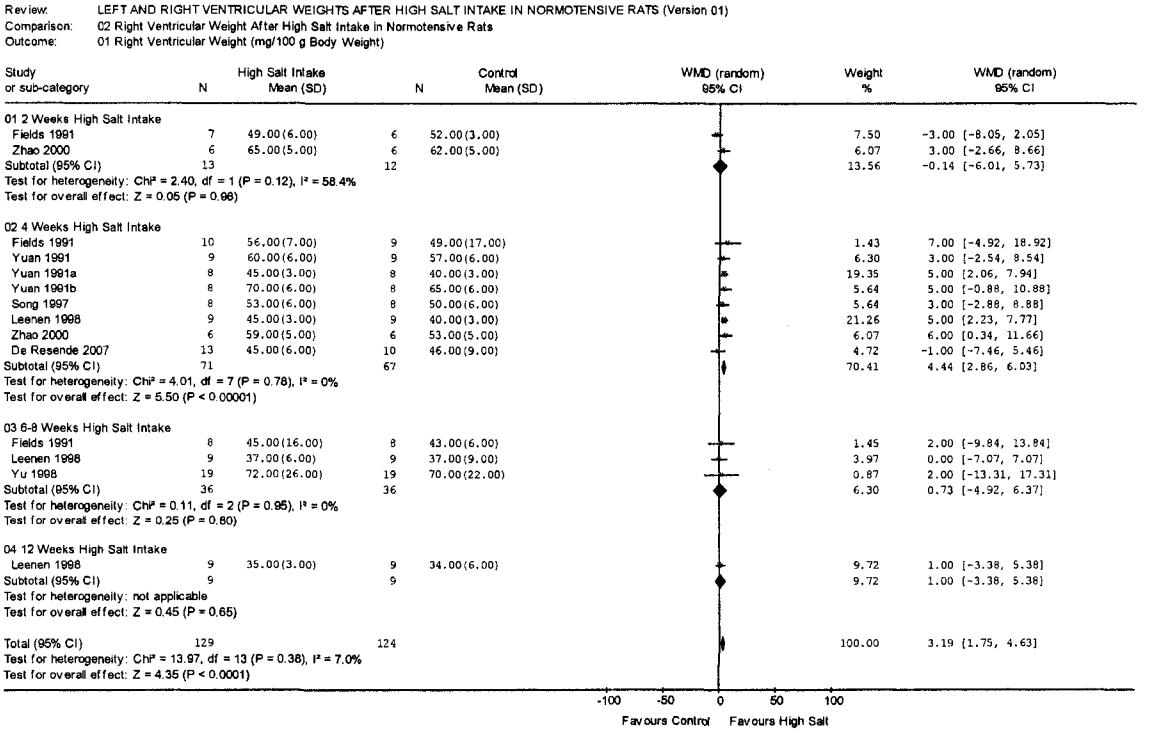
1.5.1-b High Salt and RV Weight

The data of the effect of high intake on RV weight are variable. Studies from our lab reported no change of RV weight with 2 weeks of high salt intake in Wistar (Fields et al 1991) and Dahl R rats (Zhao et al 2000). Our lab reported a tendency of increase in RV weight in Wistar rats (by 5-14%, Fields et al 1991, Yuan and Leenen 1991), Wistar-Kyoto rats (by 6-13%, Leenen and Yuan 1998, Song et al 1997, Yuan and Leenen 1991), and Dahl R rats (by 8-11%, Yuan and Leenen 1991, Zhao et al 2000). De Resende and Mill (2007) reported no change of RV weight after 4 weeks of high salt intake in Wistar rats. There was a tendency of increases of RV weight after 6 weeks of high salt intake in

Wistar rats (by 5%, Fields et al 1991). No change in RV weight was found after 8 weeks (Leenen and Yuan 1998, Yu et al 1998) and 12 weeks (Leenen and Yuan 1998) of high salt intake in Wistar-Kyoto rats.

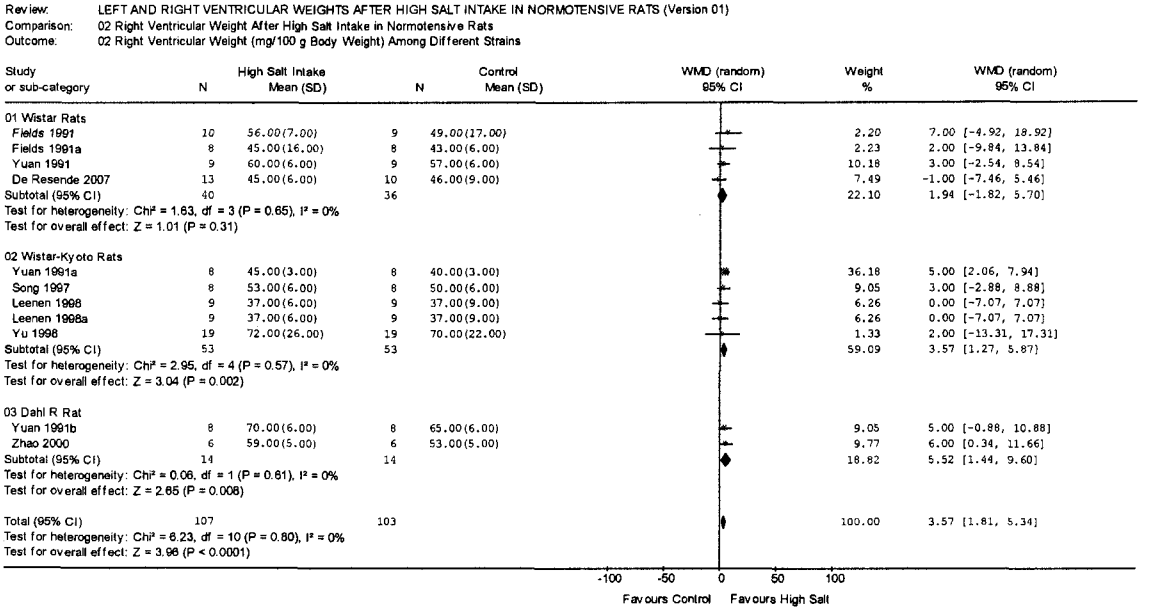
Considering the variation in the results of RV weight after high salt intake, a meta-analysis of 14 studies involving 253 normotensive rats was performed. It showed an overall small increase of RV weight after high salt intake from 2 to 12 week, WMD (random) [95% CI] = 3.2 [1.8, 4.6], ($p<0.0001$ vs control; Figure 9). Sub-group analysis of 4 week of high salt intake weeks also showed a mild increase in RV weight, WMD [95% CI] = 4.4 [2.9, 6.0] ($p<0.0001$), but sub-group analyses at 2, 6-8 and 12 weeks of high salt intake found no change in RV weight in normotensive rats (Figure 9).

Figure 9: Effect of High Salt Intake on Right Ventricular Weight in Normotensive Rats



Yuan and Leenen (1991) reported a tendency of increase in RV weight in Wistar-Kyoto rats (13%), but less in Wistar (5%) and Dahl R rats (8%) at 4 weeks of high salt intake. To evaluate if the effect of high salt intake on RV weight is strain-dependent, a meta-analysis among different strains at 4-8 weeks of high salt intake was performed.

Figure 10: Effect of 4-8 Weeks of High Salt Intake on RV Weight in Wistar, Wistar-Kyoto and Dahl R Rats



A small increase in RV weight was found after 4-8 weeks of high salt intake in Wistar-Kyoto rats, WMD (random) [95% CI] = 3.6 [1.3, 5.9], $p<0.002$ and Dahl R rats 5.5 [1.4, 9.6], $p<0.008$ (Figure 10), but not in Wistar rats, WMD (95% CI) = 1.9 [-1.8, 5.7].

1.5.1-c High Salt and Cardiomyocyte Hypertrophy

Cudnoch-Jedrzejewska et al (2005) reported a tendency of increase of LV and RV cardiomyocyte cross-sectional diameter by 4% with intake of 1% NaCl in drinking water

for 4 weeks in SD rats. In neonatal rat cultured cardiomyocytes, aldosterone caused cardiomyocyte hypertrophy and spironolactone completely blocked the effect of aldosterone, indicating that the effect of aldosterone on protein synthesis is mediated through the MR (Sato et al 1996).

1.5.1.-d Mechanism of Cardiac Hypertrophy after High Salt Intake

With regard to possible mechanisms that may explain high salt induced-LV hypertrophy (LVH), hemodynamic effects appear not to play a major role, inasmuch as resting MAP, cardiac index (cardiac output/body weight), total peripheral resistance index (MAP/cardiac index), or LVPSP and LVEDP do not rise during the initial weeks on high salt diet (Frohlich et al 1993, Yuan and Leenen 1991, Fields et al 1991, Kihara et al 1985, Song et al 1997). The effect of high salt on BP in normotensive rats has further been reviewed in section 1.5.4. RSNA was decreased after high salt intake in Wistar rats (Carillo et al 2006). Plasma norepinephrine and epinephrine and LV norepinephrine turnover rates did not change after high salt diet in Wistar-Kyoto rats (Leenen and Yuan 1998, Yuan and Leenen 1991). High salt diet did increase lumbar sympathetic nerve activity in Wistar rats (Carillo et al 2006). However, α_1- or β-adrenoceptor blockers singly or in combination failed to prevent high salt-induced LVH, but instead aggravated it (LV weight further increased by 7-9%) in Wistar-Kyoto rats (Song et al 1997), indicating that sympathetic activity may not have a role in the LVH after high salt intake.

Spironolactone completely prevented the high salt induced cardiac hypertrophy (Cordaillat et al 2005) indicating the effect of high salt diet on these parameters may be mediated through MR. Systemic infusion of aldosterone along with high salt diet induced increase of LV weight (by 12%) and tendency of increase of RV weight (by 7%).

Administration of spironolactone prevented the increase of LV and RV weights (Brilla and Weber 1992), indicating cardiac hypertrophy was mediated by MR. MR could have been activated by aldosterone, corticosterone or increase of 11β-HSD2. However, plasma corticosterone does not change and cardiac corticosterone decreases on high salt intake in Wistar rats (Gomez-Sanchez et al 2004, Gomez-Sanchez et al 2005b). The 11β-HSD2 expression and activity has been detected in the heart of normotensive rats (Mazancova et al 2005). In Dahl S rats, high salt intake decreases 11β-HSD2 activity and mRNA expression in mesenteric artery (Takeda 2003). The effect, if any, of high salt intake on 11β-HSD2 in Wistar rats has not yet been reported. Activation of cardiac aldosterone after chronic high salt intake (Takeda et al 2000) (please see section 1.5.3) could also contribute to cardiac hypertrophy through the activation of MR. Considering the production of aldosterone and expression of StAR mRNA in cultured neonatal rat cardiomyocytes (Casal et al 2003), the cardiomyocyte hypertrophy after high salt intake may be due to increased production of aldosterone in the cardiomyocytes.

1.5.2 High Salt and Cardiac Fibrosis

High salt induces cardiac fibrosis in rats. Ye et al (2002) reported 2- and 2.5-fold increases of myocardial fibrosis index after 4 weeks of intermediate (2.2% NaCl) and high (4.4% NaCl) salt diet in Wistar-Kyoto rats. Perivascular fibrosis (reflected as % of vessels displaying extension of fibrous tissue from perivascular region to the interstitium) increased by ~15-fold after high sodium diet (4.4% NaCl) consumption in Wistar-Kyoto rats. Yu et al (1998) reported increases of interstitial fibrosis by 33% in the LV and by 20% the RV in Wistar-Kyoto rats on high salt diet for 8 weeks. In the intramyocardial

coronary arteries of the LV, the fibrosis in-between the outer borders of tunica media and tunica adventitia increased by 95% after high salt intake.

Subcutaneous infusion of aldosterone and salt (in drinking water) increased type I and III pro-collagen mRNA and cardiac fibrosis in both ventricles in SD rats (Iglarz et al 2004, Brilla and Weber 1992, Nehme et al 2006, Park and Schiffrin 2002, Ramires et al 1998, Robert et al 1995, Silvestre et al 2000). These increases of collagens and interstitial and perivascular fibrosis in the LV and or RV were prevented/ attenuated by spironolactone or eplerenone (Brilla and Weber 1992, Fujisawa et al 2003, Nehme et al 2006). Aldosterone stimulates collagen synthesis, measured by ^{3}H-proline incorporation in cultured adult rat cardiac fibroblasts. Blockade of synthesis of collagen in adult rat cardiac fibroblasts in culture with spironolactone (Brilla et al 1994) supports the conclusion that MR activation mediates the increase of collagen after high salt intake. The increased cardiac production of aldosterone after high salt intake (Takeda et al 2000) could stimulate cardiac fibrosis by acting on the fibroblasts.

The cause of cardiac fibrosis after high salt intake has remained unclear so far. Several mechanisms may contribute to salt-induced cardiac fibrosis. BP is an obvious one to consider. A small rise in BP may contribute to LV fibrosis, but if hypertension is the primary factor, only the pressure-overloaded LV would manifest the fibrosis (Brilla et al 1990). In section 1.5.4, effects of high salt on BP of normotensive and "salt resistant" strains will be reviewed. Activation of cardiac RAAS after high salt intake (reviewed in section 1.5.3) could stimulate cardiac fibrosis.

1.5.3 High Salt and Cardiac RAAS

High salt decreases the activity of the plasma RAAS. PRA markedly decreased after 2-5 weeks (De Resende and Mill 2007, Ingert et al 2002, Rahmouni et al 2002, Zhao et al 2000), and 8-10 weeks (Carillo et al 2006, da Costa Lima et al 1997, De Simone et al 1993, Prada et al 2000, Takeda et al 2000, Yu et al 1998) of high salt intake in normotensive rats. An increase in NaCl reabsorption (after high salt intake) by the macula densa results in the transmission to nearby juxtraglomerular cells of signals that and decreases the renin release. High salt diet also decreases plasma levels of Ang II (Coelho et al 2006, Carillo et al 2006), Ang I (Ingert et al 2002, Zhao et al 2000) and aldosterone (Takeda et al 2000, Gomez-Sanchez et al 2004) in normotensive rats. Contrary to the decrease in activity of circulatory RAAS, 2 weeks of high salt consumption increased cardiac ACE mRNA by 2-fold and ACE activity by 3-fold in Wistar-Kyoto rats (Kreutz et al 1995). Cardiac Ang II tended to increase after high salt intake in Wistar rats at 4 weeks (De Resende and Mill 2007), Wistar-Kyoto rats at 2 weeks (Leenen and Yuan 1998) and Dahl R rats at 5 weeks (Zhao et al 2000). Cardiac expression of AT_1 receptor mRNA increased by 2-fold after 8 weeks administration of high salt in drinking water (0.9% NaCl vs. tap water) (Takeda et al 2000) and in diet (8% NaCl vs. regular salt diet) (Zhu et al 2004) and tended to increase after 4 weeks of high salt intake in Wistar rats (De Resende and Mill 2007). The cardiac expression of AT_2 receptor mRNA remained unchanged after 4 (De Resende and Mill 2007) and 8 weeks of high salt intake (Zhu et al 2004). Cardiac aldosterone synthase activity, CYP11B2 mRNA and aldosterone were increased by 2-fold after 8 weeks administration of high salt (0.9% NaCl) in drinking water compared to tap water in Wistar-Kyoto rats (Takeda et al 2000).

The mechanisms involved in high salt-induced increases of cardiac Ang II and aldosterone are not yet understood. Expression of renin mRNA and activity and angiotensinogen mRNA have been detected in the hearts of normotensive rats (Sun et al 2001, Jurkovicova et al 2001), but the effects of high salt intake on these parameters have not yet been reported. Plasma Ang I is markedly decreased after high salt intake, but cardiac Ang I remains unchanged in Dahl R rats (Zhao et al 2000). Increased cardiac ACE activity after high salt intake (Kreutz et al 1995) could increase the conversion of cardiac Ang I to Ang II. Ang II stimulates StAR and aldosterone synthase in cardiomyocytes and heart (Casal et al 2003, Silvestre et al 1998), resulting in increased aldosterone synthesis. Whether increased production of cardiac aldosterone on high salt diet is mediated through stimulation of AT_1 receptors has not yet been studied.

1.5.4 High Salt and BP in Normotensive Rats

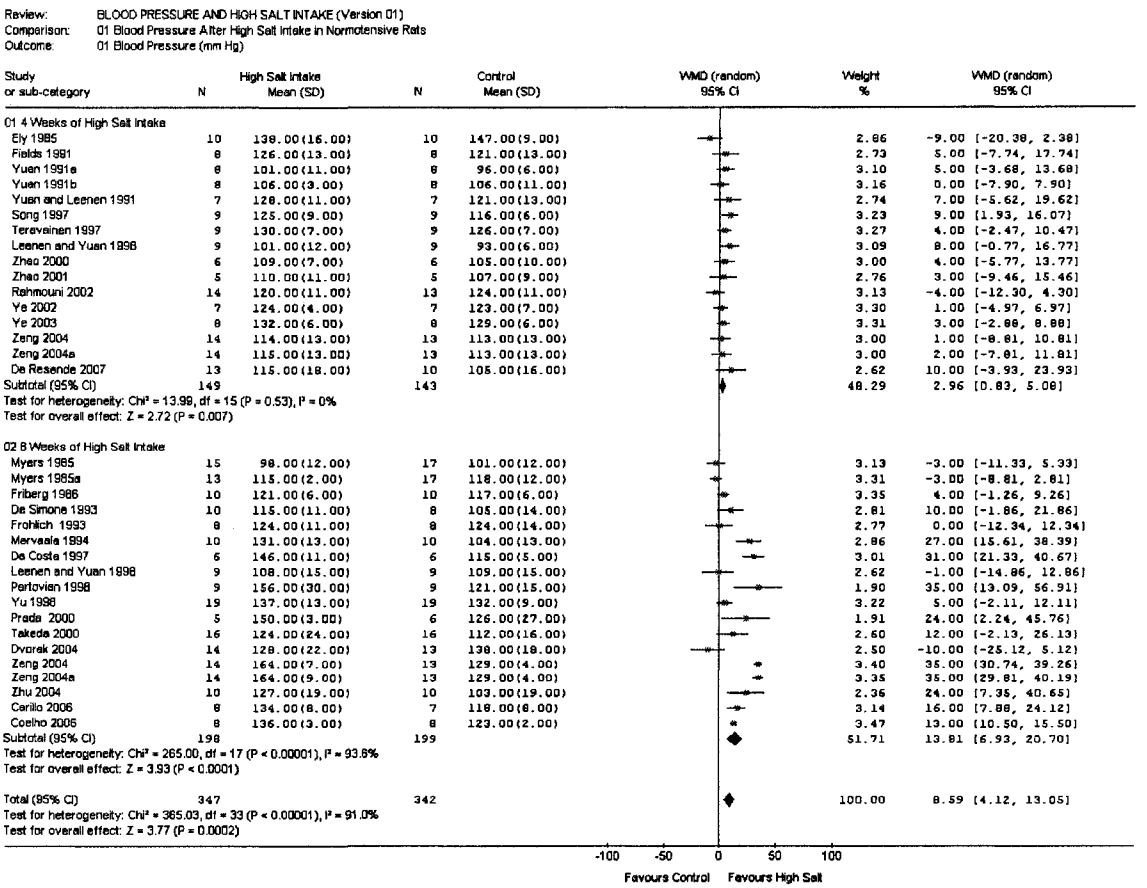
Short-term use of high salt diet does not increase BP, but chronic intake of high salt increases BP. At 4 weeks of high salt intake, our lab reported no change of BP in Wistar (Fields et al 1991, Yuan and Leenen 1991), Wistar-Kyoto (Leenen and Yuan 1998, Song et al 1997, Yuan and Leenen 1991) and Dahl R (Zhao et al 2000) rats. Other labs also reported no change of BP after 4 weeks of high salt intake in Wistar (De Resende and Mill 2007, Rahmouni et al 2002, Teravainen et al 1997, Zeng et al 2004, Zeng et al 2004a), Wistar-Kyoto (Ely et al 1985, Ye et al 2002, Ye et al 2003) and Dahl R (Yuan and Leenen 1991) rats.

Eight weeks of high salt intake generated conflicting data of BP. Some studies reported no change of BP after 8 weeks of high salt intake in Wistar-Kyoto (Friberg et al

1986, Frohlich et al 1993, Yu et al 1998) and SD rats (Dvorak et al 2004). Other studies reported a mild increase (10-20%) of BP in Wistar (Carillo et al 2006, Coelho et al 2006, de Simone et al 1993, Prada et al 2000, Zeng et al 2004, Zeng et al 2004a) and Wistar-Kyoto (Takeda et al 2000) rats, to moderate increase (21-30%) of BP in Wistar (da Costa Lima et al 1997), Wistar-Kyoto (Mervaala et al 1994, Partovian et al 1998) and Dahl R (Zhu et al 2004) rats after 8 weeks of high salt intake.

Due to variations in the BP on high salt intake in individual studies, a meta-analysis of 34 studies involving 689 normotensive rats was performed. Mainly MAP was considered for the analysis. In some studies MAP was not reported, and systolic BP considered, if reported.

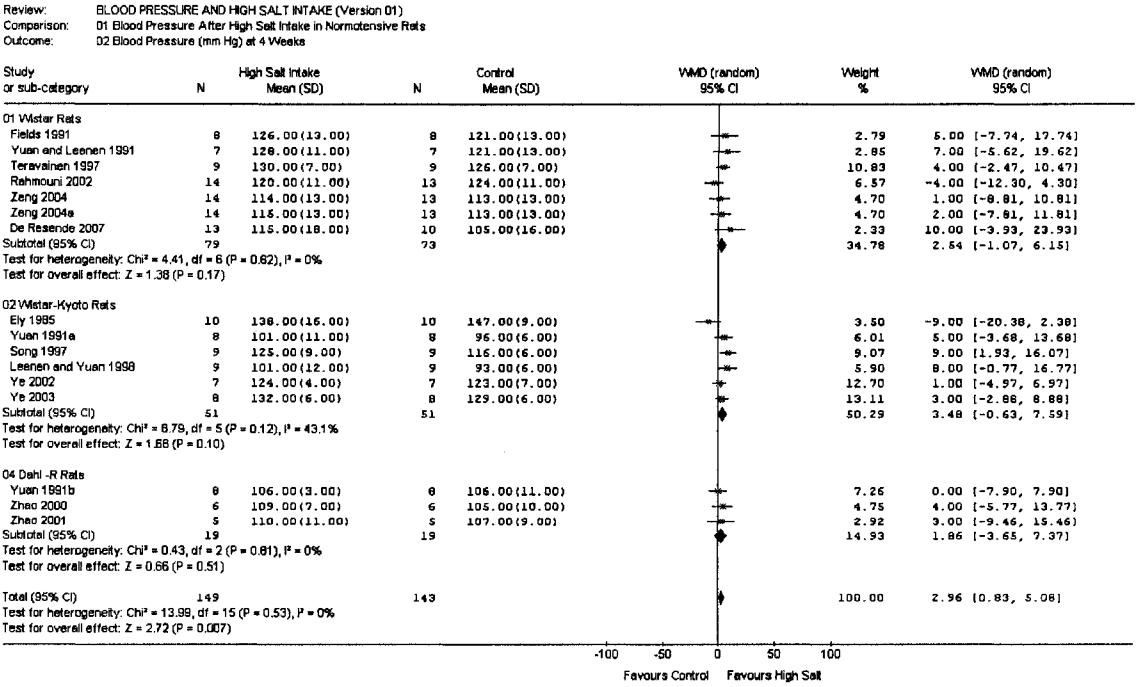
Figure 11: High Salt Intake-Induced Changes in BP of Rats at 4-8 Weeks



High salt intake induced a significant rise of BP, WMD (random) [95% CI] = 8.6 [4.1, 13.1] (p = 0.00002 vs control). Subgroup analysis at 4 week showed a minuscule increase of BP after high salt intake, WMD [95% CI] = 3.0 [0.8, 5.1](p = 0.007, 16 studies involving 292 normotensive rats), but showed a clear increase of BP at 8 weeks, WMD [95% CI] = 13.8 [6.9, 20.7](p < 0.00001, 18 studies involving 397 normotensive rats) (Figure 11).

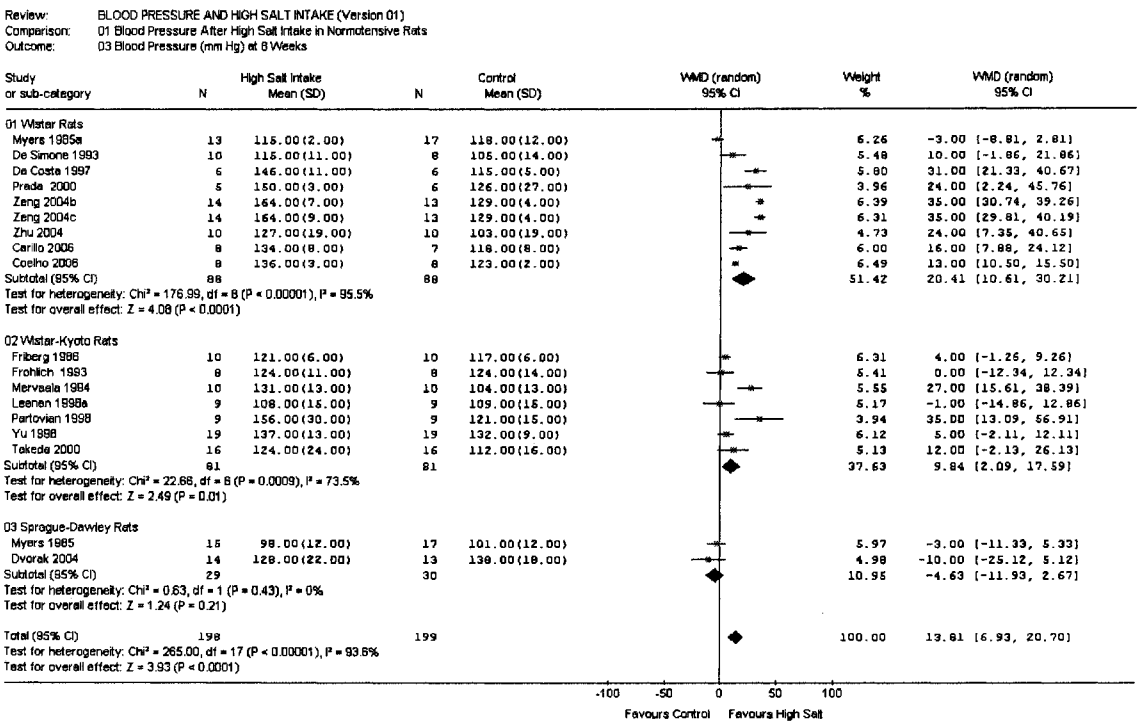
To evaluate if the effect of high salt intake in BP is strain-dependent, additional meta-analyses were performed in Wistar, Wistar-Kyoto, SD and Dahl R rats after 4 and 8 weeks of high salt intake. At 4 weeks, no increase in BP was found in any strains of rats, WMD (random) (Figure 12).

Figure 12: Effect of 4 Weeks High Salt Intake on BP of Wistar, Wistar-Kyoto and Dahl-R Rats



Eight weeks of high salt intake produced marked increase of BP in Wistar rats, WMD (random) [95% CI] = 20.4 [10.6, 30.2], ($p<0.0001$) and Wistar-Kyoto rats 9.8 [2.1, 17.6], ($p<0.01$) rats, but not in SD rats -4.6[-11.9, 2.7], ($p = 0.21$) (Figure 13).

