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Regulation of angiotensin converting enzyme and angiotensin II type 1 receptor by 17ß-estradiol in female rats: implications following experimental myocardial infarction

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Regulation of angiotensin converting enzyme and angiotensin II type 1 receptor by 17β-estradiol in female rats: implications following experimental myocardial infarction

By

Stephanie A. Dean

This thesis is submitted to the School of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the Master of Science program
in Cellular and Molecular Medicine (Pharmacology)

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DEDICATION

For my parents, Ray and Pauline

CONTRIBUTION OF COLLABORATORS

All experiments were performed under the supervision of Frans H.H. Leenen and Dr. Edward R. O'Brien at the University of Ottawa Heart Institute. All experimental work outlined in Chapter 2 - Material and Methods was carried out by Stephanie Dean except for experimental myocardial infarction induction by coronary artery ligation which were performed by Hao Wang or Monir Ahmad. Left ventricular function measurement utilizing the Millar catheter was performed in collaboration with Monir Ahmad. Plasma renin activities, and plasma and LV angiotensin I and II determinations were performed by Roselyn White. ACE and AT_1R autoradiography were performed by Junhui Tan.

AUTHORIZATION

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ABSTRACT

The present studies tested the hypothesis that 17β-estradiol (E2) downregulates ACE and AT_1R in several tissues important to cardiovascular regulation, including the brain and heart, and that this downregulation attenuates the progression of LV dysfunction following myocardial infarction (MI). In Experiment 1, female Wistar rats were randomized into one of four groups: 1) sham-ovariectomized (OVX); 2) OVX+vehicle (veh); 3) OVX+E2 replacement at physiological levels and 4) OVX+high E2. Five weeks following OVX, ACE and AT_1R were increased 15-90% in the heart, several cardiovascular nuclei of the brain, kidney, abdominal aorta, adrenal and lung. These increases were prevented in all cases by E2 replacement at physiological levels and in most cases reversed to decreases by high E2. In Experiment 2, age-matched female Wistar rats underwent 1 of 3 treatments: no surgery (ovary-intact), OVX+veh treatment for two weeks, or OVX+high E2 treatment for two weeks. Rats were then randomly assigned to sham-operation or coronary artery ligation (MI). E2 status did not affect LV function in sham rats. In MI rats, the increases in LVEDP and -dP/dt and the decreases in LVPSP and +dP/dt were similar across MI groups. Plasma renin activity and plasma Ang I were significantly decreased and plasma Ang II tended to decrease in OVX+veh sham rats compared to ovary-intact sham rats. These parameters were further decreased in OVX+high E2 sham rats compared to ovary-intact sham rats. At 3 weeks post-MI, plasma RAS components were similar in ovary-intact and OVX+veh MI rats compared to their shams, whereas OVX+high E2 MI rats exhibited increased PRA, Ang I, and Ang II compared to their shams. Levels of plasma Ang II were not different across MI groups. In all 3 MI groups, LV ACE mRNA abundance and ACE activity were increased several fold compared to their shams, and to similar levels across MI rats. LV Ang II levels were

higher in OVX+veh and OVX+high E2 MI rats than ovary-intact MI rats. ACE and AT_1R were increased post-MI in most of the brain nuclei studied. The extent of the increases were largest (20-40%) in the OVX+high E2 MI group, somewhat less (10-15%) in the ovary-intact MI group and only modestly in the OVX+veh MI group, compared to their respective sham groups. Since in sham rats brain ACE and AT_1R densities increased in the OVX+veh group and decreased in the OVX+high E2 group as compared to the ovary-intact group, actual ACE and AT_1R densities in most brain nuclei were modestly higher (20%) in OVX+veh MI rats compared to the other two MI groups. Thus E2 deficiency upregulates and E2 excess downregulates ACE and AT_1R in many tissues of otherwise healthy female rats. However, E2 status does not affect the upregulation of ACE in the LV post-MI, and modulates by 20-25% ACE and AT_1R densities in brain nuclei post-MI. However, during the early post-MI phase, E2 status does not appear to confer any benefit or detriment to the development of LV dysfunction.

TABLE OF CONTENTS

DEDICATION i

CONTRIBUTION OF COLLABORATORS ii

AUTHORIZATION iii

ABSTRACT iv

TABLE OF CONTENTS vi

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiv

ACKNOWLEDGEMENTS xvi

CHAPTER 1: INTRODUCTION 1

1.0. Overview 2

1.1. The classical renin angiotensin system 2

1.1.0. Overview 3

1.1.1. Components of the RAS 4

1.2. Tissue renin angiotensin systems 13

1.2.0. Overview 13

1.2.1. Existence and function of cardiac RAS 14

1.2.2. Existence and function of brain RAS 17

1.2.3. Existence and function of kidney RAS 19

1.2.4. Existence and function of adrenal RAS 21

1.2.5. Existence and function of vascular RAS 22

1.3. Estrogen regulation of cardiovascular function 24

1.3.0. Overview 24

1.3.1. Regulation of cellular function by estrogens 24

1.3.1.1. Estrogens and estrogen receptors 24

1.3.1.2. Estrogen 'genomic' regulation 26

1.3.1.3. Selective estrogen receptor modulators 27

1.3.1.4. Estrogen 'non-genomic' regulation 28

1.3.1.5. Summary 29

1.3.2. Regulation of cardiovascular function by estrogens 29

1.3.2.1. Regulation of blood pressure, baroreflex function, and autonomic tone by estrogens 29

1.3.3.2. Regulation of cardiac function by estrogens 31

1.3.3. Regulation of the RAS by estrogens 32
1.3.3.0. Overview 32
1.3.3.1. Regulation of circulatory RAS by estrogens 32
1.3.3.2. Regulation of cardiac RAS by estrogens 34
1.3.3.3. Regulation of brain RAS by estrogens 35
1.3.3.4. Regulation of kidney RAS by estrogens 37
1.3.3.5. Regulation of adrenal RAS by estrogens 38
1.3.3.6. Regulation of vascular RAS by estrogens 39
1.3.3.7. Summary of effects of estrogens on RASs 40
1.4. LV dysfunction following myocardial infarction (post-MI) 40
1.4.1. Progression of LV dysfunction in rats post-MI 40
1.4.2. Regulation of the circulatory RAS in male rats post-MI 42
1.4.3. Upregulation of cardiac RAS in male rats post-MI 43
1.4.4. Upregulation of brain RAS in male rats post-MI 45
1.4.5. Effects of estrogens on LV dysfunction post-MI 46
1.5. Overview and rationale for the study 50
CHAPTER 2: MATERIALS & METHODS 52
2.0. Overview 53
2.1. Experimental Methods 58
2.1.1. Animals 58
2.1.2. Ovariectomy and pellet implantation 59
2.1.3. Coronary artery ligation 60
2.1.4.0 LV function measurments 61
2.1.4.1 LV dimensions and function by echocardiography 61
2.1.4.2 LV function by direct PE-50 catheterization 62
2.1.4.3 LV function by direct catheterization with Millar catheter 62
2.1.5. Plasma and tissue collection 63
2.1.6. ACE mRNA abundance by real-time RT-PCR 68
2.1.7. ACE activity by synthetic substrate cleavage and fluorimetric detection. 74
2.1.8. AT_1R binding density by autoradiography 75
2.1.9. ACE binding density by autoradiography 76
2.1.10. Plasma renin activity by radioimmunoassay 77
2.1.11. Plasma Ang I and II concentration by HPLC separation followed by radioimmunoassay 77

2.1.12. LV Ang I and II concentration by HPLC separation followed by radioimmunoassay 78
2.1.13. Statistical analysis 79
CHAPTER 3: RESULTS 80
3.0. Overview 81
3.1. Experiment 1: Regulation of ACE and AT_1R by 17β-estradiol in female rats 81
3.1.1. Body, uterus and heart weights 81
3.1.2. ACE activities and binding densities and AT_1R binding densities in the RV and LV 84
3.1.3. ACE and AT_1R binding densities in the brain 88
3.1.4. ACE mRNA abundance, activities, binding densities and AT_1R binding densities in kidney, abdominal aorta and adrenal 92
3.1.5. Plasma ACE activity and lung ACE activity and binding density 99
3.1.6. Summary 102
3.2. Experiment 2: Regulation of circulatory, cardiac, and brain renin-angiotensin systems by 17β-estradiol in female rats with MI-induced LV dysfunction 103
3.2.1. Survival rates 3 weeks post-MI 103
3.2.2. Body weights, uterus weights, infarct sizes and heart weights 3 weeks post-MI 103
3.2.3. LV dimensions and function by echocardiography 3 weeks post-MI 110
3.2.4. LV function by direct catheterization 3 weeks post-MI 112
3.2.5. Regulation of circulatory RAS by E2 in female rats 3 weeks post-MI 115
3.2.6. ACE mRNA abundance and activity, and ACE and AT_1R density in the RV and LV of female rats 3 weeks post-MI 117
3.2.7. LV Ang I and II concentrations in female rats 3 weeks post-MI 122
3.2.8. Hypothalamic ACE mRNA abundance and activity in female rats 3 weeks post-MI 126
3.2.9. ACE and AT_1R binding densities in brain nuclei of female rats 3 weeks post-MI 128
3.2.10. Relationship between ACE and AT_1R binding densities in brain nuclei and infarct size in female rats 3 weeks post-MI 132
3.2.11. Overall summary of Experiment 2a, 2b and 2c 135
CHAPTER 4: DISCUSSION 138
4.0. Overview 139

4.1. Regulation of abdominal aorta, adrenal and kidney tissue RASs by E2 status....... 139
4.2. Regulation of circulatory RAS by E2 status and MI... 143
4.3. Regulation of cardiac ACE and AT_1R by E2 status and MI 145
4.4. Regulation of brain ACE and AT_1R by E2 status and MI 150
4.5. Regulation of LV function and heart rate by E2... 152
4.6. Potential mechanisms of regulation of ACE and AT_1R by E2 153
4.7. Pathophysiological implications of regulation of RAS by E2 155
4.8. Conclusions.. 160
4.9. Perspectives.. 161
CHAPTER 5: REFERENCES 162

LIST OF TABLES

1.1. Effects of E2 deficiency and replacement on infarct size and LV function post-MI 49

3.1. Body and heart weights of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 82

3.2. ACE activity and binding densities and AT_1R binding densities in the RV and LV of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 86

3.3. ACE and AT_1R binding densities in brain nuclei in female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation 90

3.4. ACE mRNA abundance, activity and binding densities and AT_1R binding densities in the kidney, abdominal aorta, and adrenal of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 96

3.5. ACE activity in plasma, and ACE activity and binding densities in lung of female rats 2 and 5 weeks following Sham/OVX surgery and pellet implantation 100

3.6. Body weights of female rats 3 weeks post-MI 104

3.7. Cardiac function and dimension measured by echocardiography in female rats 3 weeks post-MI 112

3.8. Left ventricular function measured by direct catheterization in female rats 3 weeks post-MI 114

3.9. Circulatory renin angiotensin system activity in female rats 3 weeks post-MI 116

3.10. Percent increases in LV ACE activity 3 weeks post-MI according to E2 status 122

3.11. ACE and AT_1R binding densities in brain nuclei of female rats 3 weeks post-MI 131

3.12. Relationships between ACE and AT_1R densities in brain nuclei relative to infarct size 3 weeks post-MI 133

LIST OF FIGURES

1.1. Renin angiotensin system cascade 5

1.2. Amino acid sequences of angiotensin peptides 5

1.3. Brain areas within which the RAS regulates cardiovascular function 19

2.1. Flowchart of Experiment 1 protocol 54

2.2. Flowchart of Experiment 2a protocol 56

2.3. Flowchart of Experiment 2b protocol 57

2.4. Flowchart of Experiment 2c protocol 58

2.5. The regions of the heart as dissected for Experiment 2 67

2.6. Dissection of the hypothalamus 67

2.7. Representative agarose gels of PCR products of ACE and PGK real-time RT-PCR 72

2.8. Representative melting curve of samples assayed by real-time RT-PCR 72

2.9. Representative standard curve for PGK-1 73

3.1. Uterus weights of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 83

3.2. Representative autoradiographs of ACE and AT_1R binding densities in hearts of female rats 5 weeks following Sham/OVX surgery and pellet implantation 85

3.3. Changes in ACE activity and binding densities and AT_1R binding densities in the left and right ventricles of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 87

3.4. Representative autoradiographs of ACE and AT_1R binding densities in brain nuclei of female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation 89

3.5. Changes in ACE and AT_1R binding densities in brain nuclei of female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation 91

3.6. Representative autoradiographs of ACE binding densities in kidney, abdominal aorta, adrenal and lung 5 weeks following Sham/OVX surgery and E2 pellet implantation...... 94

3.7. Representative autoradiographs of AT_1R binding density in abdominal aorta and adrenal 5 weeks following Sham/OVX surgery and E2 pellet implantation 95

3.8. Changes in ACE mRNA abundance, activity and binding densities, and AT_1R binding densities in kidney of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 97

3.9. Changes in ACE activity and binding densities and AT1R binding densities in abdominal aorta and adrenal 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 98

3.10. Changes in ACE activity in plasma and ACE activity and binding densities in lung of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation 101
3.11. Uterus weights of female rats 3 weeks post-MI 105
3.12. LV wet weight and LV wet weight corrected for body weight 3 weeks post-MI 108
3.13. RV wet weights and wet weights corrected for body weight 3 weeks post-MI 109
3.14. Representative autoradiographs of ACE and AT_1R binding densities in hearts of ovary-intact female rats 3 weeks post-MI 119
3.15. ACE mRNA abundance and activity in the left and right ventricles of female rats 3 weeks post-MI 120
3.16. ACE and AT_1R densities in hearts of ovary-intact female rats 3 weeks post-MI 121
3.17. Angiotensin I concentrations in the left ventricle of female rats 3 weeks post-MI 124
3.18. Angiotensin II concentrations in the left ventricle of female rats 3 weeks post-MI 125
3.19. Ratio of angiotensin II / angiotensin I concentration in left ventricle of female rats 3 weeks post-MI 126
3.20. ACE mRNA abundance and activities in hypothalamic homogenates of female rats 3 weeks post-MI 127
3.21. Representative autoradiographs of ACE and AT_1R binding densities in brain nuclei of female rats 3 weeks post-MI 130
3.22. Relationship between AT_1R binding densities in the SFO and PVN and infarct size in female rats 3 weeks post-MI 134

LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
ACE2	angiotensin converting enzyme 2
Agt	angiotensinogen
Ang	angiotensin
ANOVA	analysis of variance
AP-1	activatory protein-1
AT_1R	angiotensin II type-1 receptor
AT_2R	angiotensin II type-2 receptor
AT_3R	angiotensin II type-3 receptor
AT_4R/IRAP	angiotensin type-4 receptor/insulin regulated aminopeptidase
AV3V	anteroventral third ventricle
AVP	arginine vasopressin
BBB	blood-brain barrier
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CK2	creatine kinase 2
CP	carboxypeptidase P
CVD	cardiovascular disease
CVO	circumventricular organ
DAG	diacylglycerol
E2	17β-estradiol
EB	estradiol benzoate
EE	ethinyl estradiol
eNOS	endothelial nitric oxide synthase
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
ERT	estrogen replacement therapy
EV	estradiol valerate

HHL	hippuryl-histidyl-leucine
HL	histidyl-leucine
HPLC	high performance liquid chromatography
HR	heart rate
icv	intracerebroventricular
ip	intraperitoneal
IP_3	inositol triphosphate
iv	intravenous
IVSd	intraventricular septum thickness in diastole
IVSs	intraventricular septum thickness in systole
LV	left ventricle
LVEDP	left ventricular end diastolic pressure
LVIDd	left ventricular internal dimension in diastole
LVIDs	left ventricular internal dimension in systole
LVPSP	left ventricular peak systolic pressure
MAP	mean arterial pressure
MI	myocardial infarction
MnPO	median preoptic nucleus
mRNA	messenger ribonucleic acid
NEP	neutral endopeptidase
NFκB	nuclear factor kappa B
NO	nitric oxide
NP	nitroprusside
OVLT	organum vasculosum laminae terminalis
OVX	ovariectomized
PE	phenylephrine
PEP	prolyl endopeptidase
PGK-1	phosphoglycerate kinase-1
p-inf	peri-infarct
POP	prolyl oligopeptidase
PRA	plasma renin activity

PVN	paraventricular nucleus
PWd	posterior wall thickness in diastole
PWs	posterior wall thickness in systole
RAS	renin angiotensin system
rLV	remote left ventricle
RPM	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
RV	right ventricle
sc	subcutaneous
SERM	selective estrogen receptor modulator
SFO	subfornical organ
SP-1	stimulatory protein-1
SP-3	stimulatory protein-3
TO	thimet oligopeptidase
tPA	tissue plasminogen activator
veh	vehicle
VSMCs	vascular smooth muscle cells
ZF	zona fasiculata
ZG	zona glomerulosa

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Chapter 1:

INTRODUCTION

1.0. Overview

Observational studies provide evidence that in women under age 50, the incidence of cardiovascular disease (CVD), including myocardial infarction and stroke, is lower than in men of similar age, but the incidence in women rises significantly around the time of menopause (Wenger et al., 1993; Kannel & Wilson, 1995). Moreover, the incidence of CVD is lower in pre-menopausal women than age-matched post-menopausal women (Kannel et al., 1976; Kannel & Wilson, 1995). In addition, an increased risk of CVD occurs in women after ovariectomy (OVX) compared to age-matched ovary-intact women (Colditz et al., 1987). These studies implicate female sex hormones, including estrogens, in the protection against CVD in women before menopause and suggest that estrogen replacement therapy (ERT) may reduce the risk of CVD post-menopausally. Indeed, several observational studies have described a lower incidence of cardiovascular morbidity and mortality in post-menopausal women taking ERT compared to their untreated counterparts (Pines et al., 1997; Heckbert et al., 1997; Stampfer et al., 1991). However, recent randomized, placebo-controlled clinical trials have suggested otherwise. The Women's Health Initiative study and the Heart and Estrogen/progestin Replacement Study report that taking ERT in the form of conjugated equine estrogens + progestin is associated with no difference in secondary CVD risk (Hulley et al., 1998) and increased risk of first-time stroke and fatal and non-fatal myocardial infarction (Anderson et al., 2004; Manson et al., 2003). These opposing results warrant further study into the effects of estrogens on the cardiovascular system and potential mechanisms of these effects.

ERT has a beneficial effect on the lipid profile in post-menopausal women (Adami et al., 1993; Hong et al., 1992), and attenuates the formation of atherosclerotic plaques

(Hong et al., 1992). Pre-menopausal women have a lower incidence of hypertension than post-menopausal women (Isles et al., 1992). Several studies report that ERT reduces blood pressure in normotensive (Luotola, 1983; Beljic et al., 1998; van Ittersum et al., 1998) and hypertensive (Mercuro et al., 1997; Manhem et al., 1998) post-menopausal women, but others report no effect (Akkad et al., 1997; The Writing Group for the PEPI trial, 1995). Women taking ERT exhibit reduced left ventricle (LV) mass and dimension compared to untreated women (Lim et al., 1999).

The renin-angiotensin system (RAS) plays an important role in the maintenance of normal cardiovascular homeostasis and in the pathogenesis of hypertension, and heart failure following myocardial infarction (MI) in humans and in experimental animal models of these diseases. Both the classical, circulatory RAS and local tissue RASs contribute to the development of LV dysfunction following MI. Previous studies have suggested that E2 can affect the activity of these RASs. This review will summarize the recent evidence for E2-mediated regulation of the activity of the circulatory and tissue RASs and the importance of this regulation to normal cardiovascular physiology and left ventricular dysfunction following myocardial infarction.

1.1. The classical renin angiotensin system

1.1.0. Overview

The study of the RAS began in 1898, when Tigerstedt and Bergman discovered that extracts from the rabbit renal cortex contained a substance that caused an acute rise in blood pressure when injected intravenously. They named this substance renin (Tigerstedt & Bergman, 1898). Forty years later, renin was recognized as an enzyme involved in the formation of Angiotensin II (Ang II), the actual active pressor agent (Page, 1939; Braun-

Menendez et al., 1940; Page & Helmer, 1940). The conversion of angiotensin I (Ang I) to Ang II by angiotensin converting enzyme (ACE) was characterized later (Lentz et al., 1956; Bumpus et al., 1957). The role of the circulatory RAS in the regulation of vascular resistance and body fluid homeostasis, and therefore blood pressure, has been well characterized in the past five decades. The classical, circulatory RAS cascade begins with the production and release into the bloodstream of the protein angiotensinogen (Agt), by the liver (Fig 1.1). This circulating Agt is cleaved by kidney-derived renin to form Ang I, which has no known biological activity. As the nascent Ang I passes through the pulmonary and vascular lumen it is cleaved into Ang II by ACE. Ang II is a potent vasoactive peptide which interacts with two of the four types of Ang receptors. While Ang II is the main active peptide from this cascade, Ang III, IV, and (1-7) are also bioactive peptides produced (Fig. 1.2). A more detailed description of each individual enzymatic and peptide component of the RAS follows.

1.1.1. Components of the RAS

Angiotensinogen. Agt is the sole protein precursor of all Ang peptides. The source of plasma Agt is the liver (Morris et al., 1979; Clauser et al., 1989). The levels of plasma Agt are controlled at the level of hepatic Agt transcription and translation, as secretion from the liver is constitutive (Hackenthal et al., 1988; Clauser et al., 1983). In other words, increased production of liver Agt leads to increased secretion into the plasma. The Agt promoter region contains elements responsive to glucocorticoids, estrogen and cyclic AMP (cAMP) (Ben-Ari et al., 1989; Feldmer et al., 1991; Narayanan et al., 2000). Liver Agt production is stimulated by estrogens, glucocorticoids, cAMP and Ang II (Nakamura et al., 1990; Klett et al., 1993).

Figure 1.1. Renin angiotensin system cascade. Abbreviations of enzymes in text. AVP: arginine vasopressin; Aldo: aldosterone. Adapted from Stroth & Unger, 1999. Used with permission.

	1 2 3 4 5 6 7 8 9 10 11 12 13 14	
Agt	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser- . . .	
Ang I (1-10)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	(not bioactive)
Ang (1-9)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His	(not bioactive)
Ang II (1-8)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	
Ang III (2-8)	Arg-Val-Tyr-Ile-His-Pro-Phe	
Ang IV (3-8)	Val-Tyr-Ile-His-Pro-Phe	
Ang (1-7)	Asp-Arg-Val-Tyr-Ile-His-Pro	
Ang (1-5)	Asp-Arg-Val-Tyr-Ile	(not bioactive)

Figure 1.2. Amino acid sequences of angiotensin peptides. Adapted from Campbell, 2003. Used with permission.

Renin. Renin is a single-chain aspartyl protease expressed in high amounts in the juxtaglomerular cells of the kidney (Gomez et al., 1990). Renin converts Agt to Ang I. The transcription of renin in the kidney is upregulated by isoproterenol (Wang et al., 1999) and cAMP (Horiuchi et al., 1991) and downregulated by Ang II (Nakamura et al., 1990). However, the concentration of renin in plasma is controlled mainly by the regulation of renin secretion from the kidney (Pratt et al., 1987; Galen et al., 1984). Renin secretion is influenced by blood pressure, plasma sodium concentration, sympathetic activity and several humoral and locally generated factors. Specifically, it is increased in response to hypotension, sodium depletion, an increase in sympathetic tone, and by local effects of prostaglandins and kinins (Brown et al., 1964; Huang et al., 2001; DiBona, 2000; Holmer et al., 1997; Cheng et al., 2002; Carretero & Beierwaltes, 1984). Renin secretion is inhibited by Ang II, hypertension, sodium loading, natriuretic peptides, and arginine vasopressin (AVP) (Kammerl et al., 2002; Brown et al., 1964; Narumi et al., 1987; Konrads et al., 1978).

Angiotensin converting enzyme/Kininase II. ACE is a dipeptidyl carboxypeptidase whose classic function is to convert Ang I to Ang II. ACE is also known as kininase II, as it inactivates the potent vasodilator bradykinin (Yang et al., 1970). ACE is expressed in high abundance in lung endothelium, but is present within the plasma membrane of all vascular endothelial cells (Danilov et al., 1994) and is expressed in vascular smooth muscle cells (VSMCs) (Battle et al., 1994; Cushman & Cheung, 1971). ACE is anchored in the plasma membrane with its catalytic site exposed extracellularly. 'ACE secretase', a partially characterized metalloprotease, cleaves ACE into a soluble form that remains catalytically active. This soluble ACE is present in plasma (Ramchandran & Sen, 1995).