Figure 13: Effect of 8 Weeks High Salt Intake on BP of Wistar, Wistar-Kyoto and SD Rats



1.5.4-a Possible Mechanisms for Increase of BP on High Salt Intake

Sympathetic activity may not have any role in increasing BP after high salt intake in normotensive rats as RSNA was decreased after high salt intake in Wistar rats (Carillo et al 2006). Plasma norepinephrine and epinephrine and LV norepinephrine turnover rates did not change after high salt diet in Wistar-Kyoto rats (Leenen and Yuan 1998, Yuan and Leenen 1991). High salt diet did increase lumbar sympathetic nerve activity in Wistar

rats (Carillo et al 2006). However, α_1- or β-adrenoceptor blockers failed to decrease the BP after high salt intake in Wistar-Kyoto rats (Song et al 1997), indicating that sympathetic activity may not have a role in the increase of BP after high salt intake.

Aldosterone is produced in mesenteric artery of Wistar rats (Takeda 2004). High salt intake increases the vascular production of aldosterone and aldosterone synthase mRNA in SHR (Takeda 2004), though its effect in normotensive rats has not yet been reported. Aldosterone induces vasoconstriction of coronary artery (in-vitro) in Wistar-Kyoto rats which can be prevented by AT_1 receptor antagonists, superoxide dismutase or NADPH oxidase inhibitor, apocynin, but not by spironolactone, indicating that aldosterone induces vasoconstriction via AT_1 receptors presumably via oxidative stress (Kushibiki et al 2007). This could be a non-genomic action of aldosterone that is not prevented by spironolactone.

High salt diet from 2 weeks (Cordaillat et al 2005) to ~ 12 weeks (Zeng et al 2004, Zeng et al 2004a, Partovian et al 1998) increased medial thickness, media to lumen ratio and cross sectional area of the aorta and renal, carotid and mesenteric arteries while the lumen diameter remains unchanged in Wistar, Wistar-Kyoto and SD rats. A positive correlation between BP and the cross-sectional area of the aorta, renal and mesenteric artery ($r \geq 0.92$, $p<0.01$; Zeng et al 2004) indicates that these structural changes may be associated with a rise of BP. Two studies (Partovian et al 1998, Takahashi et al 1991) reported effects of high salt diet on collagen content of carotid artery or aorta in normotensive rats. Takahashi et al (1991) reported time-course of increase of collagen in aorta following high salt intake in Wistar rats. No difference in the collagen content in aorta was found between high salt and regular salt intake at 1 and 3 months. At 6 months,

aortic collagen tended to increase (by 30%) with high salt diet compared with regular salt diet. No increase in BP was found at 1 month of high salt intake. BP tended to increase (by 10%) at 3 months and was significantly increased at 6 months of high salt intake compared with regular salt diet (Takahashi et al 1991). Partovian et al (1998) reported tendency of increase of aortic collagen (by 8%), no change of carotid collagen and significant increase of MAP after 4 months of high salt intake in Wistar-Kyoto rats. The simultaneous increase of BP and aortic collagen after high salt intake suggests that collagen formation may be associated with a rise in BP. Medial hypertrophy and fibrosis increase the stiffness of the artery that may contribute to a rise in systolic BP. However, such changes do not affect or even decrease diastolic BP. To investigate whether the increase of BP after high salt intake is dependent on the fibrosis, additional meta-analyses of systolic and diastolic BP after high salt intake were performed.

A meta-analysis of 10 studies involving 235 normotensive rats showed an increase of systolic BP after 8 weeks of high salt intake, WMD [95% CI] = 14.6 [9.0, 20.2]. Sub-group analysis of 6 studies involving 118 Wistar rats and 3 studies involving 90 Wistar-Kyoto rats showed increases of systolic BP at 8 weeks of high salt intake, WMD [95% CI] = 16.8 [12.8, 20.8]($p<0.00001$ vs control) and 14.3 [0.3, 25.3]($p = 0.05$ vs control) respectively (Figure 14).

Only two studies (Prada et al 2000, Carillo et al 2006) reported changes in diastolic BP after chronic intake of high salt in normotensive rats. Meta-analysis of 2 studies involving 27 Wistar rats also showed a significant increase of diastolic BP 16.4 [4.7, 28.1]($p = 0.006$ vs control; Figure 15).

Figure 14: Effect of 8 Weeks High Salt Intake on Systolic BP of Wistar, Wistar-Kyoto and SD Rats

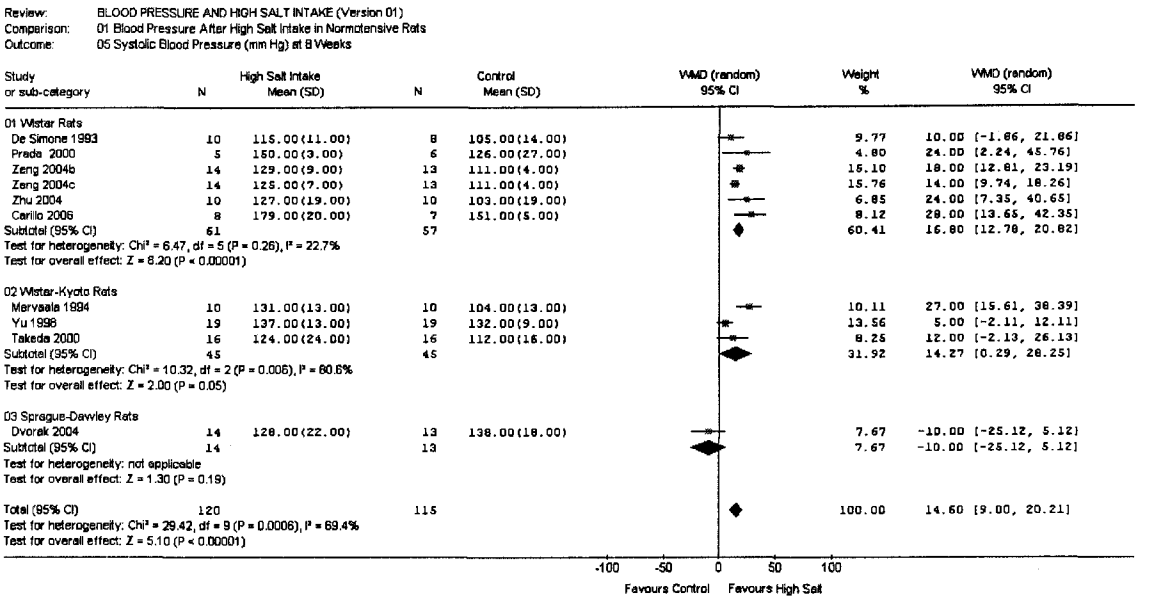
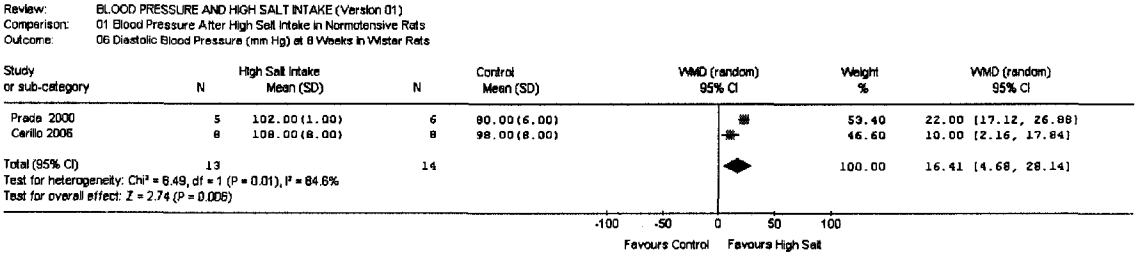


Figure 15: Effect of 8 Weeks High Salt Intake on Diastolic BP of Wistar, Wistar-Kyoto and SD Rats



In conclusion, chronic high salt intake increases MAP as well as systolic and diastolic BP in Wistar rats by mechanisms which may involve increase of vascular production of aldosterone, a direct vasoconstrictor effect of aldosterone as well as induction of vascular fibrosis and medial hypertrophy of arteries.

1.6 RATIONALE OF PRESENT STUDIES

1.6.1 High Salt Diet Induces Cardiac Fibrosis by Activating Cardiac Aldosterone

Cardiac hypertrophy is an independent predictor of cardiac morbidity and mortality (Levy et al 1990, Cooper et al 1990). High dietary salt causes LVH in animals (Frohlich et al 1993, Leenen and Yuan 1998, Fields et al 1991, Song et al 1997, Takeda 2000, Yuan and Leenen 1991) and is a powerful and independent determinant of LVH in humans (Schmieder et al 1988, Du Cailar et al 1992). High salt intake may also amplify the effect of arterial pressure on both the LV (LVH) and kidney (microalbuminuria, Du Cailar et al 2002), suggesting dietary sodium may be an independent factor for cardiovascular risk. Chronic high salt intake not only causes LVH, but also interstitial fibrosis as well as perivascular fibrosis in intramyocardial coronary arteries in the LV in normotensive and hypertensive rats (Ye et al 2002, Ye et al 2003, Yu et al 1998).

With regard to possible mechanisms that may explain high salt induced-LVH, hemodynamic effects appear not to play a major role (Yuan and Leenen 1991, Fields et al 1991, Song et al 1997). Sympathetic hyperactivity also does not appear to contribute, (Yuan and Leenen 1991, Fields et al 1991, Song et al 1997).

High salt intake decreases the expression of adrenal aldosterone synthase mRNA and plasma aldosterone and renin concentrations and activity (Ye et al 2003, Takeda et al 2000, Yu et al 1998). On the other hand, high salt increases the activity and expression of aldosterone synthase mRNA in the heart, and aldosterone production in rats (Takeda et al 2000). High salt diet increases the expression of AT_1 receptor mRNA (Takeda et al 2000) and ACE binding-density (Sun et al 2004). Cardiac Ang II also tended to increase after

high salt diet in Wistar-Kyoto rats (Leenen and Yuan 1998). Activation of the cardiac RAAS by high salt diet might therefore be contributing to LVH, independent of the circulating RAAS.

Hypothesis: We hypothesized that enhanced cardiac aldosterone production by high salt intake may increase LV weight and cardiomyocyte size and induce fibrosis in both ventricles.

Objective: To assess the possible role of cardiac aldosterone in cardiac hypertrophy and fibrosis, we evaluated the effects of high salt diet on LV and RV weight, cross-sectional cardiomyocyte diameter as well as fibrosis in Wistar rats, with and without concomitant treatment with the MR antagonist, spironolactone.

The role of spironolactone in control rats (regular salt diet) was not evaluated since MR antagonists do not affect MAP, LV weight and fibrosis in rats on regular salt diet (Cordaillat et al 2005, Bos et al 2004).

1.6.2 Brian RAAS Activates Sympathetic Activity and Circulatory and Cardiac RAAS to Influence Cardiac Remodeling

The heart may be exposed to aldosterone from the circulation as well as through its local production, which is activated after MI in animals (Silvestre et al 1999) and in humans with CHF (Mizuno et al 2001). In addition, CNS actions of aldosterone and Ang II may play a major role in cardiac remodeling post-MI.

In rats with CHF post-MI, icv infusion of spironolactone lowered increased RSNA and improved the blunted arterial baroreflex control of RSNA and HR (Francis et al 2001, Huang and Leenen 2005). In the same animal model, intracarotid injection of spironolactone lowered the increased neuronal activity in the PVN (Zhang et al 2002), indicating that peripheral administration of spironolactone may exert direct effects in the CNS. Icv infusion of an AT_1 receptor blocker also normalizes sympathetic hyperactivity and markedly attenuates LV remodeling and dysfunction post-MI. However, these approaches to assess the role of the RAAS in the brain are limited by the blockade of AT_1 receptors and MR both inside and outside the blood brain barrier and hence it will assess both the role of Ang and aldosterone locally produced in the brain (Leenen et al 1999) and the effect of circulatory Ang II and aldosterone on the brain (McKinley et al 1990). Central infusions may therefore misrepresent the actual role of local brain RAAS in the sympathetic hyperactivity and LV dysfunction or remodeling post-MI.

The role of Ang II locally produced in the brain can be assessed in TG rats deficient in brain angiotensinogen, the first transgenic animal model for the study of the role of a local brain RAS (Schinke et al 1999; see section 1.4.3-c-ii-A). For the same MI size, these TG rats maintain normal sympathetic activity and a significantly better LV function as compared to control SD rats (Wang et al 2004). Whether this better LV function is also reflected in prevention of cardiac remodeling post-MI and prevention of activation of the cardiac RAAS, specifically aldosterone, has not yet been assessed. In addition, the role of central blockade of RAAS on cardiac RAAS and remodeling has not yet been reported.

Hypothesis:

- We postulated that the effects of aldosterone in the CNS play a major role not only in sympathetic hyperactivity post-MI, but also in progressive cardiac remodeling. If so, central MR blockade by chronic icv infusion of spironolactone may induce largely similar effects to those caused by oral spironolactone.
- We also hypothesized that Ang II generated in the brain post-MI increases cardiac aldosterone that contributes to cardiac remodeling and LV dysfunction.

Objectives:

- To compare the effects of icv infusion and oral administration of spironolactone on parameters of cardiac remodeling and LV dysfunction after MI in Wistar rats.
- To investigate the role of brain angiotensins on cardiac aldosterone and cardiac remodeling post-MI by using TG rats, as an animal model with low activity of the brain RAS.
- To investigate the role of the brain aldosterone on cardiac aldosterone post-MI by central infusion of spironolactone in Wistar rats.
- The effects of MR blockade in sham rats were not evaluated since MR antagonists do not affect BP, LV functions, and LV diameter, weight and fibrosis in control rats (Cordaillat et al 2005, Bos et al 2004, Francis et al 2001).

Chapter 2

PREVENTION OF HIGH SALT DIET - INDUCED CARDIAC HYPERTROPHY AND FIBROSIS BY SPIRONOLACTONE

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Brief Communications

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ABSTRACT

Background: High salt diet causes cardiac hypertrophy and fibrosis and increases cardiac aldosterone, while decreasing plasma aldosterone. The present study assessed, in Wistar rats, the effect of high salt diet on left and right ventricle (LV and RV, respectively) weight and fibrosis both with and without aldosterone antagonist spironolactone.

Methods: Regular salt (0.6%) or high salt (8%) diet, either without or with spironolactone (20 or 80 mg/kg/day) were given to Wistar rats for 4 and 8 weeks.

Results: A modest increase in blood pressure (BP) was noted only after 8 weeks on high salt diet. Both LV weight and cardiomyocyte cross-sectional diameter were increased significantly by high salt diet after 4 and 8 weeks, whereas RV weight remained unchanged. Both LV and RV collagen as well as interstitial and perivascular fibrosis remained unchanged after 4 weeks and increased significantly after 8 weeks on high salt diet. Spironolactone at a dose of 80 mg/kg prevented increases in LV weight and cardiomyocyte cross-sectional diameter, as well as increases in LV and RV collagen and interstitial and perivascular fibrosis induced by high salt diet. In comparison, spironolactone at a dose of 20 mg/kg was somewhat less effective.

Conclusions: Chronic high salt diet increases LV weight and cardiomyocyte cross-sectional diameter and causes both LV and RV fibrosis. All of these changes are prevented by spironolactone, which is consistent with the concept that cardiac aldosterone mediates these cardiac effects of high salt diet.

Key Words: Sodium, diet, aldosterone, collagen, fibrosis, cardiomyocytes.

INTRODUCTION

High dietary salt causes left ventricular hypertrophy (LVH) in animals[1-4] and is a powerful and independent determinant of LVH in humans.[5] Chronic high salt intake causes not only LVH but also interstitial fibrosis as well as perivascular fibrosis in intramyocardial coronary arteries in the left ventricle (LV) in normotensive and hypertensive rats.[6] With regard to possible mechanisms that may explain high salt-induced LVH, hemodynamic effects do not seem to play a major role, inasmuch as resting blood pressure (BP) or LV filling pressures do not rise during the initial weeks on high salt diet.[1,2,7] Sympathetic hyperactivity also does not seem to contribute, as a high salt diet for 1 to 4 weeks does not increase cardiac norepinephrine turnover rates, and α_1- or β-adrenoceptor blockade does not prevent high salt-induced LVH in normotensive rats.[1-3]

High salt intake decreases plasma aldosterone and renin concentrations but increases aldosterone synthase activity, expression of aldosterone synthase genes, and aldosterone production in the heart.[4] Activation of the cardiac renin-angiotensin-aldosterone system by high salt diet might therefore be contributing to LVH, independent of the circulating renin-angiotensin-aldosterone system. Aldosterone increases collagen synthesis in cultured rat fibroblasts or cardiomyocytes.[8,9] Its infusion in rats causes LV hypertrophy and fibrosis in the LV and right ventricle (RV).[10]

To assess the possible role of cardiac aldosterone, we evaluated the effects of high salt diet on LV and RV weight, cross-sectional cardiomyocyte diameter, as well as fibrosis in Wistar rats, both with and without concomitant treatment with the aldosterone receptor antagonist spironolactone.

METHODS

Experimental Protocol

Male Wistar rats (body weight 100 to 125 g, approximately 4 to 5 weeks old) were obtained from Charles River Breeding Laboratories (Montreal, PQ, Canada). After 7 days of acclimatization,[3] the rats were randomly divided into 4 groups that received the following treatments: 1) regular salt diet containing 0.6% NaCl (Charles River, Montreal, PQ, Canada); 2) high salt diet containing 8% NaCl (Harlan Teklad, Madison WI, USA); 3) high salt diet and low dose of spironolactone (20 mg/kg/day in the drinking water); and 4) high salt diet and high dose of spironolactone (80 mg/kg/day in the drinking water). The treatments were continued for 4 weeks (n = 6/group) or 8 weeks (n = 8/group). The doses of spironolactone (Sigma, Chemical Co., St Louis, MO) were selected based on the study by Silvestre et al[11] on myocardial fibrosis after coronary artery ligation in Wistar rats.

Measurement of BP and Collection of Tissue

At the end of the diet and drug regimen, under halothane anesthesia, the left carotid artery was cannulated, and the BP and heart rate were recorded the next day in conscious rat.[3] The rats were then re-anesthetized with halothane and killed with 2mol/L KCl through the arterial cannula. The hearts were immediately removed, placed in ice-cold saline to remain in diastole and to remove the blood. Atria and large vessels were trimmed off. The RV were separated from the LV at the interventricular septum and were blotted dry, and weighed. Mid-level sections of the LV and RV were placed in 10% formalin for morphometric studies, and the upper and lower parts were frozen in liquid nitrogen and kept at –80°C for hydroxyproline measurement.

Measurement of Myocardial Hydroxyproline

Frozen ventricular tissues were thawed and minced, and any visible connective tissues were removed. Hydroxyproline contents of the LV and RV were determined spectrophotometrically as previously described.[12] Results were expressed as Collagen ug/mg wet weight, assuming that hydroxyproline makes up 12.7% of total collagen.

Measurement of Fibrosis

After fixation of heart tissue, transverse sections of the ventricles (4 um thick) were stained with Sirius red F3BA (0.5% in saturated aqueous picric acid). The slides of LV and RV were pictured in entirety, with a digital camera connected to the microscope, using Adobe Photoshop 4.0 imaging software (Adobe System Canada, Ottawa, ON, Canada). Interstitial fibrosis (fibrosis in interstitial spaces) and perivascular fibrosis (fibrosis around intramyocardial arteries) were determined separately,[13] using Image Pro Plus 4.1 imaging software (Media Cybernatics, Silver Spring, MD) and the results were expressed as percentages. Approximately 12-16 images for interstitial fibrosis and 10-12 images for perivascular fibrosis were analyzed. For each animal, one average value for interstitial and perivascular fibrosis for the LV and RV was calculated.

Measurement of Cardiomyocytes Diameter

Transverse sections (4 um thick) of the ventricles were stained with haematoxylin-phloxine-saffaron stain (Shandon haematoxylin, aqueous phloxine B 1%, alcohol saffron 1%). The image was captured using Adobe Photoshop 4.0 imaging software followed by analysis using Image Pro Plus 4.1 imaging software. The cross-sectional margin of cardiomyocyte was marked with the cursor and its mean diameter calculated.[13] Approximately 50 to 60 cardiomyocytes were selected from the five to

seven images captured at different sites, and one average value of the cross-sectional diameter for the LV and RV was calculated for each animal.

Statistical Analysis

Data were analyzed by one way analysis of variance and multiple comparison with Student-Newman-Keuls test. Results are expressed as mean ± SEM for each group. A value of $p < .05$ was considered to be statistically significant.

RESULTS

Body Weight

High salt diet caused a significant fall in body weight after 4 weeks but no longer after 8 weeks. Either dose of spironolactone with high salt diet did not change body weight as compared to high salt diet alone (Table 1).

Blood Pressure

After 4 weeks of high salt intake, BP was similar to that in animals on a regular salt diet, whereas it increased significantly after 8 weeks. Spironolactone with high salt diet did not affect BP after 4 weeks; however, after longer-term treatment, the BP increase was partially prevented (Table 1). The heart rate was not affected by high salt, without or with spironolactone (data not shown).

Table 1: Effect of high salt diet and spironolactone on body weight, mean arterial pressure, and absolute left and right ventricular weight.

	Regular Salt Diet	HS Diet	HS & SP, 20 mg	HS & SP, 80 mg
Body Weight (g)				
• **4 Weeks**	364 ± 11	324 ± 6*	317 ± 14*	287 ± 14*
• **8 Weeks**	470 ± 4	467 ± 11	476 ± 10	441 ± 10
MAP (mm Hg)				
• **4 Weeks**	107 ± 6	114 ± 6	103 ± 4	110 ± 4
• **8 Weeks**	113 ± 3	137 ± 4*	125 ± 4	125 ± 6
LV Weight (mg)				
• **4 Weeks**	640 ± 44	704 ± 46	636 ± 42	572 ± 24†
• **8 Weeks**	827 ± 16	950 ± 25*	908 ± 33	801 ± 39†
RV Weight (mg)				
• **4 Weeks**	202 ± 9	195 ± 12	182 ± 13	175 ± 7
• **8 Weeks**	235 ± 6	256 ± 12	239 ± 10	222 ± 9

HS = high salt diet; HS & SP = High Salt Diet and Spironolactone; MAP = Mean arterial pressure.

Values are mean ± SEM (n = 6 to 8/groups)

* $p < .05$ v regular salt diet; † $p < .05$ v high salt diet

Heart Weight

High salt diet caused a significant rise in LV weight after both 4 and 8 weeks as compared with regular salt diet (218 ± 16 v 175 ± 8 mg and 204 ± 3 v 176 ± 3 mg/100 g body weight, respectively). Spironolactone 20 mg/kg combined with high salt diet, partially prevented the increase in LV weight (200 ± 6 and 191 ± 6 mg/100 g body weight at 4 and 8 weeks, respectively). Spironolactone 80 mg/kg combined with high salt diet partially prevented the increase in LV weight after 4 weeks and fully prevented the increase after 8 weeks (199 ± 8 and 182 ± 8 mg/100 g body weight, respectively). The absolute LV weight showed a modest but non-significant increase after 4 weeks and a more clear increase ($p < .05$) after 8 weeks of high salt diet. Spironolactone prevented this increase after both 4 and 8 weeks on high salt diet (Table 1). High salt diet, without or with either dose of spironolactone, did not change RV weight after 4 and 8 weeks (Table 1).

Cardiomyocyte Diameter

In the LV, high salt diet significantly increased the cardiomyocyte cross-sectional diameter as compared with the effect of regular salt diet after 4 and 8 weeks. Spironolactone 20 mg/kg combined with high salt diet partially prevented this increase after 4 weeks but fully prevented it after 8 weeks. Spironolactone 80 mg/kg fully prevented the high salt-induced increase in cardiomyocyte diameter after 4 and 8 weeks. In the RV, high salt diet, either with or without spironolactone, did not change cardiomyocyte cross-sectional diameter after 4 and 8 weeks (Fig. 1).

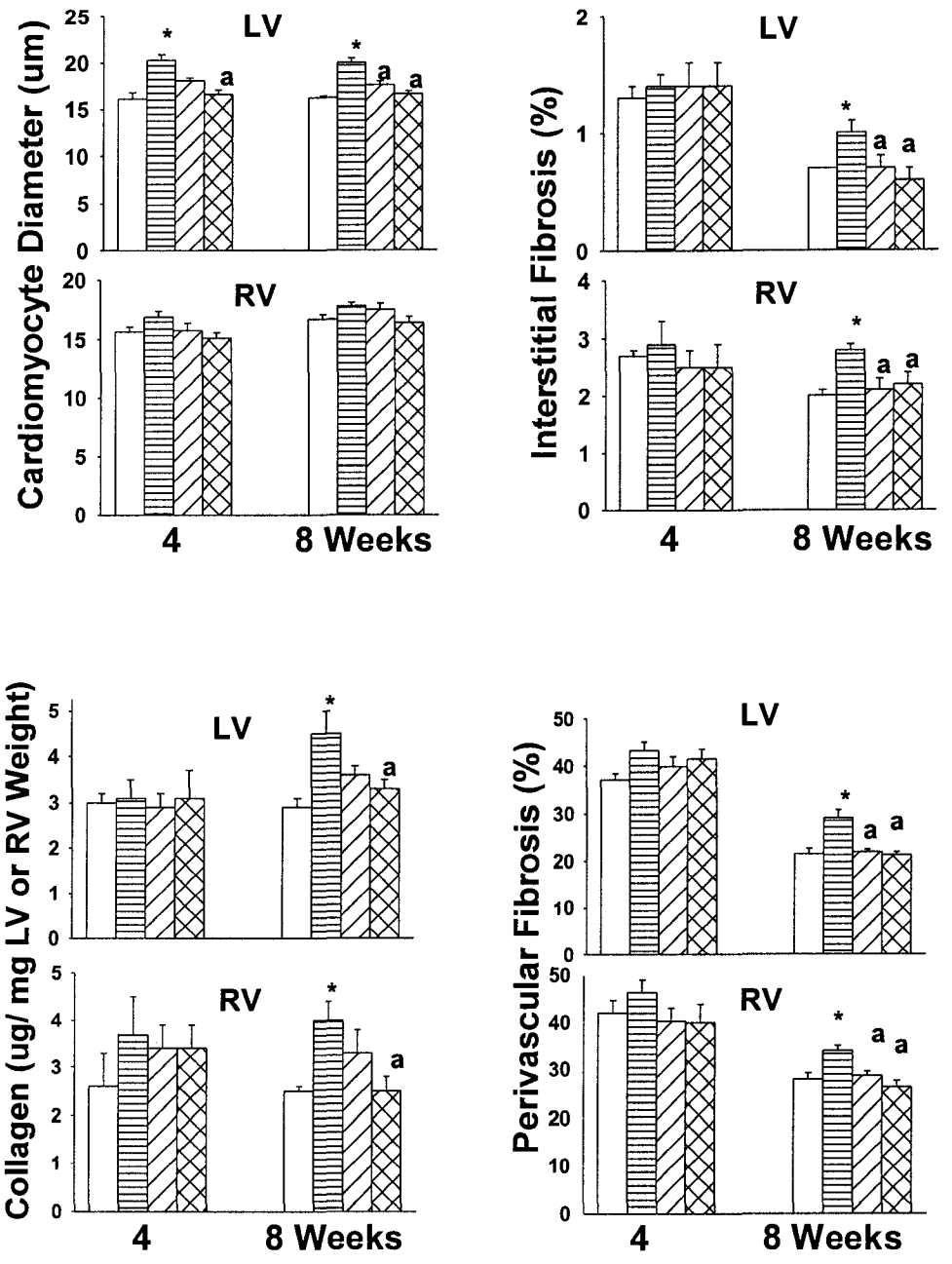

FIG. 1. Effect of high salt diet and spironolactone on left ventricle (LV) and right ventricle (RV) cardiomyocyte cross-sectional diameter, collagen, interstitial and perivascular fibrosis after 4 and 8 weeks. Values are mean ± SEM (n = 6 to 8/group). *p < .05 v regular salt diet; [a] p< .05 v high salt diet; **open bars** = regular salt diet; **horizontally striped bars** = high salt diet; **diagonally striped bars** = high salt and spironolactone 20mg/kg/day; **cross-hatched bars** = high salt and spironolactone 80 mg/kg/day.