Membrane-bound and soluble ACE are specifically inhibited by the ACE inhibitors typified by captopril and enalapril (Jerie, 1992).

ACE is regulated both at the level of mRNA transcription and stability, and at the level of cleavage-secretion from the plasma membrane. The promoter region of the ACE gene contains consensus or near-consensus response elements for stimulatory protein-1 (SP-1), glucocorticoids, activatory protein-1 (AP-1), AP-2, and cAMP (Shai et al., 1990; Kumar et al., 1991; Eyries et al., 2002). ACE mRNA and activity is increased in endothelial cells (ECs) by vascular endothelial growth factor by a tyrosine kinase-dependent mechanism (Saijonmaa et al., 2001). ACE mRNA abundance and activity are increased in rabbit ECs by β-adrenergic stimulation via a protein kinase A-, adenylyl cyclase-, Gs protein-dependent mechanism (Xavier-Neto et al., 1999). The glucocorticoid dexamethasone increases ACE mRNA abundance and stability and increases ACE activity in VSMCs (Fishel et al., 1995). Plasma ACE activity and tissue ACE expression are regulated by estrogens as discussed in detail in section 3.1.1.

The regulation of the cleavage-secretion (shedding) of ACE is not yet fully characterized. The PKC activator phorbol-12,13-dibutyrate (Ehlers et al., 1995), the mercurial compound 4-aminophenylmercuric acetate (Allinson et al., 2004), and pervanadate, an inhibitor of protein tyrosine phosphatases (Santhamma et al., 2004), all enhance ACE shedding. Casein kinase-2 (CK2) phosphorylates ACE and inhibits its shedding (Kohlstedt et al., 2002) and Compound 3, a hydroxamic acid-based inhibitor of metalloproteases, also inhibits ACE secretion (Eyries et al., 2001).

<u>Angiotensin converting enzyme 2 (ACE2).</u> A recent addition to the extended RAS family is ACE-related carboxypeptidase, or ACE2 (Donoghue et al., 2000; Tipnis et al.,

2000). ACE2 is a carboxymonopeptidase (Vickers et al., 2002), and like ACE, it is an ectoenzyme that can be cleaved from its plasma membrane position (Ramchandran & Sen, 1995; Woodman et al., 2000). ACE2 converts Ang I to the inactive Ang (1-9), precluding its conversion to Ang II, and degrades Ang II to Ang (1-7) (Fig. 1.1 and 1.2) (Donoghue et al., 2000; Vickers et al., 2002; Tipnis et al., 2000). In contrast to ACE, ACE2 activity is not inhibited by classic ACE inhibitors (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 contributes mainly to the production of Ang peptides in tissues, as it is expressed in the heart, kidney, and testis in endothelial, epithelial, and VSMCs (Crackower et al., 2002; Donoghue et al., 2000; Tipnis et al., 2000; Li et al., 2005). ACE2 knockout mice exhibit increased plasma Ang II levels in the absence of changes in Ang I levels and ACE expression, suggesting that ACE2 may regulate plasma Ang II levels by increasing its degradation (Crackower et al., 2002). Neither the transcriptional regulation nor the regulation of cleavage-secretion of ACE2 has been fully characterized.

Angiotensin effector peptides. The enzymatic components of the RAS, renin and ACE, produce the peptides that mediate the classical RAS effects. Several other non-RAS peptidases are putative Ang-forming enzymes, but most have been studied solely *in vitro*, and therefore their relative importance *in vivo* is not clearly defined. ACE is not the sole contributor of plasma Ang II, as ACE knockout mice have plasma levels of Ang II 30% of wild-type (Campbell et al., 2004). Thus non-ACE, non-renin enzymes are likely contributing to the production of plasma Ang II occurring under physiologic conditions.

Classically, *in vivo*, Agt is converted to Ang I by renin, and Ang I is then cleaved by ACE into Ang II (Fig. 1.1 & 1.2). Aminopeptidase A can then convert Ang II into the

bioactive Ang III (Sakura et al., 1983). The enzyme tonin, which is present in rat plasma (Johansen et al., 1987) has been shown *in vitro* to convert both Agt and Ang I to Ang II (Thibault & Genest, 1981). Tissue plasminogen activator (tPA), also present in rat plasma (Van Bennekum et al., 1993) can convert Agt and Ang I to Ang II (Dzau, 1989). Neutral endopeptidase (NEP), thimet oligopeptidase (TO), and prolyl endopeptidase (PEP) can cleave Ang I directly to the bioactive Ang (1-7) (Welches et al., 1993; Rice et al., 2004). The widely expressed aminopeptidase N/B can cleave Ang III into the bioactive Ang IV (Cesari et al., 2002). Ang II can be converted to Ang (1-7) by prolyl oligopeptidase (POP), carboxypeptidase P (CP) or ACE2 (Cesari et al., 2002; Tipnis et al., 2000; Rice et al., 2004). ACE or NEP can degrade Ang (1-7) into the inactive Ang (1-5) or other inactive fragments (Chappell et al., 1998; Chappell et al., 2001), and NEP can convert Ang (1-7) to Ang IV (Chappell et al., 2001). Four of these peptides are bioactive (Ang II, III, IV and (1-7)), so their interconversion has cellular/physiological consequences, depending on the expression of their receptors.

Angiotensin receptors. Ang peptides are potent ligands for at least five receptors: the AT_1 receptor (AT_1R), AT_2R, AT_3R, AT_4R, and Mas (Ang (1-7) receptor). These receptor types, for the most part, have distinct pharmacological profiles and signal transduction mechanisms. The description of the physiological effects of Ang receptor stimulation will be discussed in later sections according to tissue specificity.

AT_1R. The AT_1R subtype is a G-protein coupled seven transmembrane domain receptor that mediates most of the classical RAS effects. In rodents, but not humans, two isoforms of AT_1R exist: AT_{1A} and AT_{1B}. These AT_1R isoforms are 95% homologous at amino acid level (Inagami et al., 1994), and their affinities for Ang II and III, their main

agonists, and specific AT_1 antagonists are quite similar, but they are differentially distributed and regulated. High-affinity and specific blockade of both $AT_{1A}R$ and $AT_{1B}R$ can be achieved by the biphenylimidazoles typified by losartan (Chiu et al., 1989). Because of their similar pharmacology, the two subtypes will be discussed herein as a single entity (AT_1R) except where molecular biology techniques have distinguished between them. The signal transduction mechanisms activated by the AT_1R are well characterized. Via $G\alpha_{q/11}$, activation of AT_1R mediates the generation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by phospholipase Cβ or γ, the release of Ca^{2+} from intracellular stores and the activation of PKC (Marrero et al., 1994). Via the inhibitory $G\alpha_{i/o}$ protein, AT_1R inhibits adenylate cyclase (Inagami et al., 1992; Tian et al., 1996). Via $G\alpha_{12/13}$, AT_1R activates L-type Ca^{2+} channels (Maturana et al., 1999; Macrez et al., 1997). Through G-protein independent mechanisms, AT_1R also induces tyrosine phosphorylation of JAK and MAPK, and leads to an increase in expression of immediate early genes, such as *c-fos*, *c-jun* and *c-myc* (Marrero et al., 1995; Clark et al., 1992; Berk & Corson, 1997).

AT_1R is tightly regulated by Ang II. Ang II mediates rapid uncoupling of the G-protein from the receptor, reducing its affinity to Ang II (Boulay et al., 1994) and rapid, phosphorylation-dependent internalization of the receptor from the plasma membrane. In rat VSMCs this internalization occurs with a half-life of <2 minutes (Anderson et al., 1993). The regulation of AT_1R by Ang II also occurs via transcriptional mechanisms. In VSMCs, kidney mesangial cells and adrenal cortex, Ang II treatment inhibits transcription of and destabilizes AT_1R mRNA (Lassegue et al., 1995; Makita et al., 1992; Naville et al., 1993). In rats expressing brain-specific antisense towards Agt, brain AT_1R

is increased in brain regions inside the blood brain barrier (BBB) and decreased in areas outside the BBB (Monti et al., 2001). This suggests that a decrease in brain Ang peptides differentially modulates the brain AT_1R in a region-specific manner.

Several other substances also regulate AT_1R transcription. The AT_1R promoter contains consensus or near-consensus response elements for AP-1, SP-1, SP-3, glucocorticoids and cAMP (Holzmeister et al., 1997; Zhao et al., 2000a; Zhao et al., 2001a; Guo et al., 1995; Chen et al., 2002). In addition, norepinephrine, and nitric oxide (NO) decrease AT_1R levels (Du et al., 1996; Ichiki et al., 1998), and glucocorticoids, progesterone, mineralocorticoids all upregulate AT_1R (Nickenig et al., 2000; Della Bruna et al., 1995; Ichiki et al., 1998). Estrogens regulate the AT_1R in several tissues as discussed in detail in section 1.3.3.

$\underline{AT_2R}$. The AT_2R is a seven-transmembrane domain G-protein-coupled receptor with 32% homology with AT_1R (Mukoyama et al., 1993). Its main agonist is Ang II, and it is antagonized with high affinity by tetrahydroimidazopyrines, typified by PD-123319 (Chiu et al., 1989). AT_2R is widely expressed at high abundance in fetal tissue (Grady et al., 1991), but in adults, is limited to the adrenal medulla (Balla et al., 1991), heart (Wang et al., 1998b), kidney (Ozono et al., 1997), and specific brain regions (Reagan 1994). The signal transduction mechanisms activated by Ang II at the AT_2R include the production of NO, leading to increased production of cGMP (Gohlke et al., 1998), opening of the delayed rectifier K^+ channel (Huang et al., 1996), and inactivation of MAP kinases/ERKs (Horiuchi et al., 1998; Hansen et al., 2000). AT_2R activates phospholipase A2, and activates the Na^+/HCO_3^- symporter (Kohout & Rogers, 1995). AT_2R stimulation also increases stress proteins such as caspase-3, involved in the regulation of apoptosis

(Gallinat et al., 1999). Thus the events that occur upon activation of the AT_2R are for the most part opposite to the events following AT_1R stimulation. In further contrast to the AT_1R, the AT_2R does not internalize upon Ang II binding (Csikos et al., 1998). However, its transcription is downregulated by Ang II *in vitro* in neonatal rat cardiac myocytes and in mesenteric artery after systemic Ang II infusions in rats (Kijima et al., 1996; Bonnet et al., 2001). The AT_2R promoter region contains multiple responsive elements: AP-1, AP-2, nuclear factor kappa-B (NF-κB) and glucocorticoid and cAMP responsive elements (Murasawa et al., 1996; Ichiki et al., 1996). Norepinephrine and aldosterone downregulate AT_2R in cardiac myocytes and adrenal, respectively (Kijima et al., 1996; Matsubara & Inada, 1998; Wang et al., 1998a).

Angiotensin type 4 receptor/Insulin-regulated aminopeptidase. The AT_4R was first identified in membrane fractions from bovine adrenal and guinea pig hippocampus (Jarvis et al., 1992; Swanson et al., 1992; Harding et al., 1992), and binds Ang IV specifically and with high affinity (Swanson et al., 1992; Harding et al., 1992). AT_4R is also known as the membrane-bound insulin-regulated aminopeptidase (IRAP) (Albiston et al., 2001; Lew et al., 2003), and is expressed in brain, kidney, adrenal, heart and blood vessels (Moeller et al., 1995; Coleman et al., 1998; Harding et al., 1994; Wang et al., 1995a; Moeller et al., 1999). AT_4R/IRAP is not a typical receptor, but rather an enzyme: it specifically cleaves the N-terminal cysteine from oxytocin, arginine vasopressin (AVP), dynorphin, and other peptides (Lew et al., 2003). Ang IV inhibits the enzymatic activity of AT_4R/IRAP and thereby increases the half-life of its receptor's endogenous substrates (Albiston et al., 2001). The AT_4R/IRAP gene contains SP-1 and AP-2 promoter elements (Horio et al., 1999), but the study of its regulation has just begun.

Angiotensin (1-7) receptor (Mas). High-affinity binding sites for Ang (1-7) have been identified as the protein product of the *MAS1* proto-oncogene (Tallant et al., 1997; Santos et al., 2003). Mas is a seven-transmembrane domain G-coupled receptor (Zohn et al., 1998) that is expressed in the heart, forebrain and kidney (Metzger et al., 1995). Signaling mechanisms of Mas include the activation of phospholipase A2, which leads to increases in arachadonic acid (Andreatta-van Leyen et al., 1993), and prostacyclin, leading to increases in NO (Hilchey & Bell-Quilley, 1995). The regulation of Mas expression is still under investigation.

1.2. Tissue renin angiotensin systems

1.2.0 Overview

The classical view of the RAS has changed in the last 15-20 years. While the circulatory RAS is considered a key regulator of cardiovascular function, individual tissues contain local RASs that contain all the components required for the production and action of Ang II and other Ang peptides. Such tissues RASs exist within the kidney, heart, adrenal, blood vessel wall, and brain, and in general, are regulated separately from the circulatory RAS. However, the local systems cannot be considered completely autonomous. The activity of circulatory RAS components can affect the activity of the local systems, especially as ACE and Ang receptors are located, among other sites, at the luminal side of the blood vessels of most tissues, a prime location for interaction with circulating Ang I and II. Moreover, many tissues can sequester components of the circulatory RAS from plasma. Thus, to understand the (patho)physiological consequences of the activity of local tissue RASs, consideration must be given to its

interactions with the circulating components. The following sections describe each individual tissue RAS along with a discussion of its physiological significance.

1.2.1. Existence and function of cardiac RAS

Components. Biochemistry, molecular biology and immunology techniques have confirmed that a cardiac RAS exists and is independent of the circulatory RAS (For reviews, see Lindpaintner & Ganten, 1991; Dostal & Baker, 1999). All components have been localized to the heart at the transcript and protein levels (Boer et al., 1994; Iwai et al., 1995; Lindpaintner & Ganten, 1991; Passier et al., 1996; Pieruzzi et al., 1995). Renin mRNA is present at low levels in the ventricles of the rat (Dostal et al., 1994; Dzau et al., 1987b) and in primary culture of rat ventricular myocytes (Zhang et al., 1995). The concentration of renin in pig cardiac tissue was similar to that found in plasma. Removal of kidney-derived renin by bilateral nephrectomy caused both plasma and cardiac renin levels to fall to undetectable levels (Danser et al., 1994), indicating that under physiologic conditions, renin present in cardiac tissue is likely kidney-derived. This is consistent with the ability of heart tissue to actively sequester renin from plasma (de Lannoy et al., 1997; van Kesteren et al., 1997; Muller et al., 1998). Agt mRNA is detectable in the heart (Campbell & Habener, 1986; Dzau et al., 1987b; Lindpaintner et al., 1987), and Agt protein is present in neonatal and adult rat cardiac myocytes and fibroblasts (Sadoshima et al., 1993; Dostal et al., 1992). However, the levels of Agt in pig cardiac tissue are just 25% of that in plasma, consistent with diffusion of Agt from the plasma to the interstitial space (Danser et al., 1994; Passier et al., 1996). This suggests that Agt present in cardiac tissue, under physiological conditions, is liver-derived. ACE mRNA is readily detectable in cardiac endothelial cells and fibroblasts (Falkenhahn et al., 1995; Passier et al., 1996).

ACE protein is present in various areas in the rat heart: valves, coronary vessels, endocardium and epicardium (Yamada et al., 1991; Schunkert et al., 1990). ACE2 is expressed in VSMCs and endothelial cells of the heart (Donoghue et al., 2000; Tipnis et al., 2000). The mRNA and protein for AT_1R and AT_2R are present in cardiac tissue and in cardiac cells in culture (Fareh et al., 1996; Meggs et al., 1993; Sechi et al., 1992; Zhang et al., 1995). AT_4R/IRAP is expressed in cardiac fibroblasts and myoctyes (Wang et al., 1995a; Hanesworth et al., 1993), and Mas has been detected in the heart (Metzger et al., 1995). Ang II is secreted into the growth medium of cultured cardiac myocytes, fibroblasts, and microvascular endothelial cells (Dostal et al., 1992; Fischer et al., 1997), suggestive of independent production of Ang peptides by cardiac cells. Ang II is present in cardiac tissue (Ruzicka et al., 1993; Danser et al., 1994; Campbell et al., 1993), and in normal rats, the concentration of cardiac Ang I and II is similar to their concentrations in plasma (Ruzicka et al., 1993). In pigs following nephrectomy, the concentrations of renin in plasma and heart (and concentrations of Ang II in plasma and heart) drop to undetectable levels (Danser, 1996). Similarly, in rats, cardiac Ang II levels decrease by 80% 2 days after nephrectomy. These studies indicate that under physiologic conditions, the renin present in cardiac tissue, for the production of LV Ang I and II, is largely kidney-derived.

Function. The Ang peptides act within the myocardium to modify cardiac biochemistry and function *in vitro* and *in vivo*. Ang II induces proliferation of cultured cardiac fibroblasts (Fujisaki et al., 1995; Brilla et al., 1994) and induces hypertrophy of cultured cardiac myocytes (Miyata & Haneda, 1994). In rats, chronic intraperitoneal (ip) or subcutaneous (sc) infusion of Ang II induces cardiac myocyte injury/necrosis and

fibroblast proliferation (Tan et al., 1991; Campbell et al., 1995). Mice overexpressing rat Agt specifically in cardiac myocytes exhibited a 24% increase in heart weight that was prevented by systemic AT_1R blockade (Mazzolai et al., 1998). On the other hand, transgenic mice expressing an artificial protein in the heart that releases Ang II developed cardiac hypertrophy only when the Ang II spilled over into the circulation and caused hypertension (van Katz et al., 2001). Transfection of a human ACE transgene into the rat myocardium increased LV ACE activity by 2 fold after 3 days and was associated with an increase in cardiomyocyte size and increased interstitial collagen (Higaki et al., 2000). In mice expressing ACE in cardiac myocytes by 100 fold versus wild-type, cardiac Ang II levels were increased 4.3 fold, but no cardiac hypertrophy or fibrosis was present (Xiao et al., 2004). In contrast, transgenic rats expressing a cardiac-specific human ACE transgene exhibited a 50-fold increase in ACE activity but normal Ang II levels. These rats exhibited no cardiac phenotype at baseline, but in response to suprarenal aortic banding they developed more severe cardiac hypertrophy than wild-type rats (Tian et al., 2004). Thus moderate (<100 fold) ACE overexpression in the heart 1) may not result in increased Ang II levels and 2) may not be detrimental by itself, but may exacerbate the effects that occur to the LV in the presence of a hypertrophic stimulus.

Mice overexpressing human AT_1R (200-450 fold increase) in cardiac myocytes develop cardiac hypertrophy and increased interstitial collagen and die of heart failure (Paradis et al., 2000). However, rats overexpressing human AT_1R by 4-8 fold in cardiac myocytes did not exhibit a change in cardiac phenotype (Hoffmann et al., 2001), but exhibited more severe hypertrophy in response to systemic Ang II infusions and pressure- and volume-overload than wild-type rats (Hoffmann et al., 2001). Thus very high

expression AT_1R in cardiac myocytes may result in a hypertrophic phenotype at baseline. However, like ACE, smaller increases in AT_1R expression may be detrimental to the heart only in the presence of other hypertrophic stimuli.

Similar to Ang II, Ang IV treatment induces cardiac fibroblast proliferation *in vitro* (Wang et al., 1995b), whereas Ang (1-7) treatment decreases their proliferation (Tallant & Clark, 2003). Functional ACE2 is essential for normal cardiac function, as ACE2 knockout mice exhibit severely impaired cardiac contractility (Crackower et al., 2002). However, cardiac-specific overexpression of ACE2 in mice leads to severe conduction and rhythm abnormalities and eventually to ventricular arrhythmias and sudden death (Donoghue et al., 2004).

1.2.2. Existence and function of brain RAS

Components. Agt mRNA and protein are located mainly in astrocytes but also in neurons of the hypothalamus and brainstem (Deschepper et al., 1986; Imboden et al., 1987; Thomas et al., 1992; Healy & Prinz, 1984). Renin mRNA abundance and renin activity are high in the hypothalamus and brainstem (Fischer-Ferraro et al., 1971; Genain et al., 1985; Dzau et al., 1986). ACE is expressed widely in the brain, in endothelial cells, neurons, and neuroepithelial cells of the hypothalamus and brainstem (Chai et al., 1987; Bunnemann et al., 1992; Chai et al., 1990; Lind et al., 1985). Ang I, II and (1-7) have been observed in nuclei of the hypothalamus (Healy & Prinz, 1984; Lind et al., 1985; Block et al., 1989). Ang IV and the AT_4R/IRAP are located in brain regions involved in learning and memory (Wright & Harding, 1995). The AT_1R and AT_2R are present within the hypothalamus, though AT_2R densities are very low, and are higher in brainstem nuclei than in the hypothalamus (Roberts et al., 1995; Wright & Harding,

1995). Mas mRNA is present in the hippocampus, cortex and thalamus (Martin et al., 1992).

Function. The brain is tightly protected from substances in the circulation by the blood brain barrier (BBB). The brain RAS therefore is nearly completely separate from the circulatory RAS, as Ang peptides cannot cross the BBB (Schelling et al., 1976; Volicer & Loew, 1971). However, specialized brain regions, the circumventricular organs (CVOs), are sensitive to blood-borne substances, as they are highly vascularized with fenestrated capillaries that allow diffusion of substances across their vessel walls (Ballabh et al., 2004). These areas protect the brain from harmful circulating substances but still allow the monitoring of important hormonal constituents in the blood. The CVOs include the subfornical organ (SFO), organum vasculosum laminae terminalis (OVLT), area postrema, and median eminence (Ballabh et al., 2004). These areas are exquisitely sensitive to circulating Ang I and Ang II as they express AT_1R and ACE at high abundance (Rowe et al., 1990; Song et al., 1991). The SFO and OVLT are directly connected by angiotensinergic fibres to the paraventricular nucleus (PVN) and median preoptic nucleus (MnPO) (Fig. 1.3) (Wright et al., 1993; Li & Ferguson, 1993; Lind et al., 1985). These nuclei are inside the BBB, express AT_1R, and can produce Ang peptides (Song et al., 1991; Rowe et al., 1990). They are connected by angiotensinergic fibres to the ventrolateral medulla and the nucleus of the solitary tract; areas within which Ang II regulates sympathetic tone and baroreflex sensitivity (Sun et al., 1988; Campagnole-Santos et al., 1988). The baroreflex is a feedback mechanism by which baroreceptors in the atria and carotid arteries respond to an increase/decrease in blood pressure by mediating a compensatory increase/decrease in sympathetic tone (Head &

Mayorov, 2001). Both Ang II and III, when injected intracerebroventricularly (icv) into rats, elicit dose-dependent dipsogenic (thirst) and pressor responses. The pressor responses, blocked by losartan, occur via an increase in sympathetic tone, inhibition of baroreflex function and an increase in release of the potent vasoconstrictor AVP (Phillips, 1987). In contrast to Ang II and III, Ang (1-7) injected icv facilitates the baroreflex (Campagnole-Santos et al., 1992; Silva et al., 1993). The actions of Ang II and III are mediated through several hypothalamic nuclei, including the aforementioned PVN, MnPO, OVLT, and SFO (Jensen et al., 1992; Mangiapane & Simpson, 1980; Ku et al., 1999; Simonnet et al., 1980).

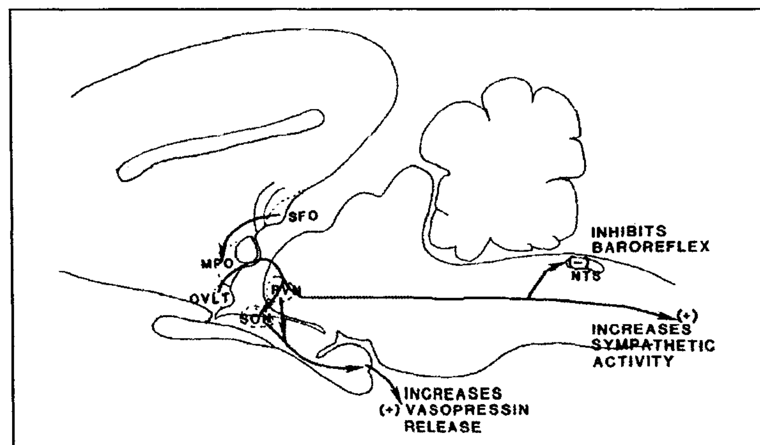


Figure 1.3. Brain areas within which the RAS regulates cardiovascular function. SFO: subfornical organ; MPO: Median preoptic nucleus; OVLT: organum vasculosum laminae terminalis; PVN: paraventricular nucleus; SON: supraoptic nucleus. From: Phillips & Kimura, 1986. Used with permission.