Myocardial Collagen

High salt diet did not change LV and RV collagen after 4 weeks but increased both after 8 weeks ($p < .05$). Spironolactone at a dose of 20 mg/kg did not change the LV and RV collagen after 4 weeks, but largely prevented the increase in high salt diet-induced collagen after 8 weeks. Spironolactone at a dose of 80 mg/kg combined with high salt diet did not affect the LV and RV collagen after 4 weeks but fully prevented the increase in high salt diet-induced collagen in both the LV and RV after 8 weeks (Fig. 1).

Myocardial Fibrosis

High salt intake did not change interstitial and perivascular fibrosis in the LV and RV after 4 weeks but caused clear increases after 8 weeks ($p < .05$). Both doses of spironolactone did not affect interstitial and perivascular fibrosis in the LV and RV after 4 weeks on high salt diet. However, after 8 weeks, both doses of spironolactone fully prevented the high salt diet-induced interstitial and perivascular fibrosis in both the LV and RV (Fig. 1).

DISCUSSION

The major findings in this study are the following. First, high salt intake initially only increases LV weight and cardiomyocyte cross-sectional diameter, but more prolonged high salt diet also increases LV as well as RV collagen, and interstitial and perivascular fibrosis. Second, spironolactone fully prevents the rise in LV weight and cardiomyocyte hypertrophy and the increases in collagen, as well as interstitial and perivascular fibrosis in both the LV and RV after chronic high salt intake.

Long-term high salt intake clearly increased myocardial collagen and fibrosis. Myocardial collagen increased by 55% to 60% in the LV and RV. Myocardial fibrosis developed both in interstitial tissue and perivascular area, not only in the hypertrophied LV but also in the non-hypertrophied RV. Interstitial fibrosis increased by 40% to 45% and perivascular fibrosis by 20% to 35% in the LV and RV. These findings are consistent with the study by Yu et al [6] showing significant interstitial fibrosis in the LV and RV and perivascular fibrosis in LV of Wistar-Kyoto rats on high salt diet for 8 weeks. High salt intake initially increased only LV weight and cardiomyocyte cross-sectional diameter and subsequently increased fibrosis as well in both the LV and RV. It appears that cardiomyocyte hypertrophy develops fairly rapidly and reaches a steady state within a few weeks, whereas fibrosis develops more slowly.

The cause of myocardial fibrosis in both the LV and RV induced by high salt diet remained unclear so far. The small rise in BP is unlikely to be sufficient to induce fibrosis. Moreover, if hypertension was the primary factor, only the pressure-overloaded LV would manifest fibrosis.[10] Alternatively, high salt diet may increase production of some common factor in the ventricles to induce a fibrotic response in both the LV and RV. Takeda et al[4] reported that administration of high salt (0.9%) in drinking water stimulates cardiac aldosterone synthesis and increases cardiac aldosterone levels in Wistar-Kyoto rats. Silvestre et al[11] also reported increased levels of cardiac aldosterone and myocardial fibrosis but no change in plasma aldosterone concentration after coronary artery ligation in Wistar rats. Aldosterone may act on mineralocorticoid receptors present on cardiac myocytes and cardiac fibroblasts to increase the synthesis of collagen in heart.[8-10] Spironolactone blocks the synthesis of collagen in adult rat cardiac fibroblasts in

culture, in response to aldosterone.[8] In the myocardial infarction model, spironolactone at doses of 20mg and 80 mg/kg/day attenuated interstitial fibrosis by 26% and 32% in the LV.[11] In the present study, high salt intake increased LV weight, cardiomyocyte cross-sectional diameter, collagen and fibrosis in both the LV and RV. Spironolactone prevented these increases, consistent with a role for aldosterone in mediating the LV hypertrophic response as well as fibrosis in both ventricles in response to high salt diet.

Short-term studies with high salt intake in Wistar or Wistar-Kyoto rats in general do not find an increase in BP; however, after more long-term high salt exposure, some studies do report moderate increases,[6] although others do not.[7,14,15] In the present study, high salt intake for 4 weeks caused LVH without an increase in BP, confirming our previous finding[1-3] that dietary salt is able to produce LVH by pressure independent mechanisms. Both doses of spironolactone attenuated the rise in BP but fully prevented LV hypertrophy and both LV and RV myocardial fibrosis induced by high salt for 8 weeks. This suggests that the dose of spironolactone required for control of BP may be different from that required for the prevention of fibrosis and cardiomyocyte hypertrophy, and that larger doses may be required for control of BP.

High salt intake lowers plasma aldosterone,[4] and renal effects of spironolactone should therefore be less on high versus regular salt diet. However, as other diuretics such as hydrochlorthiazide also prevent high salt-induced LVH,[14] and as no parameter of sodium balance was measured in the present study, we cannot exclude a role for renal effects of spironolactone in prevention of high salt-induced LVH and cardiac fibrosis. Spironolactone also may have blocked direct or indirect effects on the heart by other

steroid hormones such as progesterone and testosterone.[16] Follow-up studies with a more specific aldosterone antagonist such as eplerenone will address this possibility.

In conclusion, we provide the first published evidence that chronic high salt diet increases LV weight and cardiomyocyte cross-sectional diameter but causes interstitial and perivascular fibrosis in both ventricles, which are all fully prevented by spironolactone. These findings are consistent with the concept that cardiac aldosterone may be involved in these effects of high salt diet.

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Chapter 3

CRITICAL ROLE OF CNS EFFECTS OF ALDOSTERONE IN CARDIAC REMODELING POST - MYOCARDIAL INFARCTION IN RATS

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ABSTRACT

Background: Oral administration of spironolactone improves cardiac remodeling and its central infusion prevents the increase in sympathetic drive post-myocardial infarction (MI). We hypothesized that central actions of aldosterone contribute to cardiac remodeling post-MI.

Objective: To compare the effects of intracerebroventricular (icv) infusion and oral administration of spironolactone on cardiac remodeling and left ventricle (LV) dysfunction post-MI in rats.

Methods: Spironolactone was administered orally (80 mg/kg/day) or by icv infusion (100 ng/hr), starting 1 - 3 days post-MI in Wistar rats and continued for 6 weeks.

Results: At 6 weeks post-MI, in the rats treated with vehicle, LV peak systolic pressure (LVPSP) and LV dP/dt max were clearly decreased and LV end diastolic pressure (LVEDP) and plasma catecholamines and serum aldosterone increased. All these parameters improved with both oral and icv spironolactone. The MI-induced increases in internal circumferences of LV and right ventricle (RV), and in interstitial and perivascular fibrosis, in both the LV and RV were significantly prevented/ inhibited by both oral and icv spironolactone. Laminin, fibronectin and fibrillar collagen (visualized by scanning electron microscopy, SEM) increased in the non-infarcted part of the LV post-MI in the vehicle group, but not/ less in rats on oral or icv spironolactone.

Conclusions: Since the magnitude of beneficial effects of icv spironolactone at low doses was largely equal to that achieved with its oral administration at much higher doses, we propose that in addition to other sites of action, aldosterone appears to activate central

nervous system (CNS) pathways and thereby influences peripheral mechanisms involved in cardiac remodeling.

Key Words: Fibrosis, Heart failure, Infarction, Renin-angiotensin system, Remodeling

INTRODUCTION

Ventricular remodeling describes a series of changes in ventricular size and structure occurring after myocardial infarction (MI) that affect the infarcted and non-infarcted zone of the left ventricle (LV) as well as the right ventricle (RV).[1-3] A number of local and systemic mechanisms have been implicated in myocardial remodeling. Among these the renin-angiotensin-aldosterone system appears to be one of the major contributors. Both angiotensin II and aldosterone may contribute to the progressive remodeling and dysfunction through a variety of direct and indirect effects. The adverse effects of aldosterone are increasingly being recognized. Blockade of its effects by oral spironolactone, eplerenone or canrenone reduced LV interstitial fibrosis in rat post-MI.[2,4,5] Spironolactone and eplerenone reduced cardiac morbidity and mortality in patients with severe heart failure[6] or after acute MI complicated by LV dysfunction.[7]

The heart may be exposed to aldosterone from the circulation as well as through its local production, which is activated after MI in animals[4] and in humans with CHF.[8] Effects of aldosterone in the brain have received so far little attention. Aldosterone, either from the circulation or produced locally in the brain [9] may stimulate mineralocorticoid receptors (MR) in the central nervous system (CNS) leading to an increase in sympathetic outflow,[10] and release of arginine vasopressin.[11] Recent studies suggest that aldosterone in the brain plays a major role in regulation of sympathetic tone in CHF. In rats with

CHF post-MI, intracerebroventricular (icv) infusion of spironolactone lowered increased renal sympathetic nerve activity (RSNA) and improved the blunted arterial baroreflex control of RSNA and heart rate.[12] In the same animal model, intracarotid injection of spironolactone lowered the increased neuronal activity in the paraventricular nucleus (PVN),[13] indicating that peripheral administration of spironolactone may exert direct effects in the CNS.

In recent studies, we demonstrated that the central effects of aldosterone on sympathetic activity appear to be mediated through the stimulation of "ouabain" release.[14] The latter activates the renin-angiotensin system in the brain leading to sympathetic hyperactivity.[15] Blockade of either brain "ouabain" by icv infusion of specific Fab fragments or of brain AT_1 receptors by icv infusion of an AT_1-receptor blocker not only normalizes sympathetic hyperactivity in rats with CHF post-MI,[16,17] but also markedly attenuates LV remodeling and LV dysfunction post-MI.[18] Considering the above, we postulated that effects of aldosterone in the CNS may play a major role not only in sympathetic hyperactivity post-MI, but also in progressive cardiac remodeling. If so, central MR blockade by chronic icv infusion of spironolactone may induce largely similar effects to those caused by oral spironolactone. In the present study, we therefore compared the effects of icv infusion and oral administration of spironolactone on parameters of cardiac remodeling and LV dysfunction after MI in Wistar rats.

2. MATERIALS AND METHODS

Male Wistar rats (200-250 g body weight; 6-8 weeks of age) were obtained from Charles River Breeding Laboratories (Montreal, Canada). They were given regular chow

diet and water *ad libitum*. All procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care, which conform to NIH guidelines and approved by the University of Ottawa Animal Care Committee. MI was induced by ligation of the left coronary artery at 2-3 mm from its origin using the open chest model as previously described.[1,18,19] Buprenorphine was used to relieve the pain of surgery and MI.

2.1. Experimental protocols

2.1.1. Protocol I

One day after MI, surviving animals were randomly divided into groups for treatment with spironolactone or vehicle for 6 weeks as follows:

1) MI and oral spironolactone (80 mg/kg/day) in the drinking water. The dose of spironolactone (Sigma) was based on previous studies.[4,20] The drug was first dissolved in absolute ethanol and the required quantity based on water consumption and body weight put in the drinking water. The final concentration of ethanol was 1.5%.

2) MI and vehicle (1.5% of ethanol) in water.

3) Sham: Sham MI surgery and vehicle.

2.1.2. Protocol II

Three days after MI, an icv cannula was implanted in the left lateral cerebral ventricle.[18] The longer arm of the cannula was connected to an osmotic minipump for the infusion of spironolactone or vehicle randomly for 6 weeks as follows:

1) MI with icv spironolactone (100 ng/ h). The dose was based on previous studies.[12,21] Spironolactone at 0.4 mg/ml with 0.2% ethanol was put into Alzet osmotic minipumps (model 2004, Alza; Palo Alta, CA) for continuous infusion at a flow rate of 0.25 ul/h.

After 4 weeks the pump was replaced by a model 2002 for 2 weeks, having a flow rate of 0.5 ul/h and drug concentration of 0.2mg/ml. The pumps were placed subcutaneously behind the neck.

2) MI and icv vehicle (ethanol, 0.2%) in sterile water using similar pumps.

3) Sham: Sham MI surgery, icv ethanol (0.2%) as vehicle.

2.1.3. Protocol III

The experimental groups outlined under protocol I and II were combined into one study. At 6 weeks post-MI, these animals were used for assessment of LV function by Millar catheter, and some of the hearts used for evaluation of collagen pattern by scanning electron microscope (SEM). In a follow-up experiment, this protocol was repeated and at 6 weeks post-MI blood was collected in conscious rats from a PE-50 catheter inserted into the carotid artery for measurements of catecholamines and aldosterone. The LV was used for analysis of laminin and fibronectin.

For all protocols combined, 220 rats underwent coronary artery ligation. Of these, 64 died during/ immediately after MI, leaving 156 rats for randomization. The survival rate after 6 weeks was 100, 92 or 93% in the MI groups administered vehicle, oral or icv spironolactone. All sham rats survived.

2.2. Hemodynamics

Under halothane anesthesia, a 2F Millar mikro-tip® catheter transducer, model SPR 407 (Millar Instruments Inc, Houston, TX) was inserted through the right carotid artery for measurement, under minimal level of anesthesia, of mean arterial pressure (MAP), LV peak systolic pressure (LVPSP), LV end diastolic pressure (LVEDP), LV dP/dt max and heart rate. [19]

2.3. Catecholamines and aldosterone

Two blood samples were collected into prechilled tubes, the first containing sodium heparin, the second without additives. The plasma and serum were separated and stored at –80°C. Catecholamines were extracted with acid-washed alumina and measured by HPLC as described previously.[22] Aldosterone was measured by radioimmunoassay according to Brochu et al,[23] with as minor modification, the elution of aldosterone with 80% methanol.

2.4. Ventricular weights and infarct size

After measuring the hemodynamics, the rat was re-anesthetized with halothane and sacrificed with 1 ml of 2 M KCl to arrest the heart in diastole. The heart was removed immediately and the RV was separated from the LV at the interventricular septum. The ventricles were weighed. In the LV, the infarct size was measured as described previously.[16,18] Briefly, the LV was opened at the interventricular septum and spread out by 4-5 incisions. The infarcted and non-infarcted areas of the LV were traced on a transparent sheet, and measured by planimetry to calculate the % infarct.

2.5. Cardiac anatomy

Mid-level sections of the LV and RV were placed in 10% neutral buffered formalin for histological morphometric studies. Transverse sections of the ventricles (4 um thick) were stained with HPS stain (Shandon haematoxylin, aqueous phloxine B 1%, alcohol saffron 1%). The slides were examined under a BX 50 Olympus microscope and images were captured (magnification X20) in entirety with a digital camera. The internal circumference of LV and RV and ventricular wall thickness of LV at the interventricular septum (septum) and scar (lateral wall in Sham) and of the RV were measured.

2.6. Cardiac fibrosis

Mid-level sections of the ventricles (4 um thick) were stained with Sirius red F3BA (0.5% in saturated aqueous picric acid).[20] The images were captured (magnification X40) in entirety with a standard polarizing filter. Fibrosis was measured in the non-infarcted and infarcted LV and in the RV. For the non-infarcted LV, fibrosis in an area 2 mm outside the infarct for peri-infarct and at the septum for distant fibrosis was measured separately. About 10-12 images for interstitial fibrosis and 7-10 for perivascular fibrosis were analyzed and for each animal one average value for LV (at each site) and RV was calculated. Structure and pattern of the fibrillar collagen was examined by Bozkurt et al.[24] Briefly, the LV tissues from the peri-infarcted zone were frozen in liquid nitrogen, freeze-fractured, and thaw-fixed in paraformaldehyde and glutaraldehyde solution (2% each in 0.1 M phosphate buffer). Samples were dehydrated in ethanol, critical point dried, mounted, sputter-coated with gold and examined by SEM (XL 30 ESEM, FEI) at an accelerating voltage of 7.5 or 10 kV (magnification X 8000).

2.7. Laminin and fibronectin

Immunohistochemistry for laminin and fibronectin on LV tissues was performed using primary antibody rabbit anti-laminin and anti-fibronectin (Sigma, St Louis, Missouri) as described previously.[25,26] For laminin, 6-8 images were captured randomly from the septum at magnification (X400) and the thickness of the laminin surrounding 60-70 randomly selected cross-sectionally cut cardiomyocytes determined. For fibronectin, 5 pictures each from the septum and the peri-infarct zone of LV were captured randomly and analyzed as grade 0, 1, 2 and 3 for no, mild, moderate and

maximum staining, respectively. For negative controls, the sections were incubated with non-immune rabbit IgG.

2.8. Cardiomyocyte diameter

The HPS-stained slides of the ventricles were examined and pictures captured randomly (magnification X400) as described above. For cross-sectionally cut cardiomyocytes having intact cell walls and clear round intracytoplasmic nuclei, the margins were marked and the mean diameter was calculated.[20] Measurements were performed in the RV and LV (both peri-infarct and septum). About 50-60 cardiomyocytes were randomly selected from the 5-7 images captured randomly at different sites and one average value of its cross-sectional diameter for LV (from each site) and RV was calculated for each animal.

2.9. Statistical analysis

Results are expressed as mean ± S.E.M. The data was analyzed by One Way ANOVA followed by multiple comparisons with Student-Newman-Keuls test. A value of $p< 0.05$ was considered statistically significant.

3. RESULTS

3.1. General

Infarct size was about 30% of the LV and was similar for all MI groups. The gain in body weight was similar in all groups, except the group with oral spironolactone, which showed less increase (Table 1). Serum K^+ remained unchanged after MI and oral and icv spironolactone ranging from 4.5 to 5.2 mmol/L. Serum levels of Na^+, K^+, Cl^- and $HCO3^-$ and hematocrit also did not differ among the groups (data not shown).

Table 1

Effect of oral and icv spironolactone on body weight, LV and RV weights and wall thickness, and interstitial fibrosis in infarct

	Oral Treatment			ICV Infusion		
	Sham (n=9)	MI + Vehicle (n=9)	MI + Spir (n=6)	Sham (n=8)	MI + vehicle (n=10)	MI + Spir (n=7)
MI (%)		29.7 ± 2.1	28.3 ± 2.9		30.3 ± 2.0	31.7 ± 0.9
Gain in Body Weight (g)	206 ± 7	231 ± 15	153 ± 13*,†	203 ± 18	230 ± 13	206 ± 9
LV Weight (mg/100g Body weight)	169 ± 2	177 ± 6	194 ± 9	173 ± 13	183 ± 8	198 ± 5
RV Weight (mg/100g Body weight)	44 ± 1	84 ± 11*	49 ± 3†	44 ± 2	83 ± 10*	60 ± 4†
LV Wall Thickness (mm): At Septum	3.4 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.7 ± 0.2	3.2 ± 0.1	3.7 ± 0.3
At Scar	3.0 ± 0.1	1.0 ± 0.1*	1.2 ± 0.1*	3.4 ± 0.2	1.0 ± 0.1*	1.6 ± 0.1*,†
RV Wall Thickness (mm)	1.5 ± 0.0	1.3 ± 0.1*	1.4 ± 0.1	1.7 ± 0.1	1.3 ± 0.1*	1.5 ± 0.1
Interstitial Fibrosis in Infarct (%)	1.3 ± 0.1	62.2 ± 2.1*	50.5 ± 2.5*,†	1.0 ± 0.1	61.0 ± 2.2*	43.8 ± 2.6*,†

*p< 0.05 vs sham; † p< 0.05 vs MI + vehicle; MI + Spir = MI + Spironolactone; n = number of animals

3.2. Hemodynamics

LVPSP was decreased by about 20 mmHg at 6 weeks after MI. This decrease was clearly attenuated by icv ($p<0.05$) and to a less extent (NS) by oral spironolactone (Fig. 1). The vehicle MI groups also showed clear decreases in LV dP/dt max and MAP and increases in LVEDP. These 3 parameters significantly improved by both oral and icv spironolactone. Icv spironolactone was significantly better in improving LVPSP, LV

dP/dt max and LVEDP as compared to oral spironolactone (Fig. 1). Heart rate remained similar in all groups (data not shown).

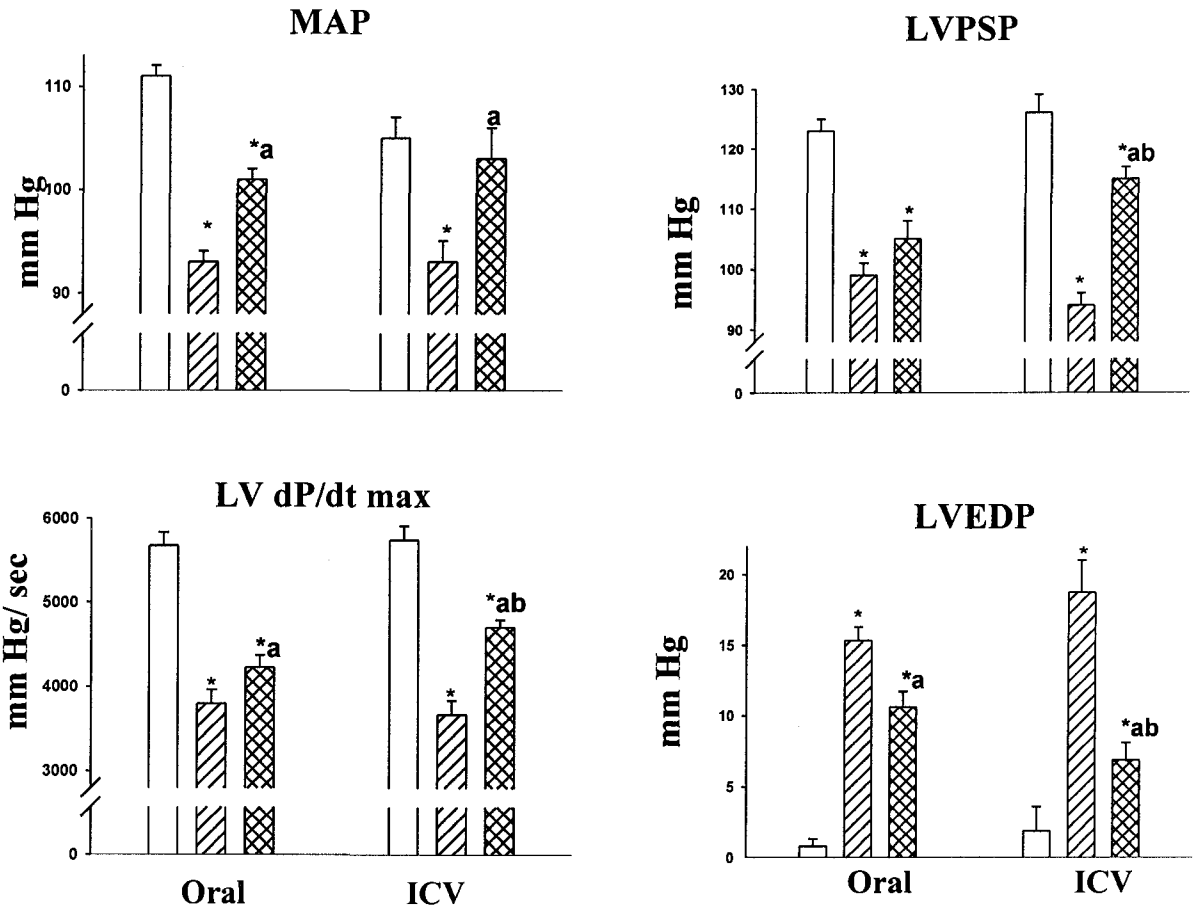

Fig. 1: Oral and icv spironolactone and MAP, LVdP/dt max, LVPSP and LVEDP at 6 weeks post-MI. Values are mean ± S.E.M. (n = 6-9 rats/group). *p< 0.05 vs Sham; a = p< 0.05 vs MI + vehicle; b=p<0.05 vs oral spironolactone. Open bars = Sham, Diagonally striped bars = MI + vehicle, Cross-hatched bars = MI + spironolactone.

3.3. Plasma catecholamines and aldosterone

Plasma norepinephrine increased by 70% in the vehicle group post-MI. This increase was attenuated by oral ($p<0.05$) and somewhat less by icv (NS) spironolactone (Fig. 2). Plasma epinephrine tended to increase by 40% post-MI in the vehicle group while it remained unchanged in the icv spironolactone group. Serum aldosterone increased by 45% post-MI in the vehicle group. This increase was prevented by both oral and icv spironolactone (Fig. 2).

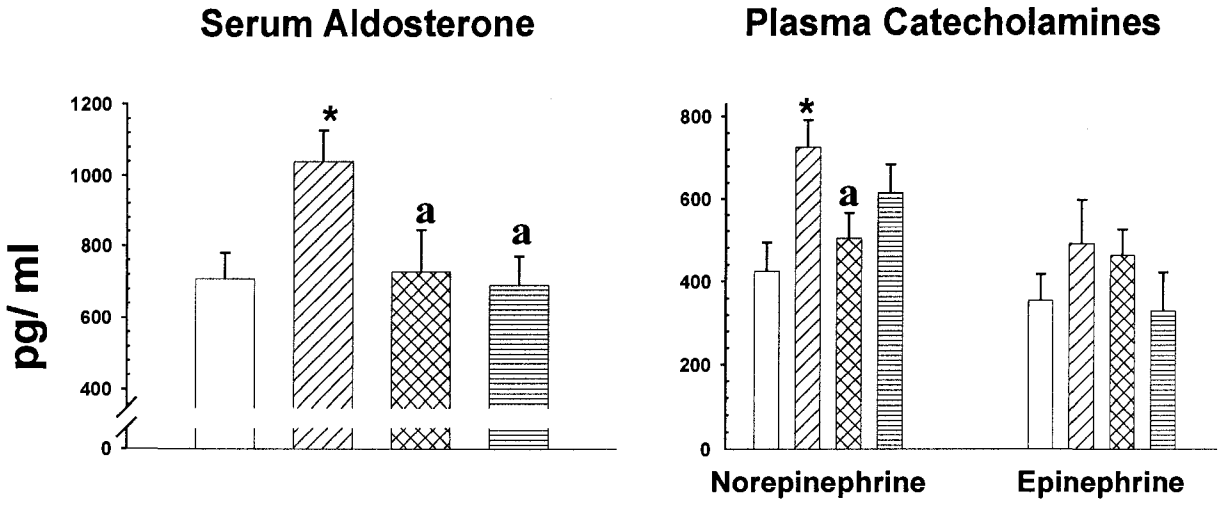

Fig. 2: Oral and icv spironolactone and serum aldosterone (n=6-9 rats/group) and plasma catecholamines (n = 4-8 rats/group) at 6 weeks post-MI. Values are mean ± S.E.M. *p< 0.05 vs Sham; a = p< 0.05 vs MI + vehicle. Open bars = Sham, Diagonally striped bars = MI + vehicle, Cross-hatched bars = MI + oral spironolactone, horizontal bars = MI + icv spironolactone.