1.2.3. Existence and function of kidney RAS

Components. Strong evidence exists for the presence of an intra-renal RAS, especially within the proximal convoluted tubules and the surrounding interstitial space. Renin is present in high abundance in the kidney in juxtaglomerular cells and is secreted into the

interstitium, where it can be taken up into the proximal tubule (Rohrwasser et al., 1999). Renin mRNA has been localized to the proximal tubule and thus may be produced there (Moe et al., 1993a; Moe et al., 1993b). Agt can be taken up from the circulation, but it is also produced in proximal tubule cells and is secreted through the apical membrane into the tubular lumen (Rohrwasser et al., 1999; Ingelfinger et al., 1990) and basolaterally to the interstitial space (Navar et al., 2002). ACE is present in high amounts in the brush border membrane of the proximal tubular epithelium where it can cleave Ang I to Ang II. Indeed, Ang II is present in tubular fluid and the interstitium at concentrations higher than in plasma (Navar et al., 1997; Ikemoto et al., 1990; Schulz et al., 1988). However, Ang II can be actively sequestered in the kidney via receptor-mediated endocytosis (Zou et al., 1996). ACE2 is present in the kidney cortex and inner and outer medulla (Li et al., 2005). Ang II can act at the AT_1R or AT_2R, as both are found throughout the kidney (Miyata et al., 1999). Mas and AT_4R/IRAP are present (Chappell et al., 2004; Handa et al., 1998) as are Ang III, IV and (1-7) (Nishiyama et al., 2002; De Silva et al., 1988; Chappell et al., 2001; Li et al. 2005).

Function. Ang II, infused at rates confined to the kidney, decreases renal blood flow, increases efferent arteriolar resistance and filtration fraction and enhances sodium reabsorption (Hall et al., 1977; Schuster et al., 1984; Dzau, 1987a). In contrast, infusion of Ang (1-7) into the renal artery increases diuresis and natriuresis (Vallon et al., 1998; Burgelova et al., 2002). Ang IV increases renal blood flow and natriuresis (Hamilton et al., 2001). The kidney RAS is also important in the development of the kidney, as both Agt and ACE knockout mice exhibit renal cortical atrophy and overall reduced kidney function (Krege et al., 1995; Tian et al., 1997).

1.2.4. Existence and function of adrenal RAS

All components of the RAS have been localized to the adrenal (Yamaguchi et al., 1990; Oda et al., 1991). Since the functions of the adrenal cortex and medulla are distinct, these areas are discussed separately.

Components, adrenal cortex. Both Agt and renin mRNA and protein are located primarily in the zona glomerulosa (ZG) but also in the zona fasiculata (ZF) of the adrenal cortex (Racz et al., 1992; Doi et al., 1984; Brecher et al., 1989). Adrenal renin concentrations increase maximally 24-36 hours post-nephrectomy, whereas plasma renin activity is undetectable just 4 hours post-nephrectomy, indicating that adrenal renin is not kidney-derived (Doi et al., 1984). ACE is expressed at low levels in the ZG only (Plunkett et al., 1985; Strittmatter et al., 1986). Both AT_1R and AT_2R are expressed, and in the rat, the adrenal cortex contains 80-90% AT_1R (Chiu et al., 1989; Balla et al., 1991). $AT_{1B}R$ is present only in the ZG, and the $AT_{1A}R$ is expressed in each cortical layer (Llorens-Cortes et al., 1994; Gasc et al., 1994). Ang II and III are present mostly in the ZG (Kim et al., 1992), and their concentrations are not reduced by nephrectomy (Aguilera et al., 1981), suggesting that Ang II is produced locally. Sequestration of Ang II via receptor-mediated endocytosis has also been demonstrated (Husain et al., 1987). Ang IV and AT_4R/IRAP is present in adrenal homogenates but not specifically to the cortex or medulla (De Silva et al., 1988; Lee et al., 2003), and Mas has not been reported present in the adrenal.

Function. Several studies have demonstrated that Ang II acts at the AT_1R in the adrenal cortex to increase the production and release of aldosterone. In isolated rat ZG cells, Ang II treatment evokes at 5-fold increase in aldosterone output. This effect is

completely prevented by pretreatment with the AT_1R antagonist losartan (Belloni et al., 1998). In similar ZG preparations, ACE inhibitors and AT_1R antagonists inhibit basal aldosterone production/secretion, and secretion in response to K^+ and adrenocorticotropic hormone (Shier et al., 1989; Horiba et al., 1990; Gupta et al., 1995).

Components, adrenal medulla. The adrenal medulla expresses all components of the RAS: renin (Racz et al., 1992; Doi et al., 1984), Agt (Racz et al., 1992), ACE (Wang et al., 2002b) and both AT_1R and AT_2R (Balla et al., 1991; Jezova et al., 2003). $AT_{1A}R$, but not $AT_{1B}R$ is present (Llorens-Cortes et al., 1994; Gasc et al., 1994). Ang II is also present (Wang et al., 2002b). Ang IV and AT_4R/IRAP are present in adrenal homogenates, but not specifically in the medulla (De Silva et al., 1988; Lee et al., 2003).

Function. The adrenal medulla produces and secretes the catecholamines epinephrine and norepinephrine (Hano et al., 1994; Bunn & Marley, 1989; Powis & O'Brien, 1991). Ang II increases the expression and activity of enzymes required for catecholamine biosynthesis, including tyrosine hydroxylase (TH) (Stachowiak et al., 1990). Systemic blockade of the AT_1R or AT_2R reduces the basal expression of adrenal TH and reduces adrenal norepinephrine levels (Jezova et al., 2003; Armando et al., 2004). In adrenal medullary fragments, the basal and Ang II-induced secretion of catecholamines was completely blocked by an AT_2R antagonist but only slightly reduced by high concentrations of an AT_1R antagonist (Belloni et al., 1998). Thus the activation of local AT_2R and perhaps AT_1R by Ang II is involved in the production/secretion of catecholamines from the adrenal medulla.

1.2.5. Existence and function of vascular RAS

Components. Agt mRNA and protein is located in the aorta and in cultured VSMCs (Cassis et al., 1988; Eggena et al., 1988). ACE is expressed in ECs and VSMCs (Iwao et al., 1988; Pipili et al., 1989). The presence of renin is debated, because renin mRNA was undetectable by reverse transcriptase polymerase chain reaction (RT-PCR) (Hilgers et al., 2001; Carey & Siragy, 2003). However, since cultured VSMCs and endothelial cells have been shown to generate Ang I and Ang II (Re et al., 1982; Dzau, 1984; Kifor & Dzau, 1987), it is likely that renin required for Ang II synthesis is taken up by vascular tissue, as has been demonstrated in the heart. AT_1R, AT_2R, AT_4R/IRAP and Mas are present in VSMCs and endothelial cells (Nickenig et al., 1998a; Dzau et al., 1991; Hall et al., 1993; Hall et al., 1995; Muthalif et al., 1998; Jaiswal et al., 1992).

Function. Ang II's potent vasoconstrictor action occurs in part by its interaction with AT_1R on VSMCs within the vascular wall (Stroth & Unger, 1999). *In vitro*, Ang II causes hypertrophy of rat aortic VSMCs (Dzau et al., 1991). *In vivo*, Ang II acting at the AT_1R, and Ang IV acting at the AT_4R are involved in neointimal formation after vessel injury (Osterrieder et al., 1991; Kawamura et al., 1993; Kino et al., 1994; Moeller et al., 1999). The stimulation of Mas by Ang (1-7) promotes the release of vasodilatory prostaglandins from, and growth inhibition of, VSMCs (Muthalif et al., 1998; Jaiswal et al., 1992; Freeman et al., 1996; Tallant & Clark, 2003), and causes the release of NO from endothelial cells (Li et al., 1997). The actions of Ang (1-7) in the vasculature are largely responsible for its antagonism of the effects of Ang II: in humans, infusion of Ang (1-7) attenuates Ang II-induced vasoconstriction (Ueda et al., 2000). The vascular RAS is therefore important in the regulation of vascular tone and the proliferation of vascular cells following vessel injury.

1.3. Estrogen regulation of cardiovascular function

1.3.0. Overview

While estrogens exert many actions within the female reproductive system, their effects on cardiovascular function are of great interest based on the observations that both gender and menopause influence the incidence of cardiovascular disease. This section will first illustrate the general cellular mechanisms of action of estrogens, and proceed to the actions of estrogens on the cardiovascular system in healthy humans and animals, specifically its actions on the circulatory and tissue RASs.

1.3.1. Regulation of cellular function by estrogens

Estrogens regulate cellular processes by two general mechanisms, distinguished by their time of onset of action. E2 exerts cellular effects that occur within minutes of application and effects that take several hours. The rapid effects of E2 are not 'genomic', i.e. do not require gene transcription and *de novo* protein synthesis. A description of estrogens, their receptors, and cellular effects mediated via both 'genomic' and 'non-genomic' mechanisms follows.

1.3.1.1. Estrogens and estrogen receptors

In the pre-menopausal female human and rat, the ovaries produce four main estrogenic compounds: 17α-estradiol, 17β-estradiol (E2), estriol, and estrone. They also produce progesterone. In the non-pregnant, pre-menopausal human and rat, E2 is the predominant circulating estrogen. Its levels fluctuate between 40-400 pg/ml during the menstrual cycle (Baird & Fraser, 1974) and between 20-90 pg/ml in the estrous cycle (Butcher et al., 1974). E2 is enzymatically converted from androstenedione or testosterone within the pre-ovulatory follicles and corpora lutea by aromatase P450 (Sasano et al., 1989).

Following surgical OVX or menopause in humans or rats, the production of E2 decreases and plasma levels drop considerably, to less than 20 pg/ml, and as low as 4 pg/ml (Longcope et al., 1986; Jones, 1992; Cauley et al., 1989).

Pharmaceutical preparations of estrogens are currently available in various forms. E2 is the main endogenous estrogen, but is readily degraded in the digestive system and therefore is not suitable for oral administration, unless in micronized form (i.e. crystals that are 1-3 μm in diameter). Derivatives of E2, such as ethinyl estradiol (EE), estradiol valerate (EV) and estradiol benzoate (EB) are more orally active due to their hydrophilic moieties, and are first-pass metabolized to E2 (Shoham & Kopernik, 2004). The most widely used estrogen preparation for ERT is Premarin™, which is a mixture of eleven conjugated estrogens isolated from the urine of pregnant mares, two of which are not present in humans. It contains mostly estrone-sulfate and equilin-sulfate (Stumpf, 1990). Since estrone and E2 are easily interconverted, Premarin™ treatment increases plasma E2 levels. To avoid first-pass metabolism altogether, parenteral administration of E2 via transdermal, percutaneous and vaginal routes is also available. Due to the lipophilicity of E2, physiologically relevant E2 concentrations are quickly obtained via these methods (Gordon, 1995). Animal studies frequently employ sc implanted E2 pellets, or sc injections of EE, EV or EB for easy estrogen replacement (e.g. Brosnihan et al., 1997a; Fregly et al., 1985; Kisley et al., 1999a; Carrière et al., 1986).

Estrogens are potent and specific ligands to two receptors: ERα and ERβ. In 1986-87, the cDNAs for human and rat ERs were cloned (Green et al., 1986; Greene et al., 1986; Koike et al., 1987). However, the complicated pharmacology and tissue selectivity of E2 and other estrogenic compounds could not be explained by the presence of a sole ER. By

the end of 1995, a second ER was cloned and named ERβ (the first became ERα) (Kuiper et al., 1996). Both receptors are expressed throughout the reproductive system of humans and rats, as required for maintenance of the menstrual and estrous cycles and pregnancy. However, many non-reproductive tissues also express ERs in a tissue-specific manner. Expression patterns will be described in sections discussing the tissue-specific actions of E2.