3.4. Ventricular weights and anatomy

At 6 weeks post-MI, overall LV weight was unchanged and spironolactone did not affect it. RV weight clearly increased after MI and this increase was similarly

inhibited by oral and icv spironolactone (Table 1). LV and RV internal circumference were both increased at 6 weeks post-MI in the vehicle groups. Oral and icv spironolactone both inhibited the increase in LV internal circumference and prevented the increase in RV internal circumference (Fig. 3).

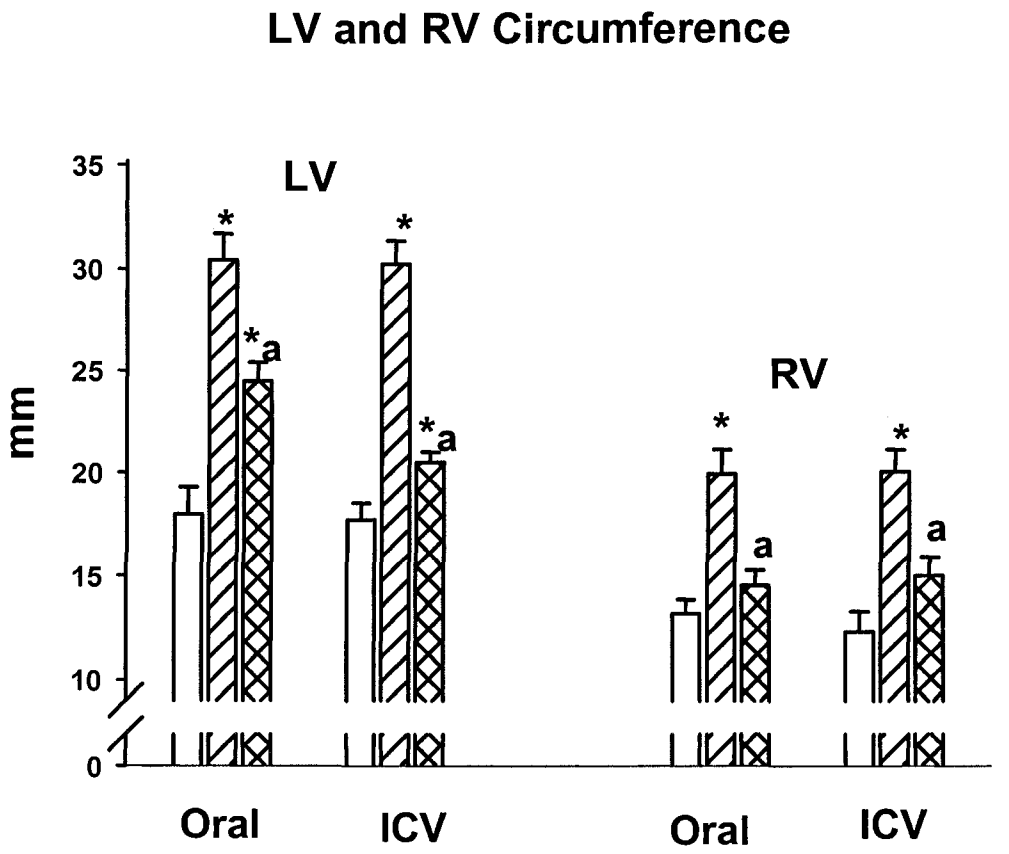

Fig. 3: Oral and icv spironolactone and LV and RV internal circumference at 6 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats/group). *p< 0.05 vs Sham; a = p< 0.05 vs MI + vehicle. Open bars = Sham, Diagonally striped bars = MI + vehicle, Cross-hatched bars = MI + spironolactone.

LV wall thickness at the septum and RV wall thickness tended to decrease in the vehicle group post-MI, but not after oral or icv spironolactone. At the infarct scar, LV

wall thickness was markedly thinned. This decrease in LV wall thickness at the scar was improved somewhat by icv spironolactone (Table 1).

3.5. Cardiomyocyte diameter

The cardiomyocyte diameter increased after MI both in the LV and RV. The increase was more pronounced in the peri-infarct area than in septum in the LV. The increase of cardiomyocyte diameter was prevented totally in the septum and the RV and partially in the peri-infarct area by both oral and icv spironolactone (Fig. 4).

3.6. Cardiac fibrosis

The MI caused interstitial fibrosis in the LV and RV. In the LV the increase was marked in the peri-infarct area and less so in the septum. Oral and icv spironolactone fully prevented the interstitial fibrosis in the septum and RV and inhibited it in the peri-infarct area (Fig. 5,6). On SEM, a markedly increased dense weave and lattice like pattern of collagen was visualized in the peri-infarct zone of the LV at 6 weeks post-MI. These changes were largely prevented by oral and icv spironolactone (Fig. 6). Perivascular fibrosis increased after MI, both in the septum and peri-infarct area in the LV and in the RV. Both oral and icv spironolactone prevented this rise in perivascular fibrosis in the LV to a similar extent. In the RV, oral spironolactone inhibited the perivascular fibrosis, whereas icv spironolactone totally prevented it (Fig. 6,7). Marked interstitial fibrosis was found in the infarct zone. Both oral and icv spironolactone attenuated the extent of fibrosis in the infarct (Table 1). No animal died of cardiac rupture.

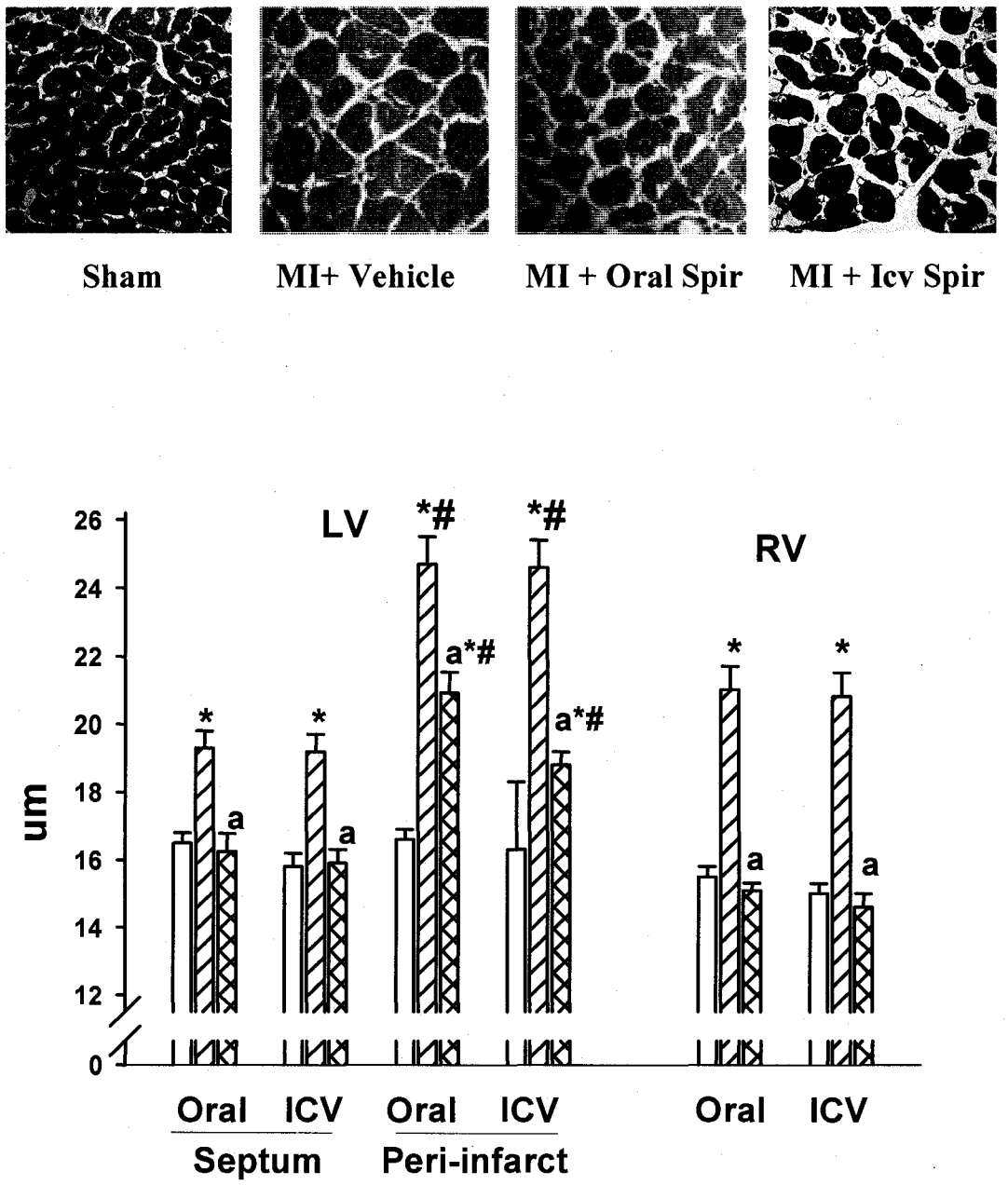

Fig. 4: Oral and icv spironolactone and LV and RV cardiomyocyte diameter at 6 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats/group). *p< 0.05 vs Sham; a = p< 0.05 vs MI + vehicle; # = p <0.05 vs Septum. On top, images of cardiomyocytes in peri-infarct zone of the LV at 6 weeks post-MI (magnification X400). Spir = spironolactone. Open bars = Sham, Diagonally striped bars = MI + vehicle, Cross-hatched bars = MI + spironolactone.

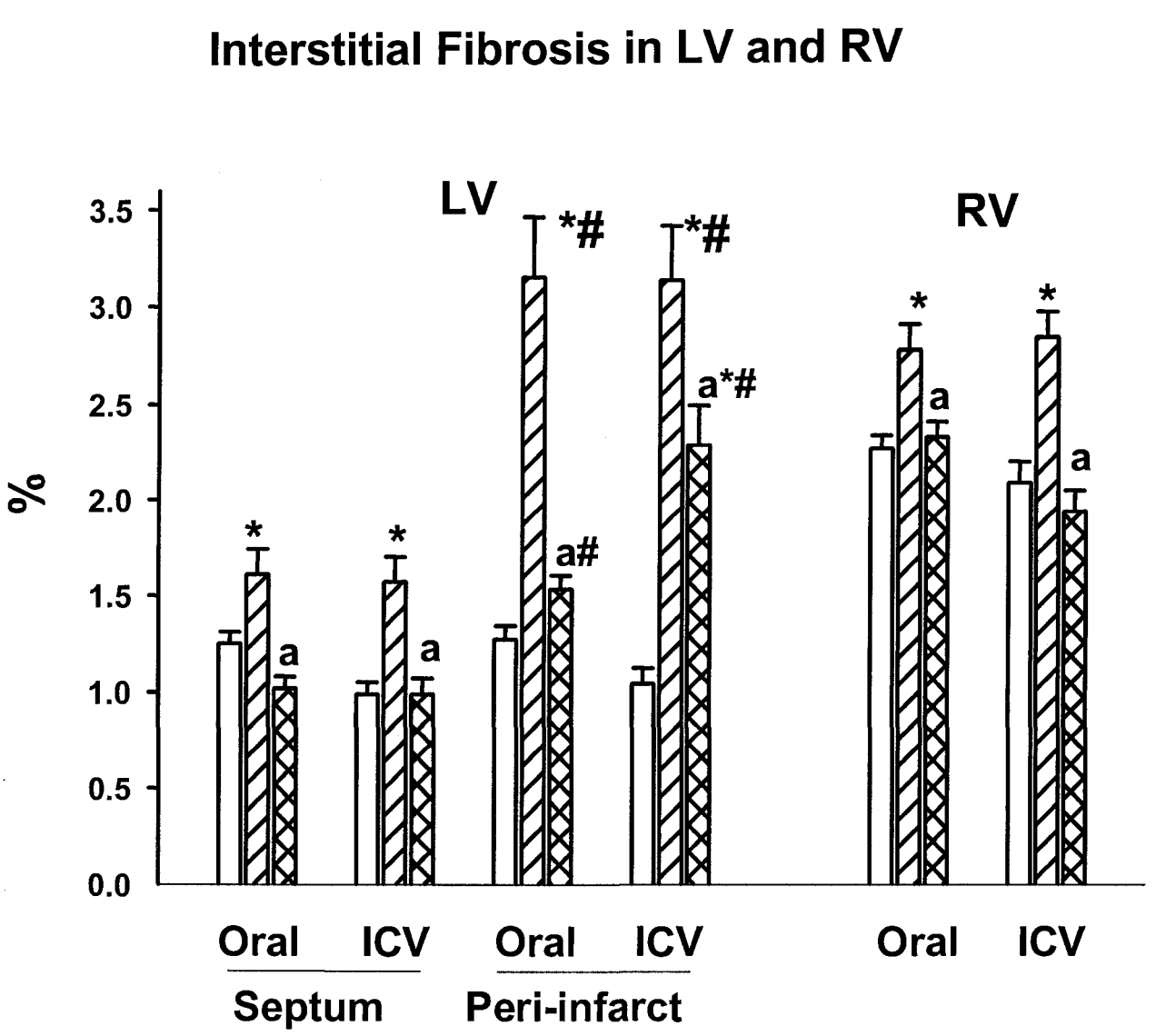

Fig. 5: Oral and icv spironolactone and LV and RV interstitial fibrosis at 6 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats/group). *p< 0.05 vs Sham; a = p< 0.05 vs MI + vehicle; # = p <0.05 vs Septum. Open bars = Sham, Diagonally striped bars = MI + vehicle, Cross-hatched bars = MI + spironolactone.

Fibrosis and Collagen in Peri-infarct Zone of LV

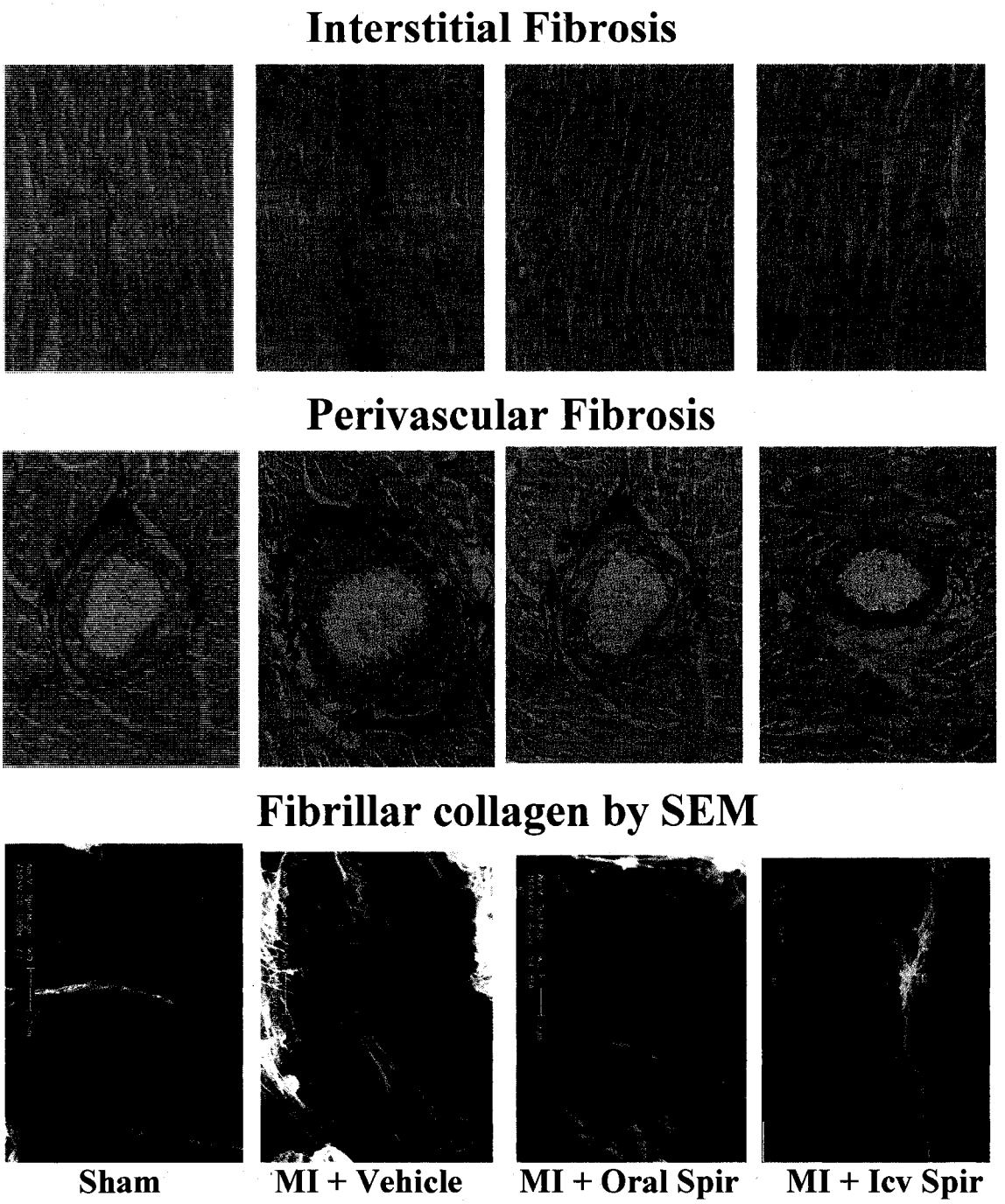

Fig. 6: Interstitial and perivascular fibrosis by light microscope (magnification X400) after picrosirius red staining and collagen by SEM (magnification X8000) in peri-infarct zone of LV, in representative hearts at 6 weeks post-MI. Spir = spironolactone

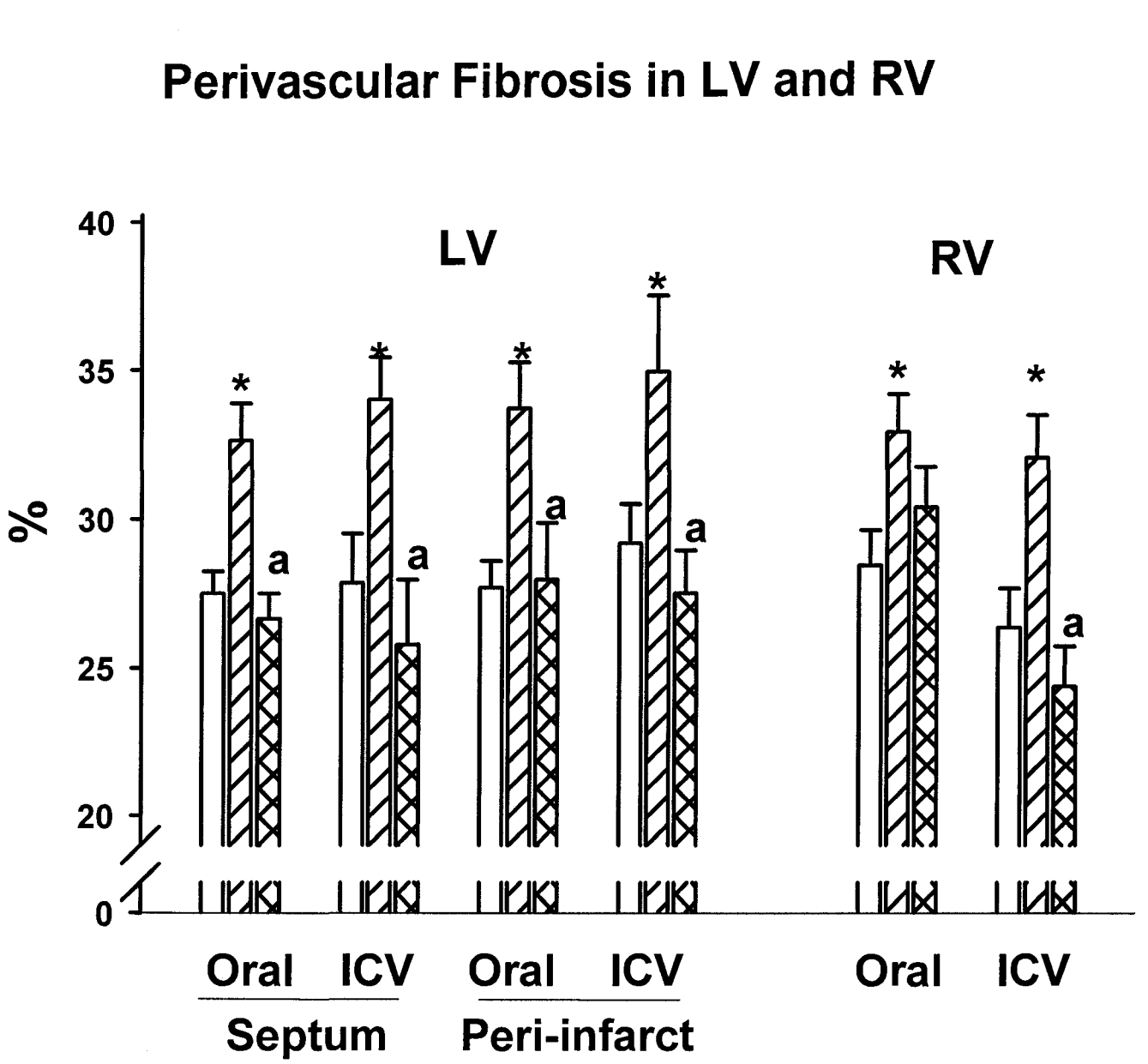

Fig. 7: Oral and icv spironolactone and perivascular fibrosis in the LV and RV at 6 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats/group). *$p < 0.05$ vs Sham; a = $p < 0.05$ vs MI + vehicle. Open bars = Sham, Diagonally striped bars = MI + vehicle, Cross-hatched bars = MI + spironolactone.

3.7. Laminin and fibronectin

Laminin around the cardiomyocytes in the non-infarcted part of the LV was significantly increased at 6 weeks post-MI. This increase was fully prevented by both oral

and icv spironolactone (Fig. 8). Fibronectin staining remained unchanged in the septum (grade 0.3-0.5), increased in the peri-infarct zone (Fig. 8) and was markedly increased in the infarct (grade 3, not shown) at 6 weeks post-MI. Both oral and icv spironolactone attenuated the increase of fibronectin in the peri-infarct zone (Fig. 8), but did not affect the increase in the infarct (not shown).

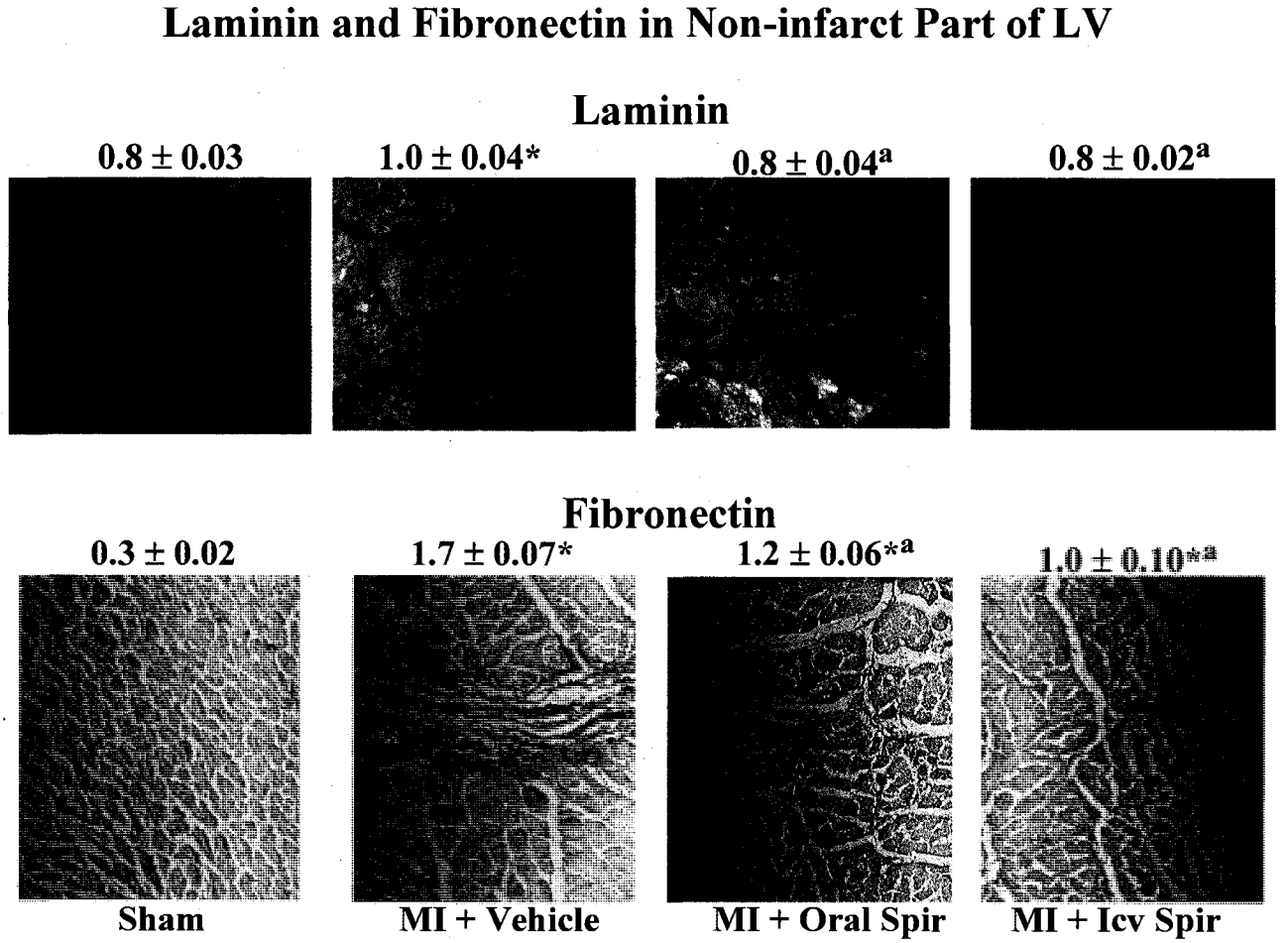

Fig. 8: Oral and icv spironolactone and laminin (magnification X400, in septum, um) and fibronectin (magnification X200, in peri-infarct zone, grades) in representative hearts at 6 weeks post-MI. Values above each image represents the mean ± S.E.M. (n = 5-7 rats/group). *p< 0.05 vs Sham; a = p< 0.05 vs MI + vehicle. Spir = spironolactone

4. DISCUSSION

As significant new finding, the present study shows that central treatment with spironolactone inhibits major components of cardiac remodeling post-MI and attenuates the decreases in LVPSP and LV dP/dt max and the increase in LVEDP. Oral treatment with spironolactone inhibited cardiac remodeling to a similar extent and LV dysfunction somewhat less.

4.1. Oral spironolactone

Consistent with previous studies,[4,5,27] oral treatment with spironolactone significantly improved LV function at 6 weeks post-MI and was remarkably effective in inhibiting most aspects of cardiac remodeling. Oral spironolactone inhibited the MI-induced increases in LV and RV internal circumference. Oral treatment with canrenone similarly inhibited the increase of LV diastolic diameter, as assessed by echocardiography.[5] This inhibition of the LV and RV dilation was associated with prevention of the cardiomyocyte hypertrophy in the septum and the RV and attenuation in the peri-infarct zone of the LV. Consistent with previous studies using MR antagonists,[2,4,5,27] oral spironolactone largely prevented the increases in interstitial and perivascular fibrosis in the non-infarcted part of the LV and the RV. The present study is the first one evaluating the effects of a MR antagonist on collagen structure by SEM and on adhesion molecules post-MI. Oral spironolactone significantly attenuated laminin and fibronectin accumulation in the septal and peri-infarct zones of the LV. By SEM, MI-rats exhibited a marked increase in collagen with dense weave and lattice like stretches in the peri-infarct zone of the LV. Oral spironolactone markedly inhibited the MI-induced increase in collagen and the normal web and strut pattern persisted.