1.3.1.2. Estrogen 'genomic' regulation

The ERs belong to the nuclear steroid hormone receptor superfamily (Beato et al., 1995) and thus classical, nuclear receptor-mediated transcriptional activation or repression is the main mode of action of E2. The highly lipophilic E2 passively diffuses across the plasma membrane. Within the nucleus, E2 interacts with the ERs present and induces tyrosine phosphorylation-mediated conformational change, homo- or heterodimerization of the receptors, and the removal of protein chaperones from the receptor complex (Pettersson et al., 1997). This complex then recruits coactivator proteins and transcription factors (Xu et al., 1999; Robyr et al., 2000; Beato & Sanchez-Pacheco, 1996). The newly activated complex can regulate transcription of genes whose promoter regions contain the classical (or nearly consensus) estrogen response element (ERE). This DNA element is not the only target for the activated ER complex. C-fos and c-jun, two immediate-early gene products, form an protein complex named AP-1 that regulates gene transcription by binding to AP-1 response elements. The activated ER dimer binds the AP-1 complex and modifies the transcriptional regulation of genes targeted by AP-1. The activated ER complex can also bind NFκB and SP-1 and inhibit

the transcription of their target genes (Stein & Yang, 1995; Porter et al., 1997; Saville et al., 2000).

The regulation of transcription by E2 is highly dependent on cell type, as cells can express ERα or ERβ or both. Thus depending on their expression, the ERs can form up to three different combinations of dimers, which will modify the effects of estrogen in a given cell type. For example, in response to E2, ERα homodimers activate, but ERβ homodimers inhibit transcription via AP-1 in cultured breast cancer cells (Paech et al., 1997). Furthermore, a comparison of the homology between the ERs provides evidence for complex and cell-type specific transcriptional regulation. ERα and β have highly homologous DNA binding domains (Kuiper et al., 1996) and thus will likely regulate transcription of the same genes. However, the transcription factors, coactivators and corepressors present in any particular cell will differentially modulate this regulation, as the activation function regions of the ERs, responsible for ligand-dependent interactions with transcription factors, exhibit low homology (Kuiper et al., 1996). Also, due to modest similarity of their ligand-binding domain (Kuiper et al., 1996), the affinities of the ERs to different estrogenic compounds differ. To summarize, estrogen bound to ERs will modulate the transcription of overlapping set of genes but differ in the degree of transcriptional activation or repression depending on 1) the combination of ERs expressed in a particular cell, 2) the type of estrogen bound by the receptors, and 3) the combination of transcription factors active within the target cell (Barkhem et al., 1998). The specific actions of E2 on the cardiovascular system, including the RAS, are discussed in detail in section 1.3.2.

1.3.1.3. Selective estrogen receptor modulators

The estrogen receptors are also targets for selective estrogen receptor modulators (SERMs). SERMs are synthetic ER ligands whose effects are highly dependent on the tissue type, and as such, the same SERM can act similarly to estrogen (i.e. estrogenic) or act as an estrogen antagonist (i.e. as an anti-estrogen) (McDonnell, 1999). The first SERM to be characterized was the compound tamoxifen, which acts as an anti-estrogen in breast tissue, but exhibits significant estrogenic activity in the skeletal and cardiovascular systems and the uterus (Love et al., 1992; Ismail, 1994). The compound raloxifene is an anti-estrogen in breast and uterus but is an ER agonist in bone, and prevents loss of bone density (Turner et al., 1994; Black et al., 1994). The complex *in vivo* pharmacology of SERMs is a function of the complex regulation of transcription by ERs. The compound ICI 182,780, also known as faslodex or fulvestrant, is considered an pure antagonist of ER with no known agonist activity. It competitively inhibits, with high affinity, the binding of E2 to the ERα (Wakeling & Bowler, 1987). It disrupts ER dimerization and therefore nuclear translocation (Fawell et al., 1990; Dauvois et al., 1993) and accelerates the degradation of ER (Nicholson et al., 1995; Peekhaus et al., 2004). Interestingly, it slows the degradation of ERβ (Peekhaus et al., 2004). Another compound, methyl-piperidino-pyrazole, also specifically antagonizes ERα (Sun et al., 2002).

1.3.1.4. Estrogen 'non-genomic' regulation

While transcriptional regulation by E2/ER occurs most often, several actions of E2 occur too rapidly to be dependent on *de novo* synthesis of protein. For example, within five minutes, E2 produces marked NO-induced vasodilation in ECs by activating endothelial nitric oxide synthase (eNOS) activity but not expression (Caulin-Glaser et al.,

1997). This action of E2 is blocked by ICI 182,780, and requires ERα with an intact E2 binding domain (Chen et al., 1999); both indications of ERα-dependent process. The short onset of action rules out mediation via the classical ER-dependent mechanism of transcriptional activation. While the precise mechanism remains under investigation, this 'non-genomic' action of E2 likely involves an atypical membrane-bound ERα that activates signal transduction pathways instead of acting as a transcriptional regulator (Caulin-Glaser et al., 1997).

1.3.1.5. Summary

Estrogens are present and active in the pre-menopausal human and rat, mediating their cellular effects with and without their receptors, ERα and β, in several reproductive and non-reproductive organs. The actions of E2 are highly tissue- and cell-specific, dependent on the combination of ERs and cofactors expressed in the target cells. Following menopause or OVX, E2 levels fall and its actions are greatly reduced.

1.3.2. Regulation of cardiovascular function by estrogens

1.3.2.1. Regulation of blood pressure, baroreflex function, and autonomic tone by estrogens

Animal studies provide extensive evidence for the regulation of cardiovascular function by E2, but also provide insights into molecular mechanisms of action of this regulation. In several studies in Wistar rats, OVX alone increases resting blood pressure (BP) by 10-23 mmHg compared to ovary-intact rats 3-10 weeks post-OVX (Mercier et al., 2002; Hernandez et al., 2000; Milsted et al., 1998; Brosnihan et al., 1997a). This increase is prevented by replacement of E2 at physiological levels. In contrast, three studies report no change in BP at 11 days, 2 and 5 weeks post-OVX (He et al., 1998;

Nickenig et al., 1998a; El-Mas & Abdel-Rahman, 1998). Female Dahl S rats fed a diet containing low to normal NaCl (0.1% or 0.5%) exhibit progressive increases in BP with age. Specifically, between 5 weeks and 20 weeks of age, systolic BP increases by 10 mmHg (Harrison-Bernard et al. 2003). Between 3 and 12 months of age mean arterial pressure increases by 30 mmHg (Hinojosa-Laborde et al., 2004). This age-induced increase in BP is exacerbated in OVX rats: systolic BP is increased by an additional 25 mmHg (Harrison-Bernard et al. 2003) and mean arterial pressure is increased by an additional 15 mmHg (Hinojosa-Laborde et al., 2004). The OVX-induced increases are prevented by E2 treatment at physiological levels (Harrison-Bernard et al., 2003) and are reversed to decreases by E2 treatment at high levels (Hinojosa-Laborde et al., 2004).

The mechanism of BP elevation post-OVX may involve endothelin, NO and/or angiotensin. Systemic treatment with an endothelin-A receptor antagonist or an AT_1R antagonist prevents the increase in BP (Mercier et al., 2002; Pham-Dang et al., 2003). In both humans and animals, E2 mediates rapid vasodilation via activation of NO production and release (Mendelsohn et al., 1999). The regulation of MAP by E2 may also be related to its regulation of sympathetic and parasympathetic tone. In OVX rats, acute intravenous (iv) injection of E2 increases vagal efferent nerve activity, decreases renal sympathetic activity, and reduces BP. These changes are associated with an enhanced baroreflex sensitivity in response to the iv administration of the pressor agent phenylephrine (PE) or the depressor agent nitroprusside (NP) (Saleh & Connell, 2000; He et al., 1998). E2 also mediates similar effects chronically. As early as 2 weeks post-OVX, rats exhibit attenuated baroreflex sensitivity in response to PE, vagal stimulation, and NP. E2 replacement at physiological levels prevents baroreflex desensitization (He

et al., 1998; El-Mas & Abdel-Rahman, 1998; Mohamed et al., 1999; Saleh et al., 2001). These studies suggest that E2 deficiency causes a shift in autonomic output towards decreased parasympathetic tone and increased sympathetic tone, and decreased baroreflex responsiveness. The presence of physiological circulating levels of E2 prevents this impairment.

1.3.2.2. Regulation of cardiac function by estrogens

In addition to the observed increase in BP, at 3 weeks post-OVX, LV peak systolic pressure (LVPSP) is increased by 27 mmHg and LV end diastolic pressure (LVEDP) is increased by 6 mmHg (Mercier et al., 2002; Pham-Dang et al., 2003), as measured by Millar catheter. The positive first derivative of LV pressure (+dP/dt) is slightly (9%, ns) increased and the negative first derivative of LV pressure (-dP/dt) is significantly increased by 17%. By six weeks after OVX, LVPSP remains increased but LVEDP is normalized. These changes are prevented by replacement of E2 at physiological levels (Mercier et al., 2002), indicating that E2 deficiency may change LV function. Another study found that 9-10 weeks after OVX, LVPSP and LVEDP were unchanged compared to ovary-intact rats (Hügel et al., 1999). Together, these studies suggest that E2 deficiency may modify LV function in a time-dependent manner.

Also apparent after OVX are changes in ventricular myocyte contractile function. Myocytes from OVX rats exhibit reduced maximal cell shortening, reduced rates of shortening and relengthening, and elevated resting intracellular [Ca^{2+}]. These changes are prevented by E2 replacement at physiological levels (Ren et al., 2003). Perhaps responsible for these changes in contractility is the increase in L-type Ca^{2+} channel density in cardiomyocytes post-OVX (Patterson et al., 1998). Together, these studies

suggest that E2 deficiency causes significant abnormalities in cardiac excitability and contractility that are ameliorated by the presence of E2.

1.3.3. Regulation of RAS by estrogens

1.3.3.0. Overview

E2 status regulates the expression of different components of the circulatory and tissue RASs in humans and animals. This section will describe this regulation and its physiological consequences.

1.3.3.1. Regulation of circulatory RAS by estrogens

Plasma Agt. Pre-menopausal women taking oral contraceptives, and postmenopausal women taking oral ERT exhibit increased plasma renin substrate (i.e. plasma Agt) compared to untreated controls (Oelkers et al., 1976; Schunkert et al., 1997; Hassager et al., 1987). However, this effect on plasma Agt does not occur with the administration of transdermal and percutaneous estrogens (Schunkert et al., 1997; Faguer de Moustier et al., 1989; Hassager et al., 1987). Agt production is activated in the liver by higher-than-physiological hepatic concentrations of estrogens after oral administration, a first-pass effect that does not occur after transdermal/percutaneous ERT administration (Fischer et al., 2002). Via a similar mechanism, high-dose estrogen via any route is likely to increase Agt production and therefore plasma Agt (Oelkers, 1996). In OVX and ovary-intact rats, acute sc/ip injection of moderate/high doses of EB or EE increases hepatic Agt mRNA abundance (Gordon et al., 1992; Hong-Brown & Deschepper, 1993; Kunapuli et al., 1987). Since the secretion of Agt from the liver is constitutive (Morris et al., 1979), it is likely that, if fully translated, this increase in Agt mRNA mediates the observed increase in plasma Agt. Indeed, whereas low-dose sc E2 does not affect plasma Agt

levels (Stavreus-Evers et al., 2001; Menard et al., 1973), chronic high-dose EE, E2 or EB treatment of OVX and ovary-intact rats increases plasma Agt (Greenland & Sernia, 2004; Stavreus-Evers et al., 2001; Krakoff & Eisenfeld, 1977; Menard et al., 1973; Hong-Brown & Deschepper, 1993; Kniefel & Katzenellenbogen, 1981).

Plasma Renin. Post-menopausal women taking oral ERT and premenopausal women taking oral contraceptives exhibit decreased plasma renin as measured by antibody detection (Schunkert et al., 1997; Derkx et al., 1986). On the other hand, plasma renin activity (PRA), the ability of plasma to cleave Ang I, may be similar to (Zacharieva et al., 2002) or even higher than (Skinner et al., 1969) untreated women, because renin activity is increased by an increase in its substrate Agt, which is elevated in women taking oral estrogens as noted above. In contrast, post-menopausal women taking transdermal ERT exhibit decreased immunoreactive renin protein in plasma (Schunkert et al., 1997; Derkx et al., 1986) but no change in PRA (Zacharieva et al., 2002). Similarly, in OVX rats, PRA is unaltered by sc replacement of E2 for 3 weeks (Brosnihan et al., 1997a).