4.2. Icv spironolactone

The effects of central MR blockade on cardiac remodeling have not been previously described. Central treatment with spironolactone substantially attenuated the fall of LVPSP and LV dP/dt max and the increase of LVEDP. Francis et al[12] reported a non-significant decrease in the LVEDP as assessed by PE-50 catheter after 4 weeks treatment with icv spironolactone. Icv spironolactone inhibited the MI-induced increases in LV and RV internal circumference and in RV weight, prevented the increase of cardiomyocyte diameter in the septum and the RV and attenuated cardiomyocyte hypertrophy in the peri-infarct zone. The increases in interstitial and perivascular fibrosis and adhesion molecules were largely prevented by icv spironolactone in the septum and the RV and attenuated in the peri-infarct zone of the LV. The MI-induced increases of dense weave and lattice like stretches in the non-infarcted LV, visualized by SEM, were also largely prevented.

Comparison of the effects of oral vs icv treatment with spironolactone on cardiac remodeling shows overall a similar pattern of responses without consistent differences. Both treatments substantially improved, but not normalized LV function. Even if cardiac remodeling would be fully prevented, one may expect some LV dysfunction to persist related to the loss of myocardium by the MI per se. Interestingly, icv spironolactone was significantly better in improving LVEDP, LVPSP and LV dP/dt max.

4.3. Mechanisms

Leakage of spironolactone from the CNS into the circulation causing peripheral MR blockade very unlikely explains the effects of central infusions at ~5-6 ug/kg/day considering that icv doses of spironolactone are 10,000-15,000 times lower than the doses used orally (20-80 mg/kg/day).[4,20] ^{3}H-labelled canrenone readily penetrates the blood brain barrier[28] and circulating spironolactone can exert direct central effects.[13] In contrast to the central infusion, peripheral infusion at the low rate of 100 ng/h had during the first 2 weeks, no effect on sympathetic activity,[29] but did decrease sympathetic activity during more prolonged infusion[12] and these authors suggested that prolonged peripheral administration of spironolactone even in small doses can produce effects through central mechanisms.

Central MR involved in cardiovascular regulation may be activated by circulating aldosterone acting on MR in periventricular or circumventricular areas.[10] Aldosterone immunoreactivity in cerebrospinal fluid correlates well with plasma aldosterone.[30] Alternatively, the central MR may be activated by aldosterone synthesized locally. Aldosterone synthase is present in the hypothalamus,[9] as is 11β hydroxysteroid dehydrogenase type 2, which makes the MR aldosterone selective.[10] Pathways involving local release of aldosterone may potentially be activated through cardiac vagal or sympathetic afferent fibers. The cardiac branch of the vagus conveys mechanosensitive and chemosensitive information to the PVN [31] and sympathetic fibers are activated in heart failure to provide excitatory input to the CNS.[32]

In the brain aldosterone appears to stimulate MR, followed by stimulation of "ouabain" release,[14] and the brain renin-angiotensin system,[15] resulting in e.g., increased

sympathetic drive[13,17] and vasopressin release.[11] Blockade of this pathway by central blockade of MR (present study), of "ouabain" or of AT_1 receptors [18] significantly attenuates cardiac remodeling post-MI. How these central blockades inhibit cardiac remodeling and progression of LV dysfunction post-MI has not been directly tested yet. Blockade of sympathetic hyperactivity may clearly play a major role. Central infusion of spironolactone normalized at both 2 and 4 weeks post-MI increased sympathetic activity and the blunted arterial baroreflex.[12] In the present study, plasma norepinephrine was clearly elevated at 6 weeks post-MI in the vehicle group and less in the groups treated with icv or oral spironolactone. The latter finding is similar to the effects of oral eplerenone.[27] These findings indicate that also oral treatment with MR antagonists lowers sympathetic activity.

Sympathetic hyperactivity may contribute to cardiac remodeling through hemodynamic effects, by direct cardiac effects, or by increasing plasma angiotensin II and aldosterone. Plasma angiotensin II shows an initial marked rise in rats post-MI and then remains elevated by 50-100% up to 2 months.[34] In the present study, serum aldosterone was increased by ~50%. Recently, we showed that blockade of the brain renin-angiotensin system prevents the increase of plasma angiotensin II post-MI[19] and the present study shows that chronic treatment with icv and oral spironolactone prevents the increase in serum aldosterone. These findings are consistent with the concept that sympathetic hyperactivity post-MI contributes to the increase in both plasma angiotensin II and serum aldosterone and that oral spironolactone may prevent these increases. However, further studies are needed to establish that effects of oral spironolactone on

sympathetic activity, angiotensin II and aldosterone are indeed a result of direct central effects and not secondary to peripheral effects on cardiac remodeling and hemodynamics. Both sympathetic drive per se, circulatory angiotensin II and aldosterone may enhance fibrosis in the heart.[35] In the heart, angiotensin II may also be locally produced, and its effects may be mediated through activation of local production of aldosterone. At 4 weeks post-MI, the non-infarcted area of the LV showed a 2-fold increase of aldosterone synthase mRNA and 4-fold increase of aldosterone production. These increases were prevented by oral treatment with an AT_1 receptor blocker.[4] It is therefore tempting to speculate that central MR blockade prevents cardiac MR stimulation by inhibiting not only circulatory levels of aldosterone, but also the cardiac production of aldosterone post-MI. Components of the extracellular matrix include collagen and adhesion molecules such as laminin and fibronectin. All can be produced by fibroblasts, under the control of aldosterone.[35,36] Consistent with previous studies, [2,4,5,26,37] marked increases were noted for all 3 components, particularly in the peri-infarct zone of the LV. The inhibition of the MI-induced increases of laminin and fibronectin by both oral and icv spironolactone suggests that central mechanisms also appear to contribute to regulation of these adhesion molecules perhaps by influencing circulatory (and possibly) cardiac aldosterone.

Post-MI, TNF-α increases in the circulation, the heart and brain,[38] whereas central infusion of spironolactone maintains normal plasma TNF-α level post-MI.[39] Pro-inflammatory cytokines such as TNF-α may contribute to progressive cardiac remodeling post-MI[24] through several actions, including further increase of sympathetic activity [40] and of cardiac angiotensin II.[41] Studies on changes in plasma vasopressin levels post-MI are inconsistent. Some reported an increase (e.g.),[33] others reported no change (e.g.),[42,43]

in plasma vasopressin levels post-MI. However, despite no increase in plasma vasopressin post-MI, conivaptan, a vasopressin (V_{1a} and V_2) antagonist increased water excretion and decreased RV weight[42] and OPC-31260, a vasopressin (V_2) antagonist increased water excretion.[43] Icv infusion of a MR antagonist in rats with CHF post-MI also increased urine volume,[12] consistent with a decrease in vasopressin release.

4.4. Limitations of study

For assessment of cardiomyocyte hypertrophy, only the cross-sectional diameter of cardiomyocytes was measured. Since LV and RV dilation was likely associated with elongation of cardiomyocytes, not measuring the length and thereby volume underestimates the actual extent of hypertrophy. However, spironolactone decreased not only the cross-sectional diameter of the cardiomyocytes, but also the LV and RV circumference. It is therefore rather likely that spironolactone also decreased cardiomyocyte length and volume. Direct renal effects of spironolactone were not assessed in the present study, but are unlikely to play a major role since serum electrolytes and hematocrit remained unaffected by oral and icv spironolactone and the chronic use of potent diuretics has little impact on cardiac remodeling post-MI.[44]

4.5. Conclusion

The present study confirms that aldosterone plays a major role in cardiac remodeling post-MI. Since the beneficial effects of icv spironolactone at low doses on LV function and remodeling were equal or better to those achieved with oral administration at high doses, we propose that in addition to its other actions, aldosterone appears to activate CNS pathways influencing peripheral mechanisms involved in cardiac remodeling. Based on the above findings, one may speculate that post-MI oral treatment

with MR blockers may provide additional benefit for outcome if lipophilic compounds and /or high enough doses are used causing central MR blockade, in addition to peripheral blockade.

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Chapter 4

Prevention of Cardiac Remodeling After Myocardial Infarction in Transgenic Rats Deficient in Brain Angiotensinogen

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ABBREVIATIONS

ACE	Angiotensin Converting enzyme
Ang	Angiotensin
AOGEN	Angiotensinogen
AT_1	Angiotensin II type 1
CHF	Congestive heart failure
CNS	Central nervous system
icv	Intracerebroventricular
LV	Left ventricle
MI	Myocardial infarction
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
RV	Right ventricle
SD	Sprague-Dawley
Septum	Interventricular septum
StAR	Steroidogenic acute regulatory protein
TG	Transgenic
TNF	Tumor necrosis factor

ABSTRACT

The brain renin-angiotensin-aldosterone system (RAAS) plays a major role in cardiac remodeling after myocardial infarction (MI). To assess the contribution of the brain RAAS in the activation of the cardiac RAAS post-MI, transgenic (TG) rats deficient in brain angiotensinogen and Wistar rats with intracerebroventricular (icv) infusion of spironolactone were studied. An MI was induced by acute coronary artery ligation. TG and control Sprague-Dawley (SD) rats were followed for 8 weeks and Wistar rats for 6 weeks. Infarct sizes, % of left ventricle (LV) area, were in the 30-33 % range. In SD rats at 8 weeks post-MI, internal circumference, interstitial and perivascular fibrosis, cardiomyocyte diameter in the LV and right ventricle (RV), laminin and fibronectin in the LV, and lung weights were increased. Aldosterone was increased markedly in both the LV and RV at 8 weeks post-MI. In TG rats, the MI-induced increases of RV internal circumference and weight were prevented and increases of lung weight and LV internal circumference significantly inhibited. In TG rats, the post-MI increases of interstitial fibrosis and cardiomyocyte diameter were prevented in interventricular septum (septum) and RV and significantly inhibited in the peri-infarct zone of the LV. The increases in perivascular fibrosis, laminin and fibronectin were prevented in the LV. In TG rats, cardiac aldosterone did not increase. In Wistar rats at 6 weeks post-MI, aldosterone was markedly increased in the LV, but not in the RV. This increase was prevented by icv infusion of spironolactone. These findings support the pivotal role of locally produced angiotensin II in the brain in cardiac remodeling post-MI. The brain RAAS appears to activate a cascade of events, among others an increase in cardiac aldosterone, which play a major role in cardiac remodeling post-MI.

Key Words: Aldosterone; Angiotensin; Brain; Cardiac remodeling; Fibrosis; Infarction

1. INTRODUCTION

Ventricular remodeling after myocardial infarction (MI) includes both geometric remodeling of the chamber as well as structural remodeling of the myocardium [1]. This remodeling leads to progressive increases in left ventricle (LV) end diastolic volume and pressure, and decreases in ejection fraction and cardiac output and the development of clinical congestive heart failure (CHF). Extensive evidence indicates that increased activity of the circulatory and cardiac renin-angiotensin-aldosterone systems (RAAS) and of cardiac-specific and generalised sympathetic activity post-MI contribute to cardiac remodeling and dysfunction post-MI through a variety of direct and indirect effects. In addition to peripheral effects, central nervous system (CNS) actions of aldosterone and angiotensin (Ang) II appear to play a major role in cardiac remodeling post-MI. Blockade of aldosterone's actions in the brain by icv infusion of spironolactone [2-4] or of Ang II type 1 (AT_1) receptors in the brain by icv infusion of an AT_1 receptor blocker [5,6] not only normalizes sympathetic hyperactivity, but also markedly attenuates LV remodeling and dysfunction post-MI. Such icv infusions will assess the central effects of Ang II or aldosterone locally produced in the brain, as well as the effects of circulating Ang II or aldosterone on the brain [7]. The role of Ang II locally produced in the brain can be assessed in transgenic (TG) rats deficient in brain angiotensinogen (AOGEN) [8]. These animals exhibit markedly reduced brain angiotensins [9] and normal plasma AOGEN concentration and resting plasma renin activity [8,10,11]. For the same MI size, these TG rats maintain a significantly better LV function as compared to control Sprague-Dawley

(SD) rats. Sympathetic hyperactivity is also prevented [11]. Whether this better LV function is also reflected in prevention of cardiac remodeling post-MI and prevention of activation of the cardiac RAAS, specifically aldosterone, has not yet been assessed.

The primary goal of the study was therefore to evaluate the role of angiotensins generated in the brain in cardiac remodeling and the increase of cardiac aldosterone post-MI using these TG rats, as an animal model with low activity of the brain renin-angiotensin system (RAS). To demonstrate that the CNS control of cardiac aldosterone post-MI is not somehow unique for the TG versus their SD parent strain, cardiac aldosterone was also evaluated in Wistar rats with and without central infusion of spironolactone [3]. Our results show that the brain RAAS plays a major role in the development of major components of cardiac remodeling post-MI and that the increase in cardiac aldosterone post-MI depends on the brain RAAS.

2. MATERIALS AND METHODS

TG rats deficient in brain AOGEN and their parent strain SD rats (both male, 300 to 350 g) were transferred from the Max-Delbruck Centre for Molecular Medicine, Berlin-Buch, Germany to the University of Ottawa Heart Institute, Canada. Male Wistar rats (200-250 g body weight; 6-8 weeks of age) were obtained from Charles River Breeding Laboratories (Montreal, Canada). Rats were housed on a 12:12- hour light-dark cycle and were given regular rat chow and tap water *ad libitum*. All procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care, which conform to NIH guidelines and approved by the University of Ottawa Animal Care Committee. After a 5 day acclimatization period, an MI was induced by coronary artery

ligation using the open chest model, as previously described. [12,13]. Briefly, under 1% halothane in oxygen anesthesia, endotracheal intubation was performed and artificial respiration was started. After opening the thorax at the left 4^{th} or 5^{th} left intercostal space, the left coronary artery was ligated at 2-3 mm from its origin with a 6-0 silk suture attached to an atraumatic needle (K801H; Ethicon). Sham control rats underwent the same surgical procedure without ligation. Buprenorphine was used for pain relief for 2 days.

The TG and SD rats were allowed to recover and were followed for 8 weeks. The impact of the MI on LV function and sympathetic activity in TG vs SD rats has been previously reported [11]. In this manuscript, we report the role of brain angiotensins in cardiac remodeling and aldosterone post-MI.

In Wistar rats, 1-3 days after MI, the surviving animals were randomly divided into 2 groups for icv treatment with vehicle or spironolactone for 6 weeks, as described previously [3]. Icv infusion of spironolactone (100 ng/h) was initiated using Alzet osmotic minipumps (model 2004, 2002, Alza; Palo Alta, CA). The dose was based on the study by Francis et al [2]. MI-control and sham rats were icv infused with vehicle. The effects of icv spironolactone on cardiac remodeling and dysfunction in Wistar rats have already been reported [3].

2.1. Ventricular Weights and Infarct Size

Rats were sacrificed with 1ml of 2 M KCl to arrest the heart in diastole. The heart was removed immediately and the right ventricle (RV) was separated from the LV at the interventricular septum (septum). The ventricles were weighed. In the LV, the infarct size

as % of the LV area was measured as described previously [3,6,13]. The lungs were removed for measurement of wet weights.

2.2. Cardiac anatomy

Mid-level sections of the LV and RV were placed in 10% neutral buffered formalin for histological morphometric studies. After fixation, transverse sections of the ventricles (4 um thick) were stained with HPS stain (Shandon haematoxylin, aqueous phloxine B 1%, alcohol saffron 1%). The slides were examined under BX 50 Olympus microscope and images were captured (magnification X20) in entirety with a digital camera using Adobe Photoshop 4.0 imaging software (Adobe System Canada, Ottawa, ON, Canada) and analyzed using Image Pro Plus 4.1 imaging software (Media Cybernetics, Silver Spring, MD). The internal circumference of LV and RV and ventricular wall thickness of LV both at septum and scar (lateral wall in sham), and of the RV were measured [3].

2.3. Cardiomyocyte diameter

The HPS-stained slides of the ventricles were examined and pictures captured randomly (magnification X400) as described above. The intact cross-sectional margins of cardiomyocytes having clear round intra-cytoplasmic nuclei were marked with the cursor by using Image Pro Plus 4.1 imaging software and the mean diameter was calculated [14]. Measurements were performed in the LV, in an area 2 mm outside the infarct for peri-infarct and in the septum for cardiomyocyte diameter in distant area separately, and in the RV. About 60-70 cardiomyocytes were randomly selected from the 5-7 images captured randomly at different sites and an average cross-sectional diameter for LV (from each site) and RV was calculated for each animal. For consistency of results, only cross-

sectionally cut cardiomyocytes having complete cell boundaries and clear round intra-cytoplasmic nuclei were measured [3].

2.4. Cardiac fibrosis

Mid-level sections of the ventricles (4 um thick) were stained with Sirius red F3BA (0.5% in saturated aqueous picric acid). The images were captured randomly (magnification X100) using Adobe Photoshop 4.0 imaging software with a standard polarizing filter and analyzed using Image Pro Plus 4.1 imaging software. Interstitial and perivascular fibrosis, % area were determined separately. Fibrosis was measured in the non-infarcted (both peri-infarct and septum) and the infarcted LV and in the RV. About 10-12 images for interstitial fibrosis and 7-10 for perivascular fibrosis were analyzed and for each animal an average value for LV (at each site) and RV was calculated [3,14].

2.5. Laminin and fibronectin

Immunohistochemistry for laminin and fibronectin in the LV was performed on the paraffin-embedded sections using primary antibody rabbit anti-laminin and anti-fibronectin (Sigma Corp, St Louis, MI, USA), as described previously [3,15,16]. Briefly, sections were deparaffinized, dehydrated, treated with 0.3% hydrogen peroxide and incubated with primary antibody for 24 hours followed by biotin-labeled secondary antibody for 30 min at room temperature. Avidin-biotinylated enzyme complex (Vector Laboratories, Burlingame, CA) was added to bind to secondary antibody followed by visualization with 3,3'-diamino benzidine. For laminin, 6-8 images were captured randomly from the septum at magnification (X400) and the thickness of the laminin surrounding 60-70 randomly selected cross-sectionally cut cardiomyocytes determined. For fibronectin, 5 images each from the septum and the peri-infarct area of LV were captured randomly and analyzed as grade 0-3 for absent, mild, moderate and maximum

staining respectively. For negative controls, the sections were incubated with non-immune rabbit IgG.

2.6. Cardiac aldosterone

Aldosterone was measured by radioimmunoassay in the RV and the non-infarcted part of the LV. Briefly, tissue was homogenized with 100% methanol, spun in the Sorvall RT 6000B, supernatant removed, dried in Savant Speed-Vac, dissolved in 3 ml 0.1% trifluroacetic acid, centrifuged, applied to preconditioned cartridge and assayed according to the method of Brochu et al [17] with as minor modification the elution of aldosterone with 80% methanol [3].

2.7. Statistical analysis

Results are expressed as mean ± S.E.M. The data was analyzed by two-way ANOVA for TG and SD rats and one-way ANOVA for Wistar rats, followed by multiple comparisons with Student-Newman-Keuls test. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. General

Body weight was similar in TG and SD rats. The infarct sizes were similar in TG and SD rats at 8 weeks post-MI. The wet weights of the lung increased markedly after MI in the SD rats, but only modestly in TG rats (Table 1). In the sham TG and SD groups, all parameters of ventricular anatomy and cardiac aldosterone levels were the same (Table 1 and Figs. 1-7).

Table 1

Body weight, infarct size, LV and RV weights and wall thickness, wet lung weight and interstitial fibrosis in infarct of SD and TG rats at 8 weeks post-MI

	SD Rats		TG Rats	
	Sham (n = 9)	**MI (n = 6)**	**Sham (n = 9)**	**MI (n = 9)**
Body Weight (g)	463 ± 19	489 ± 7	467 ± 13	486 ± 11
Infarct size (%)	--	33 ± 3	--	30 ± 3
LV weight (mg/100g body weight)	206 ± 4	215 ± 7	218 ± 3	217 ± 5
RV weight (mg/100g body weight)	58 ± 2	102 ± 12*	65 ± 2	70 ± 8†
LV wall thickness (mm):				
• **At septum**	3.7 ± 0.2	3.3 ± 0.3	3.6 ± 0.2	3.6 ± 0.1
• **At scar**	3.8 ± 0.2	1.1 ± 0.1*	3.8 ± 0.2	1.7 ± 0.1*†
RV wall thickness (mm)	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.1 ± 0.1
Lung weight (mg/100g body weight)	383 ± 24	947 ± 109*	480 ± 27	613 ± 55†
Fibrosis in Infarct (% area)	--	56 ± 1.9	--	44 ± 2.6†

* $p < 0.05$ vs sham (TG and SD rats); † = $p < 0.05$ vs MI (SD rats); n = number of animals

3.2. Ventricular weight and anatomy

At 8 weeks post-MI, the overall LV weight remained similar. RV weight markedly increased in SD rats after MI, but no increase was found in TG rats (Table 1). The internal circumference of LV and RV increased after MI in SD rats and this increase

was prevented in the RV and inhibited in the LV of TG rats (Fig. 1). The wall thickness was markedly thinned at the scar in the LV in SD rats. This decrease of LV wall thickness improved slightly in TG rats. There was no difference in the wall thickness at the septum and the RV (Table 1).

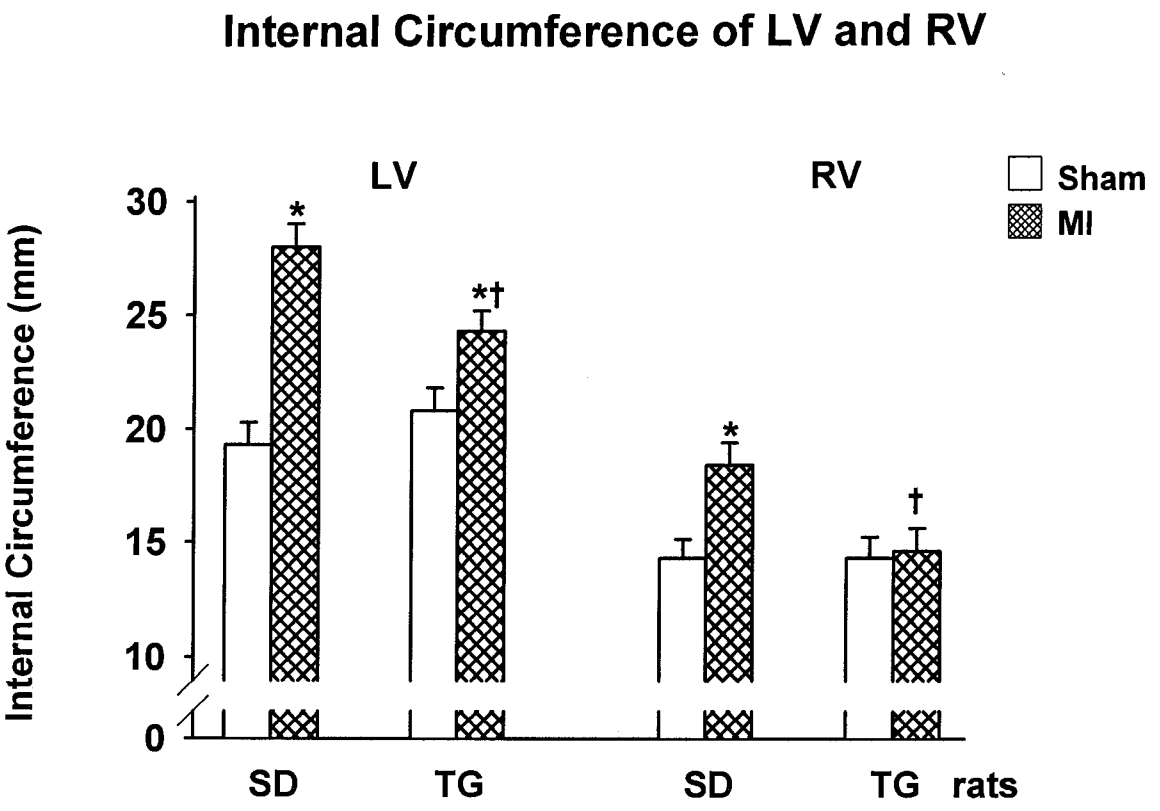

Fig. 1: LV and RV internal circumference of sham and MI groups of SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p < 0.05$ vs sham (SD and TG rats); † $p < 0.05$ vs MI (SD rats).

3.3. Cardiomyocyte diameter

The cardiomyocyte diameter increased after MI both in the LV and RV of SD rats. The increase was more pronounced in the peri-infarct area than the septum. MI-

induced increases of cardiomyocytes diameter were prevented in the septum and RV and significantly inhibited in the peri-infarct area in the TG rats (Fig. 2).

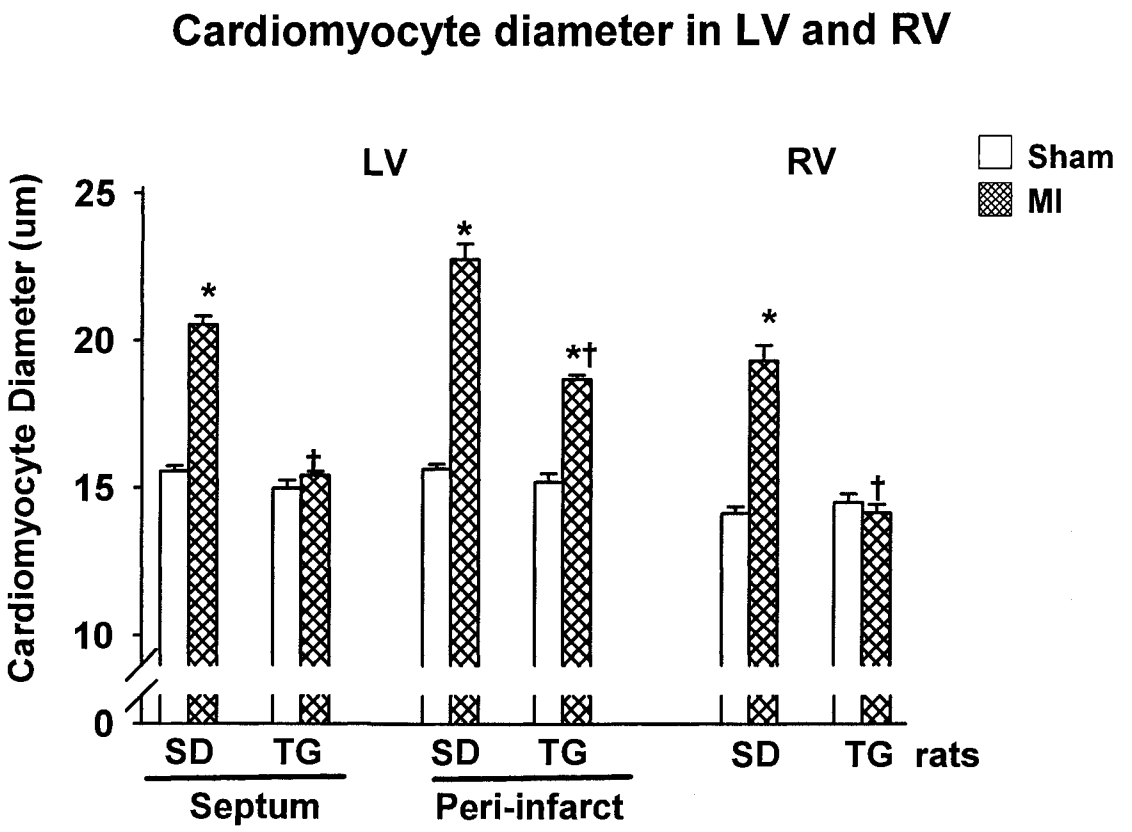

Fig. 2: Cardiomyocyte diameters in the LV and RV of sham and MI groups SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p< 0.05$ vs sham (SD and TG rats); † $p< 0.05$ vs MI (SD rats).

3.4. Cardiac fibrosis

Interstitial fibrosis increased after MI in the LV and RV of SD rats. In the LV, the increase was marked in the peri-infarct area and less so in the septum. In TG rats, the MI-

induced increases of interstitial fibrosis were prevented in the septum and the RV and significantly inhibited in the peri-infarct area (Fig. 3).

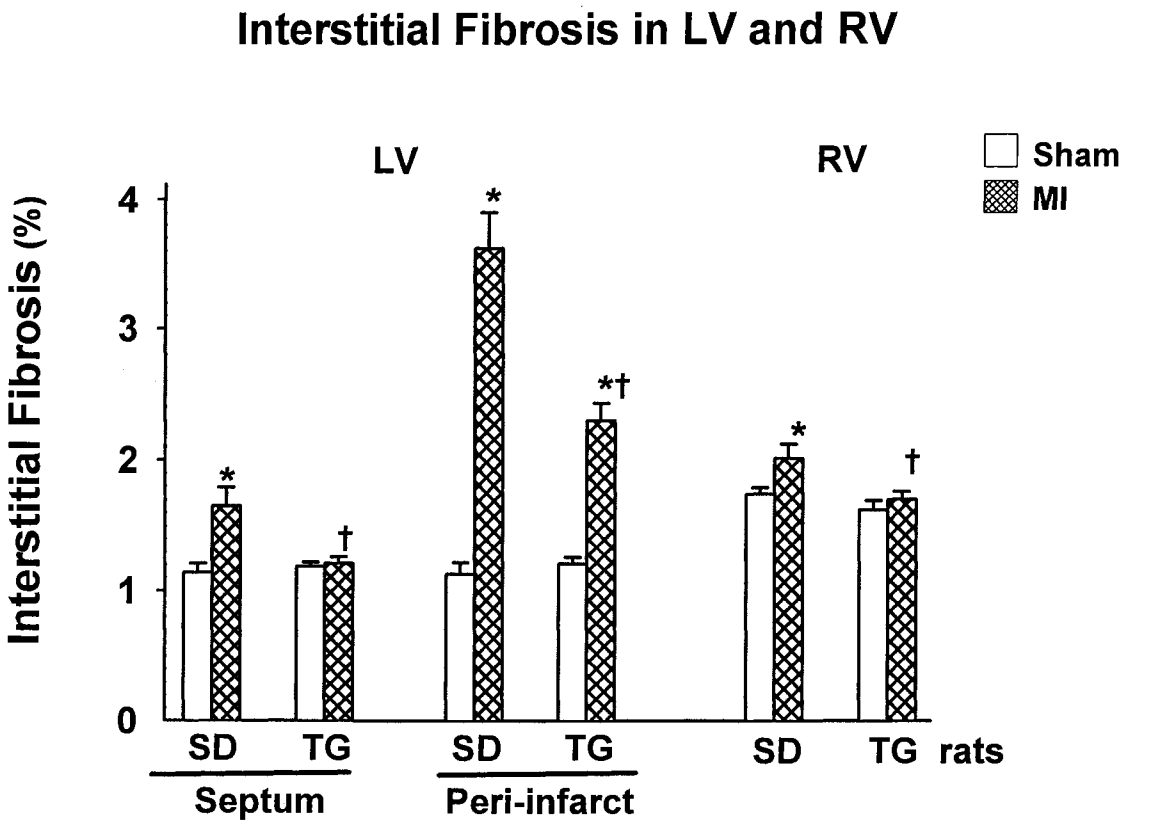

Fig. 3: Interstitial fibrosis, % area, in the LV and RV of sham and MI groups of SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p< 0.05$ vs sham (SD and TG rats); † $p< 0.05$ vs MI (SD rats).

Perivascular fibrosis increased after MI, both in the septum and peri-infarct area in the LV and tended to increase in the RV in SD rats, while no increase was found at any of these sites in TG rats (Fig. 4). A marked fibrosis was found in the infarct zone in the SD rats, which was significantly less in TG rats (Table 1).

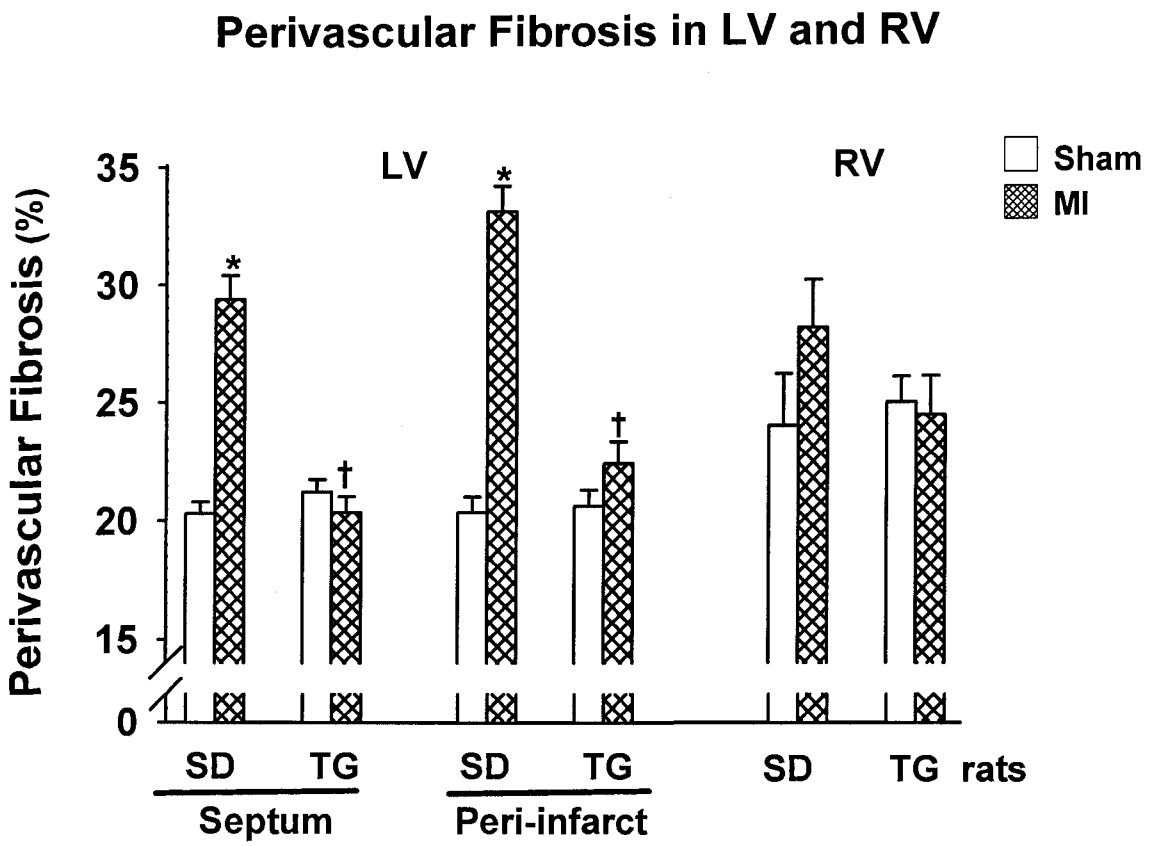

Fig. 4: Perivascular fibrosis, % area, in the LV and RV of sham and MI groups SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p < 0.05$ vs sham (SD and TG rats); † $p < 0.05$ vs MI (SD rats).

3.5. Laminin and fibronectin

Laminin around the cardiomyocytes in the non-infarcted area of the LV was significantly increased at 8 weeks post-MI in SD rats, but no increase was found in the TG rats (Fig. 5).

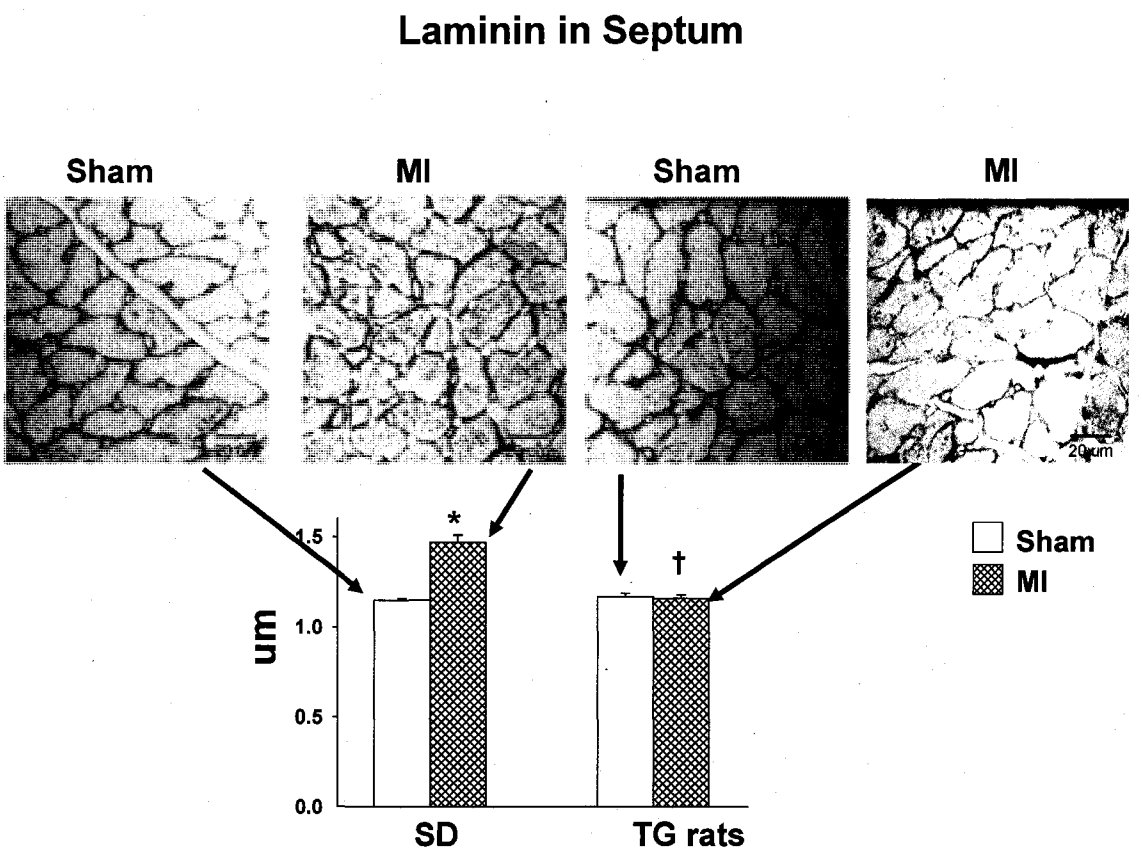

Fig. 5: Laminin thickness by immunohistochemistry (on top, images at magnification X400) in the septum of sham and MI groups of SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p < 0.05$ vs sham (SD and TG rats); † $p < 0.05$ vs MI (SD rats).

Fibronectin staining increased in the SD rats in the septum and this increase was marked in the peri-infarct area at 8 weeks post-MI. No increase was found in the septum and the increase in the peri-infarct area was significantly less in the TG than SD rats post-

MI (Fig. 6). The fibronectin staining was markedly increased in the infarct in SD and TG rats (grade 3, not shown) at 8 weeks post-MI.

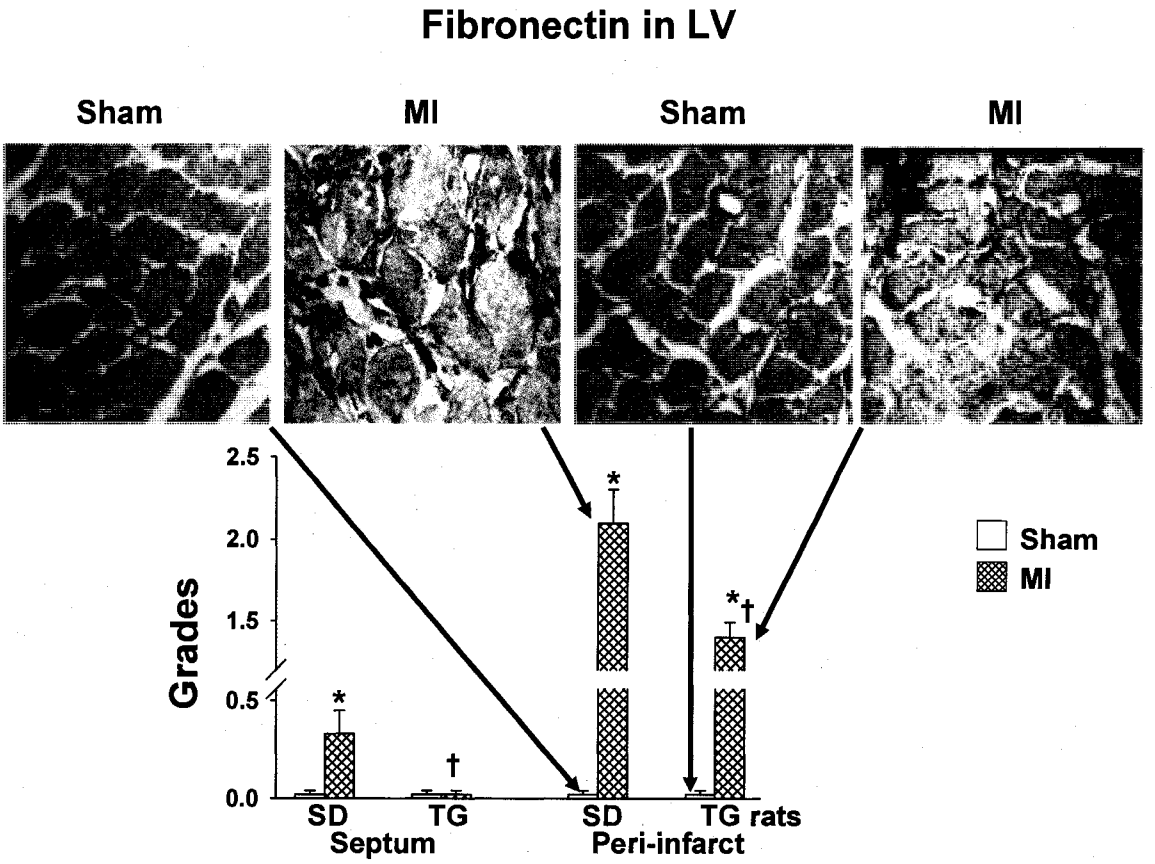

Fig. 6: Fibronectin by immunohistochemistry (on top, images at magnification X200) in the LV of sham and MI groups of SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p < 0.05$ vs sham (SD and TG rats); † $p < 0.05$ vs MI (SD rats).

3.6. Cardiac aldosterone

Aldosterone content was markedly increased in the LV and RV after MI in SD rats but no increase was found in the TG rats at 8 weeks post-MI (Fig. 7). In Wistar rats, aldosterone was increased in the LV, but not in the RV of the vehicle treated rats at 6 weeks post-MI. Icv infusion of spironolactone prevented the MI-induced increase of LV aldosterone (Fig. 8).

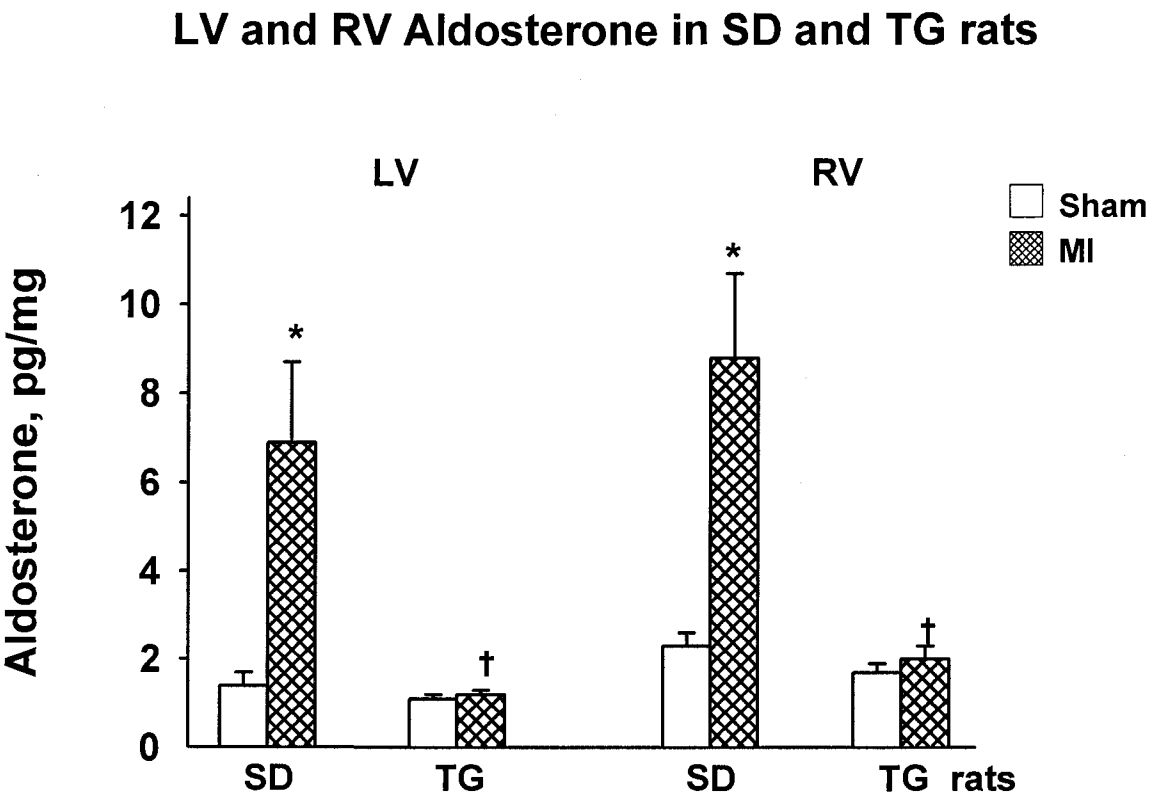

Fig. 7: Aldosterone in the non-infarcted part of LV and the RV of sham and MI groups of SD and TG rats at 8 weeks post-MI. Values are mean ± S.E.M. (Table shows number of rats per group). * $p < 0.05$ vs sham (SD and TG rats); † $p < 0.05$ vs MI (SD rats).

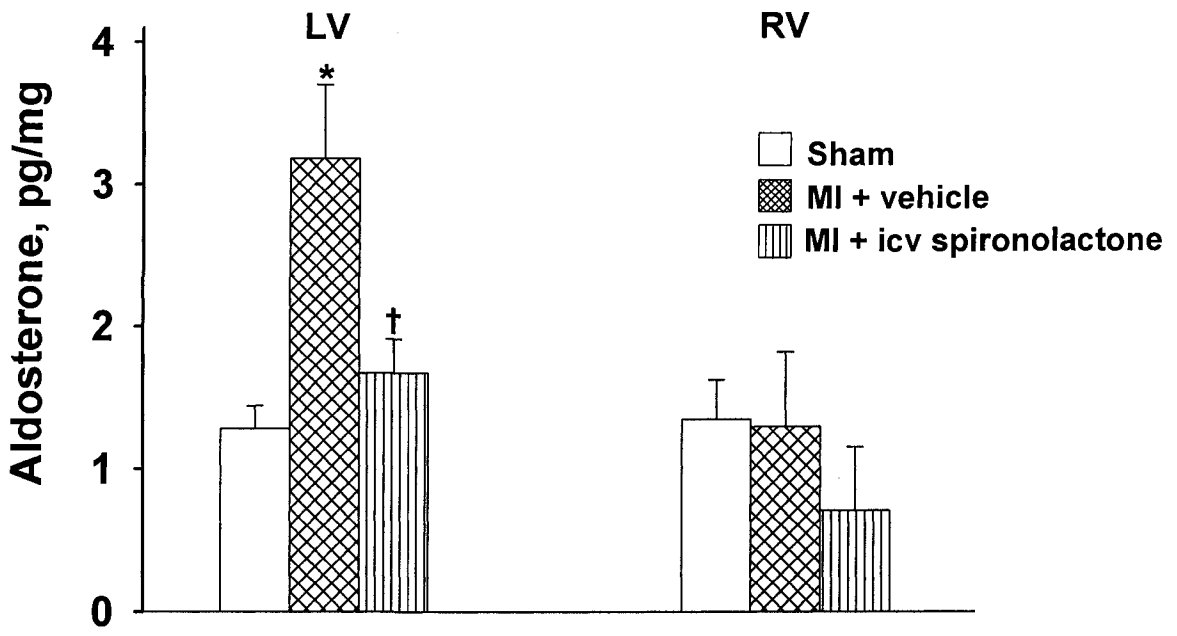

Fig. 8: Effects of icv infusion of spironolactone on aldosterone in the non-infarcted part of the LV and in the RV of Wistar rats at 6 weeks post-MI. Values are mean ± S.E.M. (number of rats 6-10 per group). * $p < 0.05$ vs sham; † $p < 0.05$ vs MI.

4. DISCUSSION

As significant new finding, the present study shows that a marked deficiency of brain AOGEN inhibits the development of major components of cardiac remodeling post-MI. This is the first study demonstrating the dependency of the increase in cardiac aldosterone post-MI on the central RAAS.

4.1. Cardiac remodeling and brain RAS post-MI

Significant cardiac remodeling had occurred in the SD rats at 8 weeks post-MI. The results are consistent with many previous studies regarding cardiac remodeling post-MI. In SD rats, RV and lung weights increased > twofold post-MI. The LV and RV internal circumferences increased by 45 and 30% and cardiomyocyte diameter increased by 30-45% in the septum and peri-infarct area of the LV, and in the RV post-MI. This (compensatory) cardiomyocyte hypertrophy is likely due to a loss of cardiomyocytes by necrosis in the infarct-zone [18] and by apoptosis [19] in the remaining LV and the RV. In the infarct, the necrosis leads to an inflammatory response and extensive fibrosis replaces the normal myocardium (18). On the other hand, in the non-infarcted LV and the RV, neurohormonal and hemodynamic stimuli contribute to interstitial and perivascular fibrosis [20]. Adhesion molecules of the extracellular matrix such as laminin and fibronectin also increased post-MI in the LV, consistent with previous reports [3,16,21].

Low activity of the brain RAS in the TG rats was associated with marked inhibition of most aspects of cardiac remodeling. The MI-induced increase in LV internal circumference was markedly inhibited and the increase in RV internal circumference fully prevented. This inhibition of the LV and RV dilation was associated with prevention of the cardiomyocyte hypertrophy in the septum and the RV and attenuation in the peri-infarct area of the LV. Similarly, the MI-induced increases in interstitial fibrosis, and of laminin and fibronectin were prevented in the septum and RV and attenuated in the peri-infarct area of LV. The increase of perivascular fibrosis was fully prevented in the LV and RV of TG rats. We previously reported (11) that this inhibition of cardiac remodeling in the TG rats is associated with marked blunting of the MI-induced decreases of LV

dP/dt_{max} and LV peak systolic pressure and increase of the LV end diastolic pressure, as well as prevention of sympathetic hyperactivity.

Chronic blockade of brain AT_1 receptors with icv losartan at the rate of 1 mg/kg/day for 8 weeks attenuated the LV dilation as assessed by the passive pressure-volume curve by 40% [6], whereas the LV dilation was inhibited by 70% in the TG rats [11]. The MI-induced increase of RV weight was attenuated by 25% with icv infusion of losartan [6] as compared to almost complete prevention in TG rats. At the 1 mg/kg/day rate of infusion, losartan produces incomplete blockade of central AT_1 receptors [11]. Increasing the rate of infusion likely would completely block central AT_1 receptors, but also causes a marked blockade of peripheral AT_1 receptors, due to leakage to the systemic circulation [22] obviously confounding the interpretation of the results. The present study in TG rats with specific marked blockade of the brain RAS establishes that Ang II locally produced in the brain plays a major role in cardiac remodeling post-MI.

4.2. Mechanisms connecting the brain and the heart

Post-MI, several mechanisms may contribute to activation of CNS pathways, which appear to mediate most of the cardiac remodeling occurring over subsequent weeks/ months. First, post-MI, cardiac vagal or sympathetic afferent fibers conveying mechanosensitive and chemosensitive information to the CNS are activated and through AT_1 receptors in the PVN, contribute to sympathetic excitation [23,24]. Secondly, post-MI the circulatory RAAS becomes activated [3,11,25] by e.g. a decrease in the blood pressure. Both Ang II and aldosterone may activate CNS pathways leading to e.g. sympathetic hyperactivity.

CNS pathways may contribute to cardiac remodeling post-MI via several mechanisms. Activation of the sympathetic nervous system may contribute through hemodynamic effects and direct cardiac effects. Increased renal sympathetic nerve activity likely plays a major role, influencing volume homeostasis either directly or indirectly by further activation of circulatory RAAS. Sympathetic hyperactivity can clearly be prevented by blockade of the brain RAAS, using either a transgenic approach [11], or icv infusions of spironolactone [2,4] or losartan [5]. Consistent with a role for renal sympathetic activity in the regulation of the circulatory RAAS post-MI, chronic activation of the circulatory RAAS post-MI appears to depend - at least in part - on the brain RAAS. At 6 weeks post-MI, Wistar rats showed a 50% increase in serum aldosterone, which was prevented by icv infusion of spironolactone [3]. In SD rats, plasma Ang II tended to be increased at 8 weeks post-MI, but not in transgenic rats [11]. Whether the more marked initial increase in plasma Ang II post-MI [25,26] is prevented, has not yet been assessed. Besides influencing volume homeostasis, circulating Ang II and aldosterone may enhance cardiac remodeling via a variety of mechanisms (for review, see reference 27). Activation of other peripheral mechanisms such as tumor necrosis factor (TNF)-α and vasopressin also appears to depend on CNS pathways involving the brain RAAS [2,28,29]. An increase of TNF-α levels in the plasma, heart and brain post-MI [30] may contribute to progressive cardiac remodeling [31] through effects on sympathetic activity [32] and cardiac Ang II [33]. Increases of plasma TNF-α levels post-MI were attenuated by icv and oral spironolactone [30,34] and of cardiac and brain TNF-α levels by oral spironolactone [30].

4.3. Central RAAS and cardiac aldosterone

In the present study, we show that activation of another peripheral mechanism - cardiac aldosterone - also depends on CNS pathways involving the brain RAAS. Aldosterone in the heart was increased markedly in SD rats in both the LV and RV at 8 weeks post-MI and to a less extent and only in the LV in Wistar rats at 6 weeks post-MI. Strain-differences in response to MI [35] or the timing post-MI (8 vs 6 weeks) may contribute to this difference. Silvestre et al [36] reported a fourfold increase of aldosterone production in-vitro and twofold increase of aldosterone synthase mRNA in the non-infarcted area of LV at 4 weeks post-MI in Wistar rats. Similar findings were reported by Xiu et al [25]. In addition, at 25 days post-MI mRNA levels for the steroidogenic acute regulatory protein (StAR) were increased twofold in the non-infarcted area of LV [37]. StAR plays a crucial role in the rate-limiting step of steroidogenesis by intramitochondrial transfer of cholesterol [38]. Considering the production of aldosterone and expression of aldosterone synthase and StAR mRNA in cultured neonatal rat cardiomyocytes [37], cardiomyocytes are a likely site for aldosterone synthesis in the heart.