Plasma ACE. In post-menopausal women taking oral ERT (with and without progesterone), plasma ACE activity is lower than in placebo-treated controls (Schunkert et al., 1997; Proudler et al., 1995; Proudler et al., 2003), but similar to Agt, plasma ACE is not affected by transdermal ERT (Proudler et al., 2003). In contrast, in OVX rats, replacement of E2 sc at physiological or supra-physiological levels for 3 weeks decreases plasma ACE activity by 40% (Gallagher et al., 1999; Brosnihan et al., 1997a). This decrease in ACE activity is associated with a 30% decrease in lung ACE mRNA abundance and activity. The reduction in lung ACE expression may lead to a decrease in secretion of ACE from the lung and therefore to lower plasma ACE levels.

Plasma Ang peptides. The regulation of plasma Agt, PRA and plasma ACE by E2 affects the concentration of Ang peptides in plasma. In OVX monkeys, the inhibitory effect on ACE activity by Premarin™ treatment is associated with an increase in plasma Ang I levels but no decrease in plasma Ang II levels (Brosnihan et al., 1997b). Similarly, in OVX rats, plasma ACE activity decreases with E2 treatment but plasma Ang II concentration is not altered (Brosnihan et al., 1997a). In OVX rats, plasma Ang (1-7) levels are unchanged by E2 replacement at physiological levels, despite the suppression of plasma ACE activity, which catabolizes that peptide (Brosnihan et al., 1997b). It is likely that despite the increase in Agt and perhaps an increase in PRA, the suppressive effect of E2 on plasma ACE activity counteracts and negates their effects on Ang II levels (Brosnihan et al., 1997b).

1.3.3.2 Regulation of cardiac RAS by estrogens

Male mice exhibit 2-fold higher LV ACE mRNA abundance and 60% higher ACE protein levels than age-matched female mice (Freshour et al., 2002). This may be an effect of both female and male sex hormones, as OVX increases LV ACE mRNA levels in female mice by 20% and castration decreases LV ACE mRNA levels in male mice by 35% (Freshour et al., 2002). Neither E2 nor testosterone replacement was performed, so it is unclear if the effects are exclusively via these hormones. In year-old ('senescent') OVX rats, LV AT_1R protein is increased by 15% and LV AT_2R protein is decreased by 30% compared to ovary-intact controls. E2 replacement at high levels reverses these changes (i.e. the increase in AT_1R to a decrease and the decrease in AT_2R to an increase) (Xu et al., 2003); physiological E2 replacement was not performed. The regulation of LV ACE, AT_1R and AT_2R by E2 is possibly ER-dependent, as both ERα and ERβ

mRNA and protein are found in adult rat and mouse heart in fibroblasts and myocytes (Kuiper et al., 1997; Grohe et al., 1998; Grohe et al., 1997). The exact mechanism of the regulation of cardiac ACE, AT_1R and AT_2R by estrogens remains unclear.

1.3.3.3. Regulation of brain RAS by estrogens

Estrogens regulate the expression of RAS components within cardiovascular regulatory regions of the brain. Three days after OVX, Agt protein is decreased in hypothalamus-thalamus tissue blocks by 22%. This decrease is prevented by treatment with high EB (Greenland & Sernia, 2004). Transcript abundance was not measured. In contrast, treatment of OVX rats with one large dose of EE decreases Agt mRNA in hypothalamus-thalamus tissue blocks by 50% (Hong-Brown & Deschepper, 1993) and a moderate dose of EB for 2 days decreases Agt mRNA abundance by 40% (Kisley et al., 1999b). Treatment of OVX rats with E2 sc at physiological levels does not alter Agt mRNA abundance in hypothalamus-thalamus-septum-midbrain tissue blocks (Healy et al., 1992). In each of the studies measuring transcript levels, protein levels were not assessed. The reasons for these opposite changes in the regulation of Agt protein and transcript levels by E2 are not evident, but may reflect post-transcriptional regulation of Agt.

E2 treatment also affects the brain AT_1R. In OVX rats, 5 days of sc E2 replacement at physiological levels results in a 3.5-fold reduction in the number of AT_1R-immunoreactive neurons in the SFO (Rosas-Arellano et al., 1999). In OVX rats, treatment with a one large sc dose of EB reduces AT_1R binding density by 20% in homogenates of the preoptic area (Jonklaas & Buggy, 1985). Similarly, in OVX rats, a moderate dose of EB sc for 2 days decreases AT_1R mRNA abundance by 40% in

hypothalamus-septum-thalamus tissue blocks and decreases AT_1R binding density in the SFO by 30% (Kisley et al., 1999b). The mechanism of this regulation has not yet been explored. However, the SFO expresses both AT_1R and ERα in the same neurons (Rosas-Arellano et al., 1999). The preoptic area and the hypothalamus contain the MnPO, OVLT and PVN; ERα is present in the OVLT, ERα and ERβ are present in the MnPO and ERβ is present in the PVN (Laflamme et al., 1998; Shughrue et al., 1998). Thus the changes in Agt and AT_1R may be mediated through ER-dependent processes.

The decrease in AT_1R expression by E2 may result in decreases in the firing rate of angiotensinergic neurons. In OVX rats, chronic treatment with moderate sc EB 1) decreases the spontaneous discharge rate of SFO neurons that synapse on the PVN by 50% and 2) decreases the responsiveness of SFO neurons to Ang II injected into the carotid artery, or microinjected into the lateral hypothalamic area or the SFO itself (Tanaka et al., 2001a; Tanaka et al., 2001b). Anteroventral third ventricle (AV3V) brain slices (containing neurons from the SFO, OVLT and MnPO) taken from OVX rats treated with chronic E2 exhibit decreased firing rate in response to perfusion with Ang II compared to those from vehicle-treated rats (Akaishi & Homma, 1996).

Estrogens are highly lipophilic and easily cross the BBB (Pardridge & Mietus, 1979). It is therefore conceivable that peripherally-administered estrogens can act within the brain to down-regulate the brain AT_1R and decrease the firing rate of angiotensinergic neurons. In doing so, they may reduce the cardiovascular responses to centrally-administered Ang II. In OVX rats, treatment with chronic moderate or high EB sc reduces the drinking response to Ang II administered icv or microinjected into the SFO or PVN (Fregly et al., 1985; Kisley et al., 1999a; Tanaka et al., 2002; Tanaka et al., 2003).

In addition, chronic sc treatment of OVX rats with low EB reduces the duration of pressor response to icv Ang II (Skoog & Kenney, 1983). Centrally-administered E2 also decreases cardiovascular responses to Ang II. Treatment of OVX rats with a one-time dose of 0.01-1 μg EB icv decreases spontaneous drinking behavior for 24 hours and, one day after treatment, attenuates dipsogenic and pressor responses in response to icv Ang II (Jonklaas & Buggy, 1984). Microinjection of EB into the medial preoptic area reduces the dipsogenic response to icv Ang II by 40% (Jonklaas & Buggy, 1985).

These studies did not address the effect of OVX alone on the brain RAS and its physiological consequences. However, they suggest that in OVX rats, E2 treatment decreases the abundance of the AT_1R in cardiovascular regulatory areas of the brain, especially the SFO, PVN and perhaps areas of the AV3V and hypothalamus, such as the OVLT and MnPO. Via this AT_1R downregulation, E2 likely attenuates angiotensinergic transmission in these areas, thereby regulating dipsogenesis, and blood pressure, perhaps via regulating sympathetic tone.

1.3.3.4. Regulation of kidney RAS by estrogens

In OVX rats, acute sc injection of a moderate dose of EB results in a transient increase in kidney Agt mRNA abundance (Gordon et al., 1992), but whether this affects kidney Agt protein levels has not been reported. Treatment of OVX rats with sc E2 at physiological levels for 3 weeks does not affect kidney ACE activity, but supra-physiological E2 treatment decreases both ACE mRNA abundance and activity by 30% (Brosnihan et al., 1997a; Gallagher et al., 1999). In Dahl salt-sensitive rats, OVX alone increases whole-kidney AT_1R protein by 50%, an increase reversed to a decrease by treatment with high E2 (Harrison-Bernard et al., 2003). Treatment of male rats with

chronic high E2 increases Ang (1-7) levels in the kidney by 2 fold and increases Ang I levels by 2.3 fold, but does not affect kidney Ang II levels significantly (Campbell, 1997). This is consistent with a decrease in ACE activity. The mechanism of kidney ACE, AT_1R, and AT_2R regulation by E2 may be receptor-mediated, as ERα is expressed in the kidney (Kuiper et al., 1997; Mohamed & Abdel-Rahman, 2000).

1.3.3.5. Regulation of adrenal RAS by estrogens

The adrenal RAS is also a target for E2 action. Eight weeks after OVX, AT_1R protein levels are increased by 38%, an effect prevented by replacement of E2 at physiological levels (Hinojosa-Laborde et al., 2004). Two week treatment of OVX dogs with E2 (1.8 mg/day) decreases AT_1R binding densities in whole-adrenal membrane fractions by 35% (Owonikoko et al., 2004). Similarly, treatment of OVX rats with moderate dose of sc E2 for 1, 2, and 4 weeks decreases AT_1R binding densities in adrenal cortical membrane fractions by 24-35% (Carrière et al., 1986; Wu et al., 2003a; Wu et al., 2003b). The mechanism of AT_1R regulation may be receptor-dependent, as ERα is located throughout the adrenal cortex, and ERβ is present in the ZG (Wu et al., 2003b). However, the decrease in AT_1R binding density is not associated with a decrease in mRNA abundance (Wu et al., 2003a; Wu et al., 2003b). Instead of regulation of the rate of transcription, in the adrenal cortex, E2 increases the activity of RNA binding proteins, which in turn bind to the 5' leader sequence of the AT_1R mRNA, causing steric hindrance, which inhibits the translation of the mRNA (Wu et al., 2003b; Krishnamurthi et al., 1999). In OVX rats, the decrease in AT_1R protein by chronic moderate E2 treatment is associated with a similar magnitude decrease in Ang II-stimulated plasma aldosterone levels. This suggests that E2 treatment may reduce Ang II-induced aldosterone secretion by down-

regulating the AT_1R in the adrenal cortex (Roesch et al., 2000). No studies have yet addressed the effects of E2 on components of the RAS in the adrenal medulla. However, in OVX rats, treatment with high-dose EB sc decreases catecholamine release from the isolated adrenal medulla (de Miguel et al., 1989). This decrease may be mediated through AT_1R- and AT_2R-dependent mechanisms, as Ang II at both AT_1R and AT_2R regulate adrenal catecholamine production/release (Jezova et al., 2003; Armando et al., 2004).

1.3.3.6. Regulation of vascular RAS by estrogens

Three weeks after OVX, aortic Agt mRNA abundance is similar compared to that of ovary-intact rats. However, in OVX rats, a sc injection of ~5 μg EB increases aortic Agt mRNA by 2.5 fold at 1 hour post-injection (Gordon et al., 1992). OVX increases ACE activity by 26% in isolated rat aorta, an effect prevented by replacement of E2 at physiological levels (Tanaka et al., 1997). Treatment of OVX rats chronically with a moderate dose of sc EV does not affect AT_1R binding density in mesenteric artery plasma membrane preparations (Carrière et al., 1986). However, 5 weeks after OVX, AT_1R mRNA abundance and binding density are increased in rat aortas by 45-55%, effects prevented by E2 replacement at physiological levels (Nickenig et al., 1998a). Similarly, in VSMCs from ovary-intact rats, E2 treatment decreases AT_1R mRNA, not by decreasing the rate of its transcription, but by decreasing its half-life (Nickenig et al., 1998a; Nickenig et al., 2000). The effects of E2 may be receptor-dependent as both aorta and VSMCs contain ERα and ERβ mRNA and protein (Couse et al., 1997; Mohamed et al., 2000; Andersson et al., 2001). In OVX rats, replacement of E2 at physiological or supraphysiological levels attenuates the pressor response to iv Ang II (Brosnihan et al.,

1997a; He et al., 1998; Conrad et al., 1994). Thus circulating levels of E2 may modulate the regulation of vascular tone by the RAS.

1.3.3.7. Summary of effects of estrogens on RASs

While perhaps upregulating plasma Agt and PRA, especially at high doses, the down-regulation of plasma ACE activity by E2 has a counterbalancing effect and thus its effects on plasma Ang II are minimal. In general, AT_1R expression is lower in tissues in E2-replete rats, and its expression is higher in rats with E2 deficiency. This regulation of the AT_1R will likely modulate the actions of both locally-derived and plasma Ang II. Significant gaps exist in the literature, including whether other components of the tissue systems, especially ACE in the brain and heart, are regulated by E2, and the physiological implications of this regulation. Of particular interest is the potential regulation of cardiac and brain ACE and AT_1R by E2 in the presence of pathology, such as following myocardial infarction, when the levels of these components are increased.