The MI-induced increase of cardiac aldosterone was prevented in TG rats deficient in brain AOGEN and also in Wistar rats icv infused with spironolactone. This consistency across strains and different approaches to blockade of the brain RAAS, strongly supports the concept that the brain RAAS mediates the increase of cardiac aldosterone post-MI. The present studies do not address the actual mechanisms connecting the brain RAAS with cardiac aldosterone. However, it appears reasonable to speculate that activation of the circulatory or cardiac RAS is part of the pathway. Firstly,

Ang II increases StAR and aldosterone synthase mRNA levels and aldosterone production in cultured neonatal rat cardiomyocytes [37] and in the isolated perfused heart [39]. Secondly, administration of losartan prevents the MI-induced increases of cardiac StAR and aldosterone synthase mRNA and aldosterone production in Wistar rats [25,36,37]. These studies support the concept that increases in circulatory and/ or cardiac Ang II through AT_1 receptor stimulation may mediate the increases of StAR and aldosterone synthase and thereby cardiac aldosterone production post-MI. Further studies on the impact of blockade of the brain RAAS on the pattern of changes in plasma and cardiac Ang II post-MI [25,26,36] are needed to confirm this concept.

4.4. Conclusion

The present findings demonstrate the pivotal role of locally produced angiotensin II in the brain in cardiac remodeling post-MI. The brain renin-angiotensin-aldosterone system appears to activate a cascade of events, among others an increase in cardiac aldosterone, which plays a major role in cardiac remodeling.

4.5. Perspectives

Chronic peripheral administration of AT_1-receptor blockers or angiotensin converting enzyme (ACE) inhibitors in 'regular' doses causes a modest improvement in parameters of cardiac remodeling and dysfunction post-MI [25,40-42]. On the other hand, in TG rats deficient of brain AOGEN, cardiac remodeling (present study) and LV dysfunction post-MI [11] were markedly improved. At the doses of 5-30 mg/kg/day, peripheral administration of an AT_1 receptor blocker such as losartan causes some blockade of the brain RAS. Central AT_1 receptor blockade becomes more prominent at higher doses (for losartan up to 100 mg/kg/day) [43]. Similarly, peripheral administration

of not only lipophilic but also hydrophilic ACE inhibitors at high doses can cause marked blockade of ACE in brain areas inside the blood brain barrier [44]. However, effects of high doses on the heart post-MI relative to their CNS effects have not yet been studied. The beneficial effects of icv infusion of spironolactone at the low rate of 100 ng/hr on cardiac remodeling and dysfunction are similar to those seen in TG rats post-MI and are equal or better to those achieved with oral administration of spironolactone at high doses, 80 mg/kg/day [3]. Since spironolactone readily crosses the blood brain barrier [45], oral spironolactone likely also causes mineralocorticoid receptor blockade in the CNS. Based on these studies, one may speculate that oral treatment with AT_1-receptor blockers, ACE inhibitors or mineralocorticoid receptor blockers will provide additional benefits for outcome post-MI, if lipophilic compounds or high doses are used also causing blockade of the central RAAS.

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Chapter 5

GENERAL DISCUSSION

5 DISCUSSION

Two major aspects of the RAAS and cardiac remodeling have been evaluated in our studies. The role of aldosterone was studied in the high salt diet-model by oral administration of spironolactone in Wistar rats. The specific role of the brain RAAS in cardiac remodeling was studied in the MI-model by icv infusion of spironolactone in Wistar rats and by using TG rats deficient in brain angiotensinogen.

5.1 ROLE OF ALDOSTERONE IN HIGH SALT-INDUCED CARDIAC HYPERTROPHY AND FIBROSIS

The major findings of our study are the following: First, high salt intake initially only increases LV weight and cardiomyocyte cross-sectional diameter, but more prolonged high salt diet also increases LV as well as RV collagen, and interstitial and perivascular fibrosis. Second, spironolactone fully prevents the rise in LV weight and cardiomyocyte hypertrophy and the increases in collagen, as well as interstitial and perivascular fibrosis in both the LV and RV after chronic high salt intake. We used high salt diet containing 8% NaCl. Lower concentrations of NaCl (1- 4%) also induce cardiac hypertrophy and fibrosis, but these may require longer duration to manifest their effects (Contreras et al 2000, Cordaillat et al 2005, Dvorak et al 2004, Ye et al 2003). The actual % of NaCl can not be extrapolated to humans as rats are high metabolizers of drugs and require higher doses of drugs and possibly salt compared to humans (Matsubara 2002, Reagan-Shaw et al 2008, FDA 2002). The effect of high salt diet in rats is relevant to humans as high salt intake is a powerful determinant of LV hypertrophy in humans (Du Cailar et al 1992).

5.1.1 High Salt Induces Cardiac Hypertrophy

Increase of LV Weight: Four weeks of high salt intake significantly increased the LV weight in Wistar rats by 25%. These findings are consistent with the results of a meta-analysis of 9 studies involving 156 normotensive rats showing a significant increase of LV weight after 4 weeks of high salt intake, WMD (random) [95% CI], 29.8 [21.4, 38.1] ($p<0.00001$ vs control; Fig 5). Eight weeks of high salt intake significantly increased the LV weight in Wistar rats by 16% in our study. This is consistent with the results of a meta-analyses of 10 studies involving 206 normotensive rats showing a significant increase of LV weight at 6-8 weeks of high salt intake, WMD (random) [95% CI], 33.9 [24.7, 43.1] ($p< 0.00001$ vs control; Fig 5).

Increase of RV Weight: In our study, 4 weeks of high salt intake tended to increase the RV weight in Wistar rats by 8%. This is consistent with other studies reporting a mild non-significant increase of RV weight in Wistar rats by 6-17% (De Resende and Mill 2007, Fields et al 1991, Yuan and Leenen 1991). Eight week of high salt intake tended to increase RV weight in Wistar rats by 10% in our study. This is consistent with other studies reporting non-significant increase of RV weight in Wistar-Kyoto rats by 9-22% (Leenen et al 1998, and Yu et al 1998). Meta-analysis of 8 studies involving 138 normotensive rats showed a small significant increases of RV weight at 4 weeks of high salt intake, WMD (random) [95% CI], 4.4 [2.9, 6.0], ($p<0.0001$ vs control), but not at 6-8 weeks of high salt intake in 3 studies involving 72 normotensive rats (Fig 9).

Cardiomyocyte Hypertrophy: In our study, cardiac hypertrophy was associated with an increase of LV cardiomyocyte cross-sectional diameter (by 26% and 23%) and tendency of increase of RV cardiomyocyte cross-sectional diameter (by 8% and 7%) at 4 and 8

weeks respectively. Cudnoch-Jedrzejewska et al (2005) reported tendency of increase of LV and RV cardiomyocyte cross-sectional diameter by 4% with intake of 1% NaCl in drinking water for 4 weeks in SD rats. The mild effect on cardiomyocyte cross-sectional diameter in the Cudnoch-Jedrzejewska et al (2005) study could be due to species variation or less intake of salt (1% NaCl) compared to 8% salt diet in our study. Cardiac and cardiomyocyte hypertrophy after high salt intake may involve several mechanisms that will be discussed in sections 5.1.3 and 5.1.4.

5.1.2 High Salt Induces Cardiac Fibrosis

Four weeks of high salt intake tended to increase interstitial fibrosis (by 8% and 7%), perivascular fibrosis (by 16% and 10%) and collagen (3% and 40%) in the LV and RV, respectively. Ye et al (2002) reported 2- and 2.5-fold increases of myocardial fibrosis index after 4 weeks of intermediate (2.2% NaCl) and high (4.4% NaCl) sodium diet consumption in Wistar-Kyoto rats. Perivascular fibrosis (reflected as % of vessels displaying extension of fibrous tissue from perivascular region to the interstitium) also increased by ~15-fold after high sodium diet (4.4% NaCl) consumption in Wistar-Kyoto rats. Their results are in the same direction as ours, but a marked increase in the fibrosis in Ye et al (2002) study could be due to species variation.

Eight weeks of high salt intake clearly increased myocardial collagen and fibrosis. Myocardial collagen increased by 55% to 60% in the LV and RV. Myocardial fibrosis developed both in interstitial tissue and perivascular area, not only in the hypertrophied LV but also in the non-hypertrophied RV. Interstitial fibrosis increased by 40% to 45% and perivascular fibrosis by 20% to 35% in the LV and RV after high salt intake. These

findings are consistent with the study by Yu et al (1998) in Wistar-Kyoto rats on high salt diet for 8 weeks. They reported increases of interstitial fibrosis by 33% in the LV and by 20% the RV. In the intramyocardial coronary arteries of the LV, the fibrosis in-between the outer borders of tunica media and tunica adventitia also increased by 95% after high salt intake.

The time course of development of cardiac hypertrophy and fibrosis indicates that cardiac hypertrophy develops fairly rapidly and reaches a steady state within a few weeks, whereas fibrosis develops more slowly. The reason for this later appearance of fibrosis (at 8 weeks) than cardiac hypertrophy (4 weeks) is not clear. It could be due to different responses of fibroblasts and cardiomyocytes to aldosterone. Robert et al (1995) reported that aldosterone-salt treatment induces cardiac hypertrophy before (2 weeks) the increase of collagen (4 weeks). Secondly, increase of BP later on (8 weeks) may also contribute to cardiac fibrosis.

Both interstitial and perivascular fibrosis appeared to be less at 8 weeks than at 4 weeks of high salt intake in all the groups, including controls. Fibrosis was measured as % of the ventricular-marked field. With the increase of body weight at 8 weeks (compared to 4 weeks), the ventricular weight increases due to increase in volumes/sizes of cardiomyocytes and to a less extent of fibroblasts. Though we did not find an increase in cardiomyocyte cross-sectional diameter at 8 weeks compared to 4 weeks, the length of cardiomyocytes likely increased. Proportionate to this, there appears less increase of collagen at 8 weeks compared to 4 weeks. This shows as lower fibrosis (% of ventricle).

The possible causes of myocardial fibrosis in both the LV and RV induced by high salt diet will be discussed in sections 5.1.3 and 5.1.4.

5.1.3 Does Cardiac Hypertrophy and Fibrosis Occur Through Increase of BP?

Several mechanisms may contribute to salt-induced cardiac and cardiomyocyte hypertrophy. BP is an obvious one to consider. High salt intake for 4 weeks caused LVH without a significant increase in BP. However, 8 weeks of high salt intake did increase BP. A small rise in BP may contribute to LV fibrosis, but if hypertension is the primary factor, only the pressure-overloaded LV would manifest the fibrosis (Brilla et al 1990). In section 5.1.3-a, an effect of high salt on BP of normotensive and "salt resistant" strains will be reviewed. In section 5.1.4, the possible role of aldosterone in mediating cardiac hypertrophy and fibrosis on high salt intake will be reviewed.

5.1.3-a Does High Salt Intake Increase BP in Normotensive Rats?

Four weeks of high salt diet did not change MAP in Wistar rats in our study, which is consistent with previous studies from our lab in Wistar (Fields et al 1991, Yuan and Leenen 1991), Wistar-Kyoto (Leenen and Yuan 1998, Song et al 1997, Yuan and Leenen 1991) and Dahl R (Zhao et al 2000, Yuan and Leenen 1991) rats. Chronic consumption of high salt for 8 weeks induced a significant increase of MAP by 22% (systolic and diastolic BP increased by 18% and 28%) in Wistar rats in our study.

Meta-analysis of 16 studies involving 292 normotensive rats showed a miniscule rise in BP (mainly MAP) on 4 weeks of high salt intake, WMD (random) [95% CI], 3.0 [0.8, 5.1] ($p = 0.007$ vs control). After 8 weeks of high salt intake, a meta-analysis of 18 studies involving 397 normotensive rats showed a more clear increase of BP (mainly MAP), WMD (random) [95% CI], 13.8 [6.9, 20.7] ($p<0.00001$ vs control; Fig 11) and

the increase of BP was marked in Wistar rats, 20.4 [10.6, 30.2], ($p<0.00001$ vs control) and Wistar-Kyoto rats 9.8 [2.1, 17.6], ($p<0.01$ vs control) rats, but not in SD rats -4.6 [-11.9, 2.7], ($p = 0.21$ vs control) (Fig 13). To investigate whether systolic and diastolic BP change similarly, meta-analyses of systolic and diastolic BP after 8 weeks of high salt intake were also performed. Meta-analysis of systolic BP in 6 studies involving 118 Wistar rats and 3 studies involving 90 Wistar-Kyoto rats showed similar increases, WMD [95% CI], 16.8 [12.8, 20.8]($p<0.00001$ vs control) and 14.3 [0.3, 25.3]($p = 0.05$ vs control) respectively. Only two studies (Prada et al 2000, Carillo et al 2006) reporting changes in diastolic BP after chronic intake of high salt in normotensive rats were found. Meta-analysis of these studies involving 27 Wistar rats also showed a significant increase of diastolic BP after 8 weeks of high salt intake, WMD [95% CI], 16.4 [4.7, 28.1]($p = 0.006$ vs control).

In conclusion, chronic high salt intake increases MAP as well as systolic and diastolic BP in Wistar rats.

5.1.3-b Possible Mechanisms for Increase of BP on High Salt Intake and Effects of Spironolactone

RSNA was decreased after high salt intake in Wistar rats (Carillo et al 2006). Plasma norepinephrine and epinephrine and LV norepinephrine turnover rates did not change after high salt diet in Wistar-Kyoto rats (Leenen and Yuan 1998, Yuan and Leenen 1991). High salt diet did increase lumbar sympathetic nerve activity in Wistar rats (Carillo et al 2006). However, α_1- or β-adrenoceptor blockers failed to decrease the BP

after high salt intake in Wistar-Kyoto rats (Song et al 1997), indicating that sympathetic activity may not have a role in the increase of BP after high salt intake.

In our study, MAP was increased by 24 mmHg (from 113 mmHg with regular salt diet to 137 mmHg with high salt diet) after 8 weeks of high salt intake. Systolic BP was increased by 33 mmHg (from 128 ± 5 mmHg with regular salt diet to 151 ± 4 mmHg with high salt diet) and diastolic BP by 29 mmHg (from 102 ± 3 mmHg with regular salt diet to 131 ± 5 mmHg with high salt diet) after 8 weeks of high salt intake. The high salt intake-induced rise in MAP at 8 weeks was attenuated to 125 mmHg by spironolactone at both, 20 and 80 mg/kg/day. Systolic BP tended to be reduced to 145 ± 5 and 143 ± 7 and diastolic BP to 115 ± 4 and 116 ± 4 mmHg by 20 and 80 mg/kg/day of spironolactone respectively. One cannot exclude that larger doses of spironolactone may be required for control of BP.

The effect of spironolactone in attenuating the salt-induced rise in BP was likely mediated by blocking the MR which could have been activated by aldosterone, corticosterone or increase of 11β-HSD2. However, plasma corticosterone does not change and cardiac corticosterone decreases on high salt intake in Wistar rats (Gomez-Sanchez et al 2004, Gomez-Sanchez et al 2005b). In Dahl S rats, high salt intake decreases 11β-HSD2 activity and mRNA expression in mesenteric artery (Takeda 2003). The effect of high salt intake on 11β-HSD2 in Wistar rats has not yet been reported. Aldosterone is produced in mesenteric artery of Wistar rats (Takeda 2004). High salt intake increases the vascular production of aldosterone and aldosterone synthase mRNA in SHR (Takeda 2004), though its effect in normotensive rats has not been reported.

High salt diet from 2 weeks (Cordaillat et al 2005) to ~ 12 weeks (Zeng et al 2004, Zeng et al 2004a, Partovian et al 1998) increased medial thickness, medial to lumen ratio and cross sectional area of the aorta and renal, carotid and mesenteric arteries while the lumen diameter remains unchanged in Wistar, Wistar-Kyoto and SD rats. A positive correlation between BP and the cross-sectional area of the aorta, renal and mesenteric artery ($r \geq 0.92$, $p<0.01$; Zeng et al 2004) indicates that these structural changes may be associated with a rise of BP. Collagen contents of the aorta tended to increase after high salt intake in Wistar and Wistar-Kyoto rats (Partovian et al 1998, Takahashi et al 1991). Medial hypertrophy and fibrosis increase the stiffness of the artery that may contribute to a rise in systolic BP. However, such changes do not affect or even decrease diastolic BP. The increase of both systolic and diastolic BP after 8 weeks of high salt diet in our study indicates the involvement of other factors/mechanisms in addition to arterial stiffness. Moreover, total prevention of increase in collagen versus partial prevention of the increase in BP with the MR blocker, spironolactone also indicates the involvement of some other mechanism/factor in the rise of BP besides arterial fibrosis. It could be a non-genomic effect of aldosterone (Chai et al 2005). Aldosterone-induced vasoconstriction of coronary artery (in-vitro) in Wistar-Kyoto rats was prevented by AT_1 receptor antagonists (valsartan and candesartan), superoxide dismutase or NADPH oxidase inhibitor, apocynin, but not by spironolactone, indicating that aldosterone induces vasoconstriction via AT_1 receptors presumably via oxidative stress (Kushibiki et al 2007).

In summary, a variety of mechanisms such as vascular aldosterone synthesis, increase of collagen and medial thickness and stimulation of AT_1 receptors may contribute to increase of BP after high salt intake in normotensive rats.

5.1.4 Is Cardiac Hypertrophy and Fibrosis Aldosterone-Mediated?

5.1.4-a Is Cardiac Hypertrophy Aldosterone-Mediated?

Spironolactone completely prevented the high salt induced cardiac (our study, Cordaillat et al 2005) and cardiomyocyte hypertrophy (our study) indicating the effect of high salt diet on these parameters may be mediated through MR. Systemic infusion of aldosterone along with high salt diet induced LV hypertrophy (by 12%) and tended to increase RV weight (by 7%). Administration of spironolactone prevented the increase of LV and RV weights (Brilla and Weber 1992), indicating cardiac hypertrophy was mediated by MR. In neonatal rat cultured cardiomyocytes, aldosterone caused cardiomyocyte hypertrophy (measured as [^{3}H] leucine incorporation) and spironolactone completely blocked the effect of aldosterone, indicating that the effect of aldosterone on protein synthesis is mediated through the MR (Sato et al 1996).

5.1.4-b Is Cardiac Fibrosis Aldosterone-Mediated?

The appearance of myocardial fibrosis in both the LV and RV on high salt diet indicates some common factor in the ventricles that induces fibrosis in both the LV and RV. Spironolactone fully prevented the high salt-induced collagen and fibrosis indicating that the effect may be mediated through MR.

Subcutaneous infusion of aldosterone and salt (in drinking water) increased type I and III pro-collagen mRNA and cardiac fibrosis in both ventricles in SD rats (Iglarz et al 2004, Brilla and Weber 1992, Nehme et al 2006, Park and Schiffrin 2002, Ramires et al 1998, Robert et al 1995, Silvestre et al 2000). These increases of collagens and interstitial and perivascular fibrosis in the LV and or RV were prevented/ attenuated by spironolactone or eplerenone (Brilla and Weber 1992, Fujisawa et al 2003, Nehme et al 2006). Aldosterone stimulates collagen synthesis, measured by ^{3}H-proline incorporation in cultured adult rat cardiac fibroblasts. Blockade of synthesis of collagen in adult rat cardiac fibroblasts in culture (Brilla et al 1994) and prevention of the increase of cardiac fibrosis with spironolactone (our study) further supports the conclusion that MR activation mediates the increase of collagen after high salt intake.

5.1.4-c High Salt Diet and Cardiac RAAS

Spironolactone prevented the high salt induced increases of LV weight and cardiomyocyte diameter, collagen and fibrosis in LV and RV. The above findings are consistent with a role for MR in mediating the LV hypertrophic response as well as fibrosis in both ventricles in response to high salt diet. MR activation could occur by excess of aldosterone, corticosterone or 11β-HSD2. However cardiac corticosterone decreases and plasma corticosterone does not change after high salt intake in Wistar rats (Gomez-Sanchez et al 2004). The effect of high salt on 11β-HSD2 in Wistar rats has not yet been reported.

High salt decreases the activity of the plasma RAAS. PRA markedly decreased after 2-5 weeks (De Resende and Mill 2007, Ingert et al 2002, Rahmouni et al 2002, Zhao

et al 2000), and 8-10 weeks (Carillo et al 2006, da Costa Lima et al 1997, De Simone et al 1993, Prada et al 2000, Takeda et al 2000, Yu et al 1998) of high salt intake in normotensive rats. High salt diet also decreases plasma levels of Ang II (Coelho et al 2006, Carillo et al 2006), Ang I (Ingert et al 2002, Zhao et al 2000) and aldosterone (Takeda et al 2000, Gomez-Sanchez et al 2004) in normotensive rats. Contrary to the decrease in activity of circulatory RAAS, 2 weeks of high salt consumption increased cardiac ACE mRNA by 2-fold and ACE activity by 3-fold in Wistar-Kyoto rats (Kreutz et al 1995). Cardiac Ang II tended to increase after high salt intake in Wistar rats at 4 weeks (De Resende and Mill 2007), Wistar-Kyoto rats at 2 weeks (Leenen and Yuan 1998) and Dahl R rats at 5 weeks (Zhao et al 2000). Cardiac expression of AT_1 receptor mRNA increased by 2-fold after 8 weeks administration of high salt in drinking water (0.9% NaCl) (Takeda et al 2000) and in diet (8% NaCl) (Zhu et al 2004) and tended to increase after 4 weeks of high salt intake in Wistar rats (De Resende and Mill 2007). The cardiac expression of AT_2 receptor mRNA remained unchanged after 4 (De Resende and Mill 2007) and 8 weeks of high salt intake (Zhu et al 2004). Cardiac aldosterone synthase activity, CYP11B2 mRNA and aldosterone were increased by 2-fold after 8 weeks administration of high salt (0.9% NaCl) in drinking water in Wistar-Kyoto rats (Takeda et al 2000).

The mechanisms involved in high salt-induced increases of cardiac Ang II and aldosterone are not yet understood. Expression of renin mRNA and activity and angiotensinogen mRNA have been detected in the hearts of normotensive rats (Sun et al 2001, Jurkovicova et al 2001), but the effects of high salt intake on these parameters have not yet been reported. Plasma Ang I is markedly decreased after high salt intake, but

cardiac Ang I remains unchanged in Dahl R rats (Zhao et al 2000). Increased cardiac ACE activity after high salt intake (Kreutz et al 1995) could increase the conversion of cardiac Ang I to Ang II. Ang II stimulates StAR and aldosterone synthase in cardiomyocytes and heart (Casal et al 2003, Silvestre et al 1998), resulting in increased aldosterone synthesis. Whether increased production of cardiac aldosterone on high salt diet is mediated through stimulation of AT_1 receptors has not yet been studied.

Ang II contributes to development of cardiac fibrosis, at least in part through the activation of NADPH oxidase, generation of reactive oxygen species and subsequent modulation of redox-sensitive signaling pathways (Griendling and FitzGerald 2003, 2003a). Of the 5 different NADPH oxidase forms, Nox2 is expressed in the heart. Administration of aldosterone (sc) or Ang II (sc) increased heart weight and myocardial NADPH oxidase activity, interstitial fibrosis, expression of collagen I and fibronectin in wild type mice, but not in Nox2-/- mice. Spironolactone inhibited the Ang II-induced cardiac changes in the wild type mice. This indicates that aldosterone and Ang II may induce cardiac remodeling by involving Nox2 oxidase (Johar et al 2006).

High salt and aldosterone treatment (6 weeks) increased LV weight, fibrosis, collagen 1 mRNA and collagen III mRNA and TNF-α mRNA, and NADPH oxidase activity in Wistar and SD rats. Administration of spironolactone attenuated these cardiac changes, indicating that cardioprotective effects of MR antagonist could also be through NADPH oxidase pathway (Nakano et al 2005, Sun et al 2002). The high salt-induced increases of BP, LV weight and fibrosis, and NADPH oxidase activity and decreases of PRA and plasma aldosterone in Dahl-S rats were attenuated by eplerenone, indicating

that the beneficial effects of MR antagonist are likely attributable, at least in part, to the attenuation of myocardial oxidative stress (Nagata e tal 2006).

5.1.5 Are Other Effects of Spironolactone Involved in Preventing the Cardiac Fibrosis and Hypertrophy?

High salt intake lowers activity of plasma RAAS (Coelho et al 2006, Carillo et al 2006, da Costa Lima et al 1997, De Simone et al 1993, Gomez-Sanchez et al 2004, Ingert et al 2002, Lima et al 2006, Prada et al 2000, Rahmouni et al 2002, Takeda et al 2000, Yu et al 1998, Zhao et al 2000) and renal effects of spironolactone should therefore be less on high versus regular salt diet. Indeed, no increase of urinary excretion of sodium, but marked increase in glomerular filtration pressure and filtration fraction occurs with administration of spironolactone to SD rats fed a high salt diet for 2 weeks (Cordaillat et al 2005). Diuretics such as hydrochlorthiazide increased the excretion of water and sodium as compared to control and also prevented the LVH after 8 weeks of high salt diet intake in Wistar-Kyoto rats (Mervaala et al 1994). As no parameter of sodium balance was measured in the present study, we cannot exclude a role for renal effects of spironolactone in prevention of high salt-induced LVH and cardiac fibrosis. Diuretics probably reduce high salt induced cardiac hypertrophy through improvement in hemodynamics.

In addition to blocking aldosterone, spironolactone also blocks the effects of other steroid hormones such as progesterone and testosterone (Weinberger et al 2002, Thum et al 2002, Zillich and Carter 2002). Medroxyprogesterone aggravated the perivascular fibrosis induced by aldosterone and salt treatment and blocked the inhibitory effects of

17β-estradiol on BP, perivascular fibrosis, and cardiomyocyte hypertrophy in female Wistar rats (Arias-Loza et al 2006). In an in-vitro study, progesterone inhibited fibroblast hypertrophy and ^{3}H-proline incorporation (Dubey et al 1998). Administration of testosterone induces cardiac (Koenig et al 1982, Nahrendorf et al 2003) and cardiomyocyte (Marsh et al 1998) hypertrophy. No parameter of progesterone or testosterone involvement was evaluated in our study.

5.1.6 Conclusion

We provide the first published evidence that chronic high salt diet (4 weeks) increases LV weight and cardiomyocyte cross-sectional diameter without any increase of BP, which are fully prevented by spironolactone. Prolonged use of high salt diet (8 weeks) did not further increase LV weight and cardiomyocyte cross-sectional diameter, but caused an increase in BP and induced interstitial and perivascular fibrosis and increased collagen in both the ventricles, which were prevented/ attenuated by spironolactone. Since high salt intake increases cardiac aldosterone and decreases cardiac corticosterone, these findings are consistent with the concept that cardiac aldosterone may be involved in these effects of high salt diet.

5.2 ROLE OF BRAIN RAAS IN CARDIAC REMODELING POST-MI

As significant new findings, our studies show that icv treatment with spironolactone inhibits cardiac remodeling post-MI and attenuates the decreases in LVPSP and LV dP/dt max and the increase in LVEDP to a similar extent or somewhat

better than oral treatment with spironolactone. The development of major components of cardiac remodeling post-MI was inhibited in TG rats with marked deficiency of brain angiotensinogen. MI-induced increase of cardiac aldosterone is prevented with icv infusion and oral administration of spironolactone and in TG rats deficient in brain angiotensinogen, indicating that the increase in cardiac aldosterone post-MI is dependent on the central RAAS.

5.2.1 Cardiac Remodeling Post-MI

Significant cardiac remodeling had occurred in Wistar rats at 6 weeks and in SD rats at 8 weeks post-MI.

RV weight was increased by 2-fold in Wistar rats at 6 weeks and in SD rats at 8 weeks post-MI. The results are consistent with previous studies reporting increase of RV and/ or cardiac weight at 6-8 weeks post MI in Wistar rats (Fraccarollo et al 2003, Hu et al 1996, Hu et al 2001, Leenen et al 1999, Leenen et al 1999a, Milanez et al 1997, Michel et al 1988, Nawata et al 1999, Sandmann et al 2006, Tan et al 2004, Zelis et al 1994) and SD rats (Geng et al 2006, Ocaranza et al 2006, Schieffer et al 1994, Wang et al 2004). Other studies also reported an increase of RV or cardiac weight at 4 weeks (28 studies) and at $\geq$ 12 weeks (8 studies) post-MI. The internal circumferences of the LV and RV increased by 1.5-2 fold both in Wistar at 6 weeks and in SD rats at 8 weeks post-MI. Others also found increase of LV internal circumference in Wistar rats (Kalkman et al 1995, Michel et al 1988, Sandmann et al 2006) and SD rats (Cittadini et al 2003, Delyani et al 2001, Ocaranza et al 2006) post-MI.

Cardiomyocyte diameter increased by ~1.5-fold in the septum and peri-infarct area of the LV, and in the RV post-MI, both in Wistar and SD rats in our studies. LV cardiomyocyte size increased by 1.5-fold in Wistar rats at 6 weeks post-MI (Nawata et al 1999) and by ~2-fold in Wistar rats (Enomoto et al 2005 and Matsumoto et al 2004) and SD rats (Dedkov et al 2007, Milik et al 2006) at 4 weeks post-MI. This (compensatory) cardiomyocyte hypertrophy is likely due to a loss of cardiomyocytes by necrosis in the infarct-zone (Sun and Weber 2000) and by apoptosis (Takemura and Fujiwara 2004, Takeda et al 2007) in the remaining LV and RV. MI-induced LV cardiomyocyte hypertrophy could have increased the LV weight post-MI as cardiomyocytes constitute about 80% weight of the heart. However, due to loss (infarct) of a significant portion of the LV after myocardial infarction, on balance, there was no or little increase in LV weight (~ 5%) in our studies.

A marked increase of interstitial and perivascular fibrosis was found in the septum and peri-infarct area of the LV (1.5-fold and 3-fold) and the RV (~20%), both in Wistar and SD rats post-MI in our studies. Significant increases in LV interstitial and / or perivascular fibrosis have been reported in Wistar rats (Enomoto et al 2005, Kalkman et al 1995, Masson et al 2004, Matsumoto et al 2004, Michel et al 1988, Nakamura et al 2004, Nawata et al 1999, Sandmann et al 2006, Silvestre et al 1999, Takeda et al 2007) and SD rats (Cittadini et al 2002, Cittadini et al 2003, Dedkov et al 2007, Delyani et al 2001, Geng et al 2006, Milik et al 2006, Schieffer et al 1994, Schieffer et al 1995, Takeda et al 2007) post-MI. RV also showed significant increase of interstitial fibrosis in Wistar rats post-MI (Kalkman et al 1995). Collagen I and III mRNA increased in the non-infarcted LV in Wistar rats (Enomoto et al 2005, Hanatani et al 1998, Matsmoto et al

2004, Nawata et al 1999, Tekeuchi et al 1999) and SD rats (Jin et al 2002, El-Sabban et al 2000) post-MI. Hydroxyproline contents of the non-infarcted LV and/ or the RV also increased in the Wistar rats (Fraccarollo et al 2003, Milanez et al 1997, Mills et al 2003) and SD rats (Cittadini et al 2002, Cittadini et al 2003) post-MI. In our study, visualization of the structure of fibrillar collagen under scanning electron microscope (SEM), showed a marked increase in collagen with dense weave and lattice like stretches in the peri-infarct zone of the LV in Wistar rats post-MI.

Adhesion molecules of the extracellular matrix such as fibronectin and laminin bind to collagen and provide structural support in the heart. Fibronectin is distributed homogenously in the extracellular spaces in which cardiomyocytes and collagen are located. Laminin is located in the basement membrane of the cardiomyocytes, fibroblasts and endothelial cells where it binds to collagen I, III and IV. We found significant increases of fibronectin expression in the non-infarcted LV in our studies in Wistar and SD rats. Hanatani et al (1998), Jin et al (2002), Nawata et al (1999), and Ulrich et al (1997) reported increased expression and mRNA of fibronectin in Wistar and SD rats post-MI. Laminin also increased post-MI in our studies in Wistar and SD rats. Morishita et al (1996) also reported increased expression of laminin in the non-infarcted LV after MI in SD rats.

The increases of cardiomyocyte diameter, interstitial and perivascular fibrosis and fibronectin were more marked in the peri-infarct area of the LV than the septum both in Wistar and SD rats post-MI. Ang I, Ang II, and ACE activity/expression, AT_1 receptor expression and TNF-α are also higher in the peri-infarct zone of the LV than the remote LV (septum) or RV post-MI (Dean et al 2006, Tan et al 2004, Irwin et al 1999). ACE and

TNF-α may increase the production of Ang II (Flesch et al 2003) in the peri-infarct zone of the LV. Ang II, through the involvement of AT_1 receptors may increase the production of aldosterone in the peri-infarct zone of the LV (Silvestre et al 1999) and thereby larger responses of fibroblasts and cardiomyocytes in inducing cardiomyocyte hypertropy and increasing extracellular matrix.

5.2.2 Cardiac Dysfunction Post-MI

At 6 weeks post-MI (MI size ~30%), in our study LVPSP was decreased by ~20 mmHg, MAP by 15 mmHg, and LV dP/dt max by 35% and LVEDP increased from 1 mmHg to 15 mmHg in Wistar rats . The results are consistent with previous studies (with large MI, >30%) reporting decreases of LVPSP (mean 22 mmHg, range 16-24 mmHg), MAP (mean 23 mmHg, range 10-30 mmHg), and LV dP/dt max (mean 38%, range 20-50%) and increase of mean LVEDP from 4 mmHg to 17 mmHg at 6-8 weeks post MI in Wistar rats (Fraccarollo et al 2003, Hu et al 1996, Hu et al 2001, Leenen et al 1999, Leenen et al 1999a, Milanez et al 1997, Michel et al 1988, Nawata et al 1999, Sandmann et al 2006, Tan et al 2004) and SD rats (Geng et al 2006, Ocaranza et al 2006, Wang et al 2004). With small MI (<30%), LVPSP was decreased by 18 mmHg and LVEDP increased from 3 mmHg to 7 mmHg in Wistar rats at 8 weeks post-MI (Tan et al 2004). Wang et al (2004) reported a non-significant deterioration of LV function in SD rats with small MI (<30%) at 8 weeks post-MI. Other studies also reported deterioration of LV function at 4 weeks (21 studies) and at ≥ 12 weeks (5 studies) post-MI in normotensive rats.

5.2.3 Mechanisms Connecting the Heart with the Brain

Post-MI, several mechanisms may contribute to activation of CNS pathways, which appear to mediate most of the cardiac remodeling occurring over subsequent weeks/ months. Firstly, cardiac vagal or sympathetic afferent fibers conveying mechanosensitive and chemosensitive information to the CNS are activated post-MI and through AT_1 receptors in the PVN, contribute to sympathetic excitation (Ma et al 1997, Zhu et al 2004). Secondly, circulatory RAAS becomes activated post-MI with an increase in PRA and plasma Ang II (Leenen et al 1999a, Schunkert et al 1993). Ang II may stimulate aldosterone secretion from the adrenals. Both Ang II and aldosterone may activate CNS pathways. The activation of plasma Ang II starts at 6 h post-MI and remains significantly high until 2 months (Leenen et al 1999a). Lindley et al (2004) suggested that increased plasma Ang II post-MI may activate AT_1 receptors in circumventricular organs such as the SFO or OVLT, which signal downstream nuclei such as PVN and SON, activating sympathetic excitatory pathways.

No increase in plasma aldosterone appears to occur until 4 weeks post-MI though subsequent increases were found from 6 to 20 weeks Post-MI (Fig 4, Meta-Analysis). Central MR involved in cardiovascular regulation may be activated by circulating aldosterone acting on MR in periventricular or circumventricular areas (Gomez-Sanchez 1997). Aldosterone increases the binding of Ang II to AT_1 receptors in the PVN (De Nicola et al 1993) and also upregulates the expression of ACE (Harda et al 2001). Aldosterone may therefore amplify the influence of circulating and locally produced Ang II in the brain.

MI and heart failure are characterized by high circulating levels of pro-inflammatory cytokines such as TNF-α. These are markers of disease severity and a harbinger of adverse outcome (Francis et al 2002, Feldman et al 2000, Schultz et al 2004). Cytokines are too large to readily cross the blood brain barrier. It has been proposed that circulating cytokines may signal the brain through activation of sensory neurons in the circumventricular organs and through the activation of visceral sensory afferent nerves, particularly the vagus (Buller 2001, Goehler et al 1997). Cytokines could also activate receptors on perivascular and endothelial cells of blood brain barrier to induce cyclooxygenase-2 activity and synthesis of prostaglandin E_2 (PGE_2) (Engblom et al 2002, Rivest et al 2000). PGE_2 enters the brain and may activate hypothalamic neurons mediating neuroendocrine and sympathetic functions (Engblom et al 2002, Rivest et al 2000). Administration of the cytokine inhibitor, etanercept (1 mg/kg/day intra-peritoneal), lowered the MI-induced increases of plasma TNF-α and IL-β levels, as well as COX-2 expression in the penetrating vessels of the PVN, CSF levels of PGE_2 and Fra-Li-positive PVN neurons (Kang et al 2006).

5.2.4-a Icv Infusion of Spironolactone and Cardiac Remodeling Post-MI

The effects of central MR blockade on cardiac remodeling have not been previously described. Central treatment with spironolactone substantially attenuated the fall of LVPSP and LV dP/dt max and the increase of LVEDP in Wistar rats. This was confirmed by another study from our lab, showing marked improvement of cardiac function after MI, reflected as higher LVPSP, LV dP/dt max and lower LVEDP with icv infusion of spironolactone (Huang and Leenen 2005). Francis et al (2001a) reported a

non-significant decrease in the LVEDP as assessed by PE-50 catheter after 4 weeks treatment with icv spironolactone. However, Francis et al (2001a) measured LVEDP by using a polyethylene catheter and a regular pressure transducer under pentobarbital anesthesia at 4 weeks post-MI. In our and Huang and Leenen (2005) studies, hemodynamics were measured using high-fidelity micromanometer (Millar) catheter under minimal level of halothane-anesthesia. Different infarct sizes may also play a role. The infarct size was 47% in Francis et al (2001a) study vs ~30% in our and Huang and Leenen (2005) studies. Central blockade may be less effective in improving cardiac function in case of severe damage of the LV by a large MI.

Icv spironolactone inhibited the MI-induced increases in LV and RV internal circumference and in RV weight, prevented the increase of cardiomyocyte diameter in the septum and the RV and attenuated cardiomyocyte hypertrophy in the peri-infarct zone. The increases in interstitial and perivascular fibrosis and adhesion molecules were largely prevented by icv spironolactone in the septum and the RV and attenuated in the peri-infarct zone of the LV. The MI-induced increases of dense weave and lattice like stretches in the non-infarcted LV, visualized by SEM, were also largely prevented in Wistar rats post-MI.

5.2.4-b Oral Treatment of Spironolactone and Cardiac Remodeling Post-MI

In Wistar rats, oral treatment with spironolactone significantly improved LV function at 6 weeks post-MI and was remarkably effective in inhibiting most aspects of cardiac remodeling, which is consistent with previous studies. MR antagonists, spironolactone, canrenone and eplerenone improved the MI-induced increases of cardiac

weight and LVEDP and decreases of MAP, LVPSP and LVdP/dt max or min (De Resende et al 2006, Enomoto et al 2005, Fraccarollo et al 2003, Masson et al 2004, Milanez et al 1997, Matsumoto et al 2004, Mill et al 2003, Milliez et al 2005, Takeda et al 2007).

Oral spironolactone inhibited the MI-induced increases in LV and RV internal circumference. This inhibition of the LV and RV dilation was associated with prevention of the cardiomyocyte hypertrophy in the septum and the RV and attenuation in the peri-infarct zone of the LV. MI-induced increase of cardiomyocyte cross-sectional area was fully prevented with canrenone or spironolactone (Matsumoto et al 2004) and inhibited with eplerenone (Enomoto et al 2005). Spironolactone significantly prevented the MI-induced increase of interstitial and perivascular fibrosis in the LV and RV in Wistar rats. This is consistent with previous studies reporting prevention/ attenuation of the MI-induced increases in interstitial and perivascular fibrosis and hydroxyproline contents in the non-infarcted part of the LV and the RV with oral administration of MR antagonists such as spironolactone, canrenone or eplerenone (Cittadini et al 2002, Cittadini et al 2003, Delyani et al 2001, Enomoto et al 2005, Fraccarollo et al 2003, Masson et al 2004, Milanez et al 1997, Matsumoto et al 2004, Silvestre et al 1999, Takeda et al 2007).

The present study is the first one evaluating the effects of a MR antagonist on collagen structure by SEM and on adhesion molecules post-MI. Oral spironolactone markedly inhibited the MI-induced increase in dense weave and lattice like stretches of collagen in the peri-infarct zone of the LV, visualized by SEM and the normal web and strut pattern persisted. Oral spironolactone significantly attenuated laminin and fibronectin accumulation in the septal and peri-infarct zones of the LV.

Comparison of the effects of oral vs icv treatment with spironolactone on cardiac remodeling and LV dysfunction in Wistar rats shows overall a similar pattern of responses. Both treatments substantially improved, but did not normalize LV function. Even if cardiac remodeling would be fully prevented, one may expect some LV dysfunction to persist related to the loss of myocardium by the MI *per se*. Interestingly, icv spironolactone was significantly better compared to oral spironolactone in improving MI-induced changes in hemodynamics (LVEDP, LVPSP and LV dP/dt max), LV wall thickness and interstitial fibrosis in the infarct. A better effect of icv spironolactone could be due to specific central effects. Spironolactone crosses the blood brain barrier (Schimiedek et al 1973), but larger oral doses may be required to obtain the central effects achieved with icv spironolactone. Alternatively, large doses may have other peripheral effects (in addition to blocking the MR) such as blocking testosterone and progesterone (discussed in 5.1.5), possibly adversely affecting LV function. Both oral and icv spironolactone did not decrease infarct size, because these drugs were administered 1 to 3 days after the MI when most of pathological chages had already occurred/set in the heart. Drugs have to be administered before the MI to evaluate the effect of these drugs on the MI size, if these really have.

5.2.4-c Cardiac Remodeling Post-MI in Transgenic Rats Deficient in Brain Angiotensinogen

Low activity of the brain RAS in the TG rats was associated with marked inhibition of most aspects of cardiac remodeling. The MI-induced increase in LV internal circumference was markedly inhibited and the increase in RV internal circumference

fully prevented. This inhibition of the LV and RV dilation was associated with prevention of the cardiomyocyte hypertrophy in the septum and the RV and attenuation in the peri-infarct area of the LV. The MI-induced increase of lung weight was attenuated. Similarly, the MI-induced increases in interstitial fibrosis, and of laminin and fibronectin were prevented in the septum and RV and attenuated in the peri-infarct area of LV. The increase of perivascular fibrosis was fully prevented in the LV and RV of TG rats. Our lab previously reported (Wang et al 2004) that this inhibition of cardiac remodeling in the TG rats is associated with marked blunting of the MI-induced decreases of LV dP/dt max and LVPSP and increase of the LVEDP, as well as prevention of sympathetic hyperactivity. The effects of icv and oral spironolactone in Wistar rats and TG rats deficient in brain angiotensins were less in the peri-infarct area of the LV than the septum or the RV, possibly due to more marked activation of RAAS and TNF-α in the peri-infarct area than the septum and the RV (Discussed in section 5.2.1).

Chronic blockade of brain AT_1 receptors with icv losartan at the rate of 1 mg/kg/day for 8 weeks attenuated the LV dilation as assessed by the passive pressure-volume curve by 40% (Leenen et al 1999), whereas the LV dilation was inhibited by 70% in the TG rats (Wang et al 2004). The MI-induced increase of RV weight was attenuated by 25% with icv infusion of losartan (Leenen et al 1999) as compared to almost complete prevention in TG rats. At the 1 mg/kg/day rate of icv infusion, losartan produces incomplete blockade of central AT_1 receptors (Wang et al 2004). Increasing the rate of icv infusion likely would completely block central AT_1 receptors, but also causes a marked blockade of peripheral AT_1 receptors, due to leakage to the systemic circulation (Kawano et al 1994) obviously confounding the interpretation of the results. The present

study in TG rats with specific marked blockade of the brain RAS establishes that Ang II locally produced in the brain plays a major role in cardiac remodeling post-MI.

5.2.5 Central versus Peripheral Effects of Spironolactone

Leakage of spironolactone from the CNS into the circulation causing peripheral MR blockade, very unlikely explains the effects of central infusions at ~6 ug/kg/day considering that icv doses of spironolactone are 3,000-13,000 times lower than the 20-80 mg/kg/day of doses used orally (Silvestre et al 1999). ^{3}H-labelled canrenone readily penetrates the blood brain barrier in dogs (Schmiedek et al 1973). In rats with CHF post-MI, intra-carotid injection of spironolactone lowered the increased neuronal activity in the PVN (Zhang et al 2002), indicating that peripheral administration or circulating spironolactone may exert direct central effects. In contrast to the central infusion, peripheral infusion at the low rate of 100 ng/h had during the first 2 weeks, no effect on sympathetic activity (Francis et al 2002), but did decrease sympathetic activity during more prolonged infusion (Francis et al 2001a) and these authors suggested that prolonged peripheral administration of spironolactone even in small doses can produce effects through central mechanisms. Oral spironolactone likely causes MR blockade in the CNS by crossing the blood brain barrier (Schmiedek et al 1973) and by direct central effects in the PVN (Zhang et al 2002).

5.2.6 Mechanisms Connecting the Brain with the Heart

CNS pathways may contribute to cardiac remodeling post-MI via several mechanisms.

Sympathetic Activity: Activation of the sympathetic nervous system may contribute through hemodynamic effects and direct cardiac effects. Increased renal sympathetic nerve activity may also play a major role, influencing volume homeostasis either directly or indirectly by further activation of circulatory RAAS.

In the present study, plasma norepinephrine was clearly elevated at 6 weeks post-MI in Wistar rats in the vehicle group and less in the groups treated with icv or oral spironolactone. The latter finding is similar to the effects of oral eplerenone (Kang et al 2006, Fraccarollo et al 2003). Oral treatment with canrenone markedly decreased myocardial norepinephrine contents compared with MI and sham rats (Cittadini et al 2003). These findings indicate that oral treatment with MR antagonists lowers sympathetic activity, possibly by entering the brain and producing central effects.

Central infusion of spironolactone normalized the increased sympathetic activity and blunted the arterial baroreflex (Francis et al 2001a, Huang and Leenen 2005), but not its intraperitoneal administration (same dose as used for icv) at 2 weeks post-MI (Francis et al 2002), indicating central action of spironolactone may be involved in lowering the sympathetic hyperactivity post-MI. It has not yet been evaluated whether post-MI circulation-derived or locally produced aldosterone or other MR agonists increase binding to MR in the brain

AT_1 receptor stimulation in the CNS appears to play a crucial role. Icv infusion of losartan, at doses devoid of effects when infused peripherally, prevents sympathetic hyperactivity and improves baroreflex function in rats post-MI (DiBona et al 1995, Zhang et al 1999). Blockade of forebrain AT_1 receptor with losartan attenuates the increased neuronal activity of PVN and decreases sympathetic nerve activity in rats with

CHF post-MI (Zhang et al 2002). Blocking the brain RAS by enalaprilat prevents sympathetic hyperactivity (Francis et al 2004), and injection of AT_1 receptor mRNA antisense into the PVN normalizes the enhanced cardiac sympathetic afferent and decreases resting RSNA (Zhu et al 2004). The transgenic rats specifically lacking brain angiotensinogen in the brain showed that Ang II locally produced in the brain play a major role in the CNS pathways leading to sympathetic hyperactivity post-MI (Wang et al 2004). These findings suggest that circulation-derived Ang II may act on SFO or OVLT, but that in the central pathways, locally produced Ang II acts as an essential neurotransmitter.

Circulatory RAAS: Consistent with a role for renal sympathetic activity in the regulation of the circulatory RAAS post-MI, chronic activation of the circulatory RAAS post-MI appears to depend - at least in part - on the brain RAAS. At 6 weeks post-MI, Wistar rats showed a 50% increase in serum aldosterone, which was prevented by oral treatment and icv infusion of spironolactone to a similar extent, indicating that the effect of oral spironolactone may be centrally-mediated. In SD rats, plasma Ang II tended to be increased at 8 weeks post-MI, but not in transgenic rats (Wang et al 2004). Besides influencing volume homeostasis, circulating Ang II and aldosterone may enhance cardiac remodeling via a variety of mechanisms (see Literature Review, section 1.4.3-a).

TNF--α: An increase of TNF-α levels in the plasma, heart and brain post-MI (Kang et al 2004) may contribute to progressive cardiac remodeling (Bozkurt et al 1998) through effects on sympathetic activity (Zhang et al 2003) and cardiac Ang II (Flesch et al 2003). Intraperitoneal administration of etanercept (TNF-α antagonist) prevented the MI-induced activation of PVN neurons and increase of plasma norepinephrine (Kang et al

2006). Increases of plasma TNF-α levels post-MI were attenuated by icv and oral spironolactone (Kang et al 2004, Francis et al 2003) and of cardiac and brain TNF-α levels by oral spironolactone (Kang et al 2004).

Vasopressin: Studies on changes in plasma vasopressin levels post-MI are inconsistent. Some reported an increase (e.g., Francis et al 2001), others reported no change (e.g., Naitoh et al 2002, Burrell et al 1998), in plasma vasopressin levels post-MI. However, despite no increase in plasma vasopressin post-MI, conivaptan, a vasopressin (V_{1a} and V_2) antagonist increased water excretion and decreased RV weight (Naitoh et al 2002) and OPC-31260, a vasopressin (V_2) antagonist increased water excretion (Burrell et al 1998). Icv infusion of a MR antagonist in rats with CHF post-MI also increased urine volume (Francis et al 2001a), consistent with a decrease in vasopressin release.

Cardiac aldosterone: In the present study, we show that activation of another peripheral mechanism - cardiac aldosterone - also depends on CNS pathways involving the brain RAAS. Aldosterone in the heart was increased markedly in SD rats in both the LV and RV at 8 weeks post-MI and to a less extent and only in the LV in Wistar rats at 6 weeks post-MI. Strain-differences in response to MI (Rudolph et al 2000) or the timing post-MI (8 vs 6 weeks) may contribute to this difference. In the heart, angiotensin II may also be locally produced, and its effects may be mediated through activation of local production of aldosterone.

Silvestre et al (1999) reported a 4-fold increase of aldosterone production in-vitro and 2-fold increase of aldosterone synthase mRNA in the non-infarcted area of LV at 4 weeks post-MI in Wistar rats. Similar findings were reported by Geng et al (2006) and Xiu et al (2002). In addition, at 25 days post-MI StAR mRNA was increased by 2-fold in

the non-infarcted area of LV (Casal et al 2003). StAR plays a crucial role in the rate-limiting step of steroidogenesis by intramitochondrial transfer of cholesterol (Stocco et al 1996). Considering the production of aldosterone and expression of aldosterone synthase and StAR mRNA in cultured neonatal rat cardiomyocytes (Silvestre et al 1998), cardiomyocytes are a likely site for aldosterone synthesis in the heart.

The MI-induced increase of cardiac aldosterone was prevented in TG rats deficient in brain angiotensinogen and also in Wistar rats icv infused with spironolactone. This consistency across strains and different approaches to blockade of the brain RAAS, strongly supports the concept that the brain RAAS mediates the increase of cardiac aldosterone post-MI. The present studies do not address the actual mechanisms connecting the brain RAAS with cardiac aldosterone. However, it appears reasonable to speculate that activation of the circulatory or cardiac RAS is part of the pathway. Firstly, Ang II increases StAR and aldosterone synthase mRNA levels and aldosterone production in cultured neonatal rat cardiomyocytes (Casal et al 2003) and in the isolated perfused heart (Silvestre et al 1998). Secondly, administration of losartan prevents the MI-induced increases of cardiac StAR and aldosterone synthase mRNA and aldosterone production in Wistar rats (Xiu et al 2002, Silvestre et al 1999, Casal et al 2003). These studies support the concept that increases in circulatory and/ or cardiac Ang II through AT_1 receptor stimulation may mediate the increases of StAR and aldosterone synthase and thereby cardiac aldosterone production post-MI. Further studies on the impact of blockade of the brain RAAS on the pattern of changes in plasma and cardiac Ang II post-MI (Xiu et al 2002, Leenen et al 1999a, Silvestre et al 1999) are needed to confirm this concept.

5.2.7 Conclusion

Since the beneficial effects of icv spironolactone at low doses on LV function and remodeling were equal or better to those achieved with oral administration at high doses, we propose that in addition to its other actions, aldosterone appears to activate CNS pathways influencing peripheral mechanisms involved in cardiac remodeling. In addition, locally produced angiotensin II play a pivotal role in the brain in cardiac remodeling post-MI. The brain RAAS appears to activate a cascade of events, among others an increase in circulatory and cardiac aldosterone, which plays a major role in cardiac remodeling.

5.2.8 Perspectives

Chronic peripheral administration of AT_1-receptor blockers or ACE inhibitors in 'regular' doses causes a modest improvement in parameters of cardiac remodeling and dysfunction post-MI (Ishiyama et al 2004, Xiu et al 2002, Matsumoto et al 2004, Ruzicka et al 1999, Nakamura et al 2003, Michel et al 1988). On the other hand, in TG rats deficient of brain angiotensinogen, cardiac remodeling (present study) and LV dysfunction post-MI (Wang et al 2004) were markedly improved. At the doses of 5-30 mg/kg/day, peripheral administration of an AT_1 receptor blocker such as losartan causes some blockade of the brain RAS. Central AT_1 receptor blockade becomes more prominent at higher doses (for losartan up to 100 mg/kg/day) (Wang et al 2003). Similarly, peripheral administration of not only lipophilic but also hydrophilic ACE inhibitors at high doses can cause marked blockade of ACE in brain areas inside the blood brain barrier (Tan et al 2005). However, effects of high doses on the heart post-MI relative to their CNS effects have not yet been studied. The beneficial effects of icv infusion of spironolactone at the low rate of 100 ng/h and oral treatment (80 mg/kg/day)

on cardiac remodeling and dysfunction in Wistar rats are similar to those seen in TG rats post-MI. Since spironolactone readily crosses the blood brain barrier (Schmiedek et al 1973) and circulating spironolactone can exert central effects (Zhang et al 2002), oral spironolactone likely also causes MR blockade in the CNS. Based on these studies, one may speculate that oral treatment with AT_1-receptor blockers, ACE inhibitors or MR blockers will provide additional benefits for outcome post-MI, if lipophilic compounds or high doses are used causing blockade of the central RAAS, in addition to peripheral blockade.

Chapter 6

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