1.4. LV dysfunction following myocardial infarction (post-MI)

1.4.1. Progression of LV dysfunction in rats post-MI

In rats, myocardial infarction can be induced by permanent ligation of the left anterior descending coronary artery (Pfeffer et al., 1979). This leads to extensive ischemia in the area normally perfused by this artery, affecting an area 20-60% of the LV free wall. Immediately, a multitude of cellular/biochemical processes begin to repair the damaged tissue to maintain the structural integrity of the ventricle. In the early stages of this 'LV remodeling', the infarcted tissue is invaded with inflammatory cells, and nascent fibroblasts, called myofibroblasts, proliferate to replace necrotic/apoptotic myocytes (Lipper et al., 1980). This infarct scar 'healing' is completed over approximately three

weeks in rats (Jugdutt et al., 1996). The nascent myofibroblasts mediate the deposition of collagen within the scar and in areas remote from the scar (Volders et al., 1993). In the remote LV, increased diastolic wall stress (Weisman et al., 1985) results in myocyte enlargement and progressive hypertrophy of the RV and remaining viable LV (Nahrendorf et al., 2003). Both tissue damage and remodeling result in impairment in LV function, measured as a progressive, time-dependent increase in LVEDP and decrease in LVPSP (DeFelice et al., 1989). The progression of LV dysfunction post-MI is highly dependent on the area of the LV affected by the initial ischemic insult, i.e. the size of the infarct scar (Pfeffer, 1991).

The remodeling that results from the local tissue damage is only one mediator of the progressive LV dysfunction post-MI. The early decrease in cardiac output post-MI activates several compensatory neurohormonal pathways. In both humans and rats, this includes an increase in sympathetic drive (Leimbach et al., 1986; Francis et al., 2001), a decrease in parasympathetic drive (Eckberg et al., 1971; Du et al., 1998) activation of the circulatory RAS via renin release from the kidney (Curtiss et al., 1978; Francis et al., 2001), and an increase in plasma AVP, endothelin and natriuretic peptides (Yamane, 1968; Francis et al., 2001; Cherng et al., 1998; Baxter, 2004). Together, these adaptations result in arterial vasoconstriction, sodium retention and increase in plasma volume. Initially, both *in situ* LV remodeling and neurohormonal activation are essential in maintaining the structural integrity of the LV and cardiovascular homeostasis. However, over time these 'adaptive' processes contribute to the progression of LV dysfunction post-MI. If left untreated, these processes lead inevitably to overt heart failure (Packer, 1992).

1.4.2. Regulation of circulatory RAS in male rats post-MI

Levels of plasma renin substrate (a.k.a plasma Agt) are essentially unchanged post-MI in rats with small infarct sizes (Yamagishi et al., 1993; Kelly et al., 1997; Duncan et al., 1997; Schunkert et al., 1992), but are decreased by ~50% in rats with large infarcts (Hirsch et al., 1999; Wang et al., 2004; Huang et al., 1994). PRA is increased in the early stages (within 6 hours to 1 day post-MI) (Leenen et al., 1999a; Duncan et al., 1997) but normalizes, variably, by 3 days post-MI (Leenen et al., 1999a) to 4-5 weeks post-MI (Sun et al., 2001; Lindpaintner et al., 1993; Duncan et al., 1997). The early increase in PRA is associated with a parallel increase in plasma Ang I concentration (Duncan et al., 1997; Leenen et al., 1999a). In contrast to PRA, plasma ACE activity is unchanged in the earlier phase (0-6 weeks) post-MI (Duncan et al., 1997; Yoshida et al., 1998; Kelly et al., 1997; Schieffer et al., 1994), but is increased in the later chronic stages (~12 weeks post-MI) in proportion to infarct size and degree of LV dysfunction (Gaertner et al., 2002; Huang et al., 1994). Interestingly, lung ACE mRNA abundance and activity are <u>decreased</u> at 4 and 12 weeks post-MI, proportional to infarct size (Huang et al., 1994; Kelly et al., 1997; Gaertner et al., 2002). The paradoxical increase in plasma ACE, at a time when lung ACE is decreased has been attributed to an increase in ACE shedding from the infarct scar (Huang et al., 1994; Gaertner et al., 2002). Like PRA, plasma Ang II is increased at 6 hours post-MI (Leenen et al., 1999a), but the increases weeks later tend to be very small (Leenen et al., 1999a; Duncan et al., 1997) or not present (Yamagishi et al., 1993; Huang et al., 1994; Schunkert et al., 1992). Plasma Ang (1-7) is increased at 1 week post-MI but is normalized by 4 weeks (Duncan et al., 1997).

In summary, in the early stages post-MI, plasma Agt is not changed, but PRA and plasma Ang I and Ang II are increased. Plasma Agt decreases in the later stages, whereas plasma ACE activity is increased. PRA and plasma Ang I and II normalize by the later stages post-MI.

1.4.3. Upregulation of cardiac RAS in male rats post-MI

Following MI in the male rat, components of the cardiac RAS are upregulated in a region-specific and time-dependent manner. Agt mRNA is increased transiently at 5 days post-MI but not by 25 days post-MI in the remote LV (Lindpaintner et al., 1993). Its protein concentration post-MI has not been reported. Renin mRNA abundance and activity are increased in macrophages and myofibroblasts within the infarct scar, but not the remote LV post-MI. Renin mRNA is increased at one day post-MI (Passier et al., 1996), and its activity is increased at 7 days to at least 4 weeks post-MI (Sun & Weber, 1996). ACE mRNA abundance is increased as early as 4 days post-MI and remains increased to at least 7 days post-MI; its activity is increased by 7 days and remains increased at 12-13 weeks post-MI (Passier et al., 1995; Sanbe et al., 1995; Schieffer et al., 1994; Kobayashi et al., 1998). The increases in ACE are highest within the scar and peri-infarct area (Busatto et al., 1997; Gaertner et al., 2002) in myofibroblasts, endothelial cells and macrophages (Passier et al., 1995; Gaertner et al., 2002; Sun & Weber, 1996; Falkenhahn et al., 1995). The reported effects of right ventricle (RV) ACE post-MI are variable: some studies report an increase in ACE mRNA abundance and activity (Sanbe et al., 1995; Schieffer et al., 1994; Hirsch et al., 1991) and several report no change (Busatto et al., 1997; Passier et al., 1995). Measurement of ACE binding density using highly sensitive in vitro autoradiography demonstrates that ACE density is increased at 4

and 8 weeks post-MI in all ventricular areas, including the scar, the remote LV, the septum and the RV (Tan et al., 2004; Johnston et al., 1991; Sun & Weber, 1996).

The increases in LV Agt, renin, and ACE post-MI are associated with increases in LV Ang I and II. At 3 days post-MI, Ang I is increased in remote LV and the scar and remains increased at 3 months post-MI. Ang II is increased in the remote LV and the scar by 6 hours post-MI. In the remote LV these levels normalize by 3 days and the infarct scar by 4 weeks post-MI (Leenen et al., 1999a; Duncan et al., 1997; Yamagishi et al., 1993). A marked increase in RV Ang II occurs at 7 days post-MI but normalizes after 4 weeks (Duncan et al., 1997). Ang (1-7) is increased within myocytes in remote LV and the scar border zone 4 weeks post-MI; its concentration in the LV correlates positively with severity of LV dysfunction (Averill et al., 2003). Not only are Ang peptides increased in the heart post-MI, but so are their receptors, likely leading to increased sensitivity to the Ang II present. AT_{1A} receptor mRNA abundance is increased in the remote LV and the scar 1 week and 4 weeks post-MI, whereas AT_{1B} receptor mRNA levels are unchanged (Nio et al., 1995; Iijima et al., 1998). Ligand binding to the AT_1R is not increased in the scar 18h post-MI (Lefroy et al., 1996), but by 1 week post-MI, consistent with the increase in mRNA abundance, LV AT_1R binding is increased (Nio et al., 1995; Lefroy et al., 1996). AT_1R density remains increased at 4 weeks, 8 weeks and 8 months post-MI, in the infarct area, remote LV and in the RV (Lefroy et al., 1996; Tan et al., 2004; Sun & Weber, 1994). This increase in AT_1R density is located within myofibroblasts in the infarct area (Lefroy et al., 1996). AT_2R mRNA abundance is also increased 1 week post-MI (Nio et al., 1995) but a recent report suggests that its mRNA may be transiently reduced at 4 weeks and normalized by 20 weeks post-MI (Lax et al.,

2004). AT_2R binding is increased in LV membrane fractions at 1 week post-MI (Nio et al., 1995; Lefroy et al., 1996).

Thus a time-dependent activation of the cardiac RAS occurs in the cardiac ventricles post-MI. The functional and morphological differences in the remote LV and scar are significant, but both exhibit activation of the RAS post-MI. Importantly, ACE activity in the remote LV, and within the scar itself, and the amount of ACE secreted from explanted scars, is directly proportional to the severity of LV dysfunction (Gaertner et al., 2002; Pinto et al., 1993).

1.4.4. Upregulation of brain RAS in male rats post-MI

A clear activation of components of the RAS occurs in several cardiovascular regulatory nuclei post-MI, including the PVN and SFO. In male rats, at both 4 and 8 weeks post-MI, ACE and AT_1R binding densities increase 8-15% in the SFO, 14-22% in the OVLT, 20-34% in the PVN and 13-15% in the MnPO. The degree of activation of ACE and AT_1R is proportional to the size of the infarct scar, and therefore, indirectly, to LV dysfunction (Tan et al., 2004). The increase in RAS components in these nuclei post-MI may lead to increased neuronal activity in these nuclei. Consistent with this, PVN and SFO neurons are chronically activated post-MI (Vahid-Ansari & Leenen, 1998) and neuronal firing of the PVN is normalized by intra-carotid injections of losartan or captopril (Zhang et al., 2002). The activation of these angiotensinergic neurons may mediate the sympathetic hyperactivity post-MI, as acute and chronic icv infusion of losartan normalizes sympathetic hyperactivity and baroreflex function (DiBona et al., 1995; Zhang et al., 1999) and prevents a significant component of LV remodeling and dysfunction post-MI (Leenen et al., 1999b). In addition, transgenic rats expressing <10%

normal levels of brain Agt and with 50% of normal levels of hypothalamic Ang I and II exhibit decreased sympathetic activation, reduced LV fibrosis, and reduced LV dysfunction post-MI (Wang et al., 2004; Huang et al., 2001). These studies suggest that increased stimulation of the AT_1R in the PVN, and perhaps the SFO, OVLT, and MnPO is involved in the sympathetic hyperactivity, and the progression of LV dysfunction post-MI.

1.4.5. Effects of estrogens on LV dysfunction following myocardial infarction

Nearly all studies post-MI have been performed on male rodents. The few studies performed on females suggest that gender differences exist in the degree of LV dysfunction that occurs post-MI. Male and ovary-intact female rats exhibit similar infarct sizes (45 and 48%, respectively) at 6 weeks post-MI. They develop similar ~70% increases in LV internal dimension and ~25% increases in LVEDP (Litwin et al., 1999). Males exhibit larger increases in the non-infarcted posterior wall thickness and larger increases in myocyte cross-sectional area than females (Litwin et al., 1999). At similar infarct sizes (37% in females and 40% in males), by 2 weeks post-MI male mice exhibit decreased survival, increased myocyte cross sectional area, and a greater reduction in ejection fraction than female mice (Cavasin et al., 2004).

Some components of the RAS are regulated similarly in male and female rats post-MI but most have not been studied. Changes in plasma Agt, Ang I, Ang II and ACE in female rats post-MI have not been reported. Like in male rats, PRA is not changed in ovary-intact female rats 4 weeks post-MI (Dean et al., 2003; Naitoh et al., 2002). Similar to male rats, ovary-intact female rats exhibit increases in ACE binding densities in the RV, remote LV and infarct scar post-MI (Fabris et al., 1990; Kohzuki et al., 1996) and an

increase in LV AT_1R mRNA and protein abundance (Staufenberger et al., 2001; El-Sabban et al., 2000). Changes in the brain RAS post-MI have not yet been reported in female rats. AT_1R antagonists or ACE inhibitors are effective at attenuating the LV dysfunction in both male and female rats post-MI (Youn et al., 1999; Pfeffer et al., 1991). It is not known whether the degree of improvement of LV function is greater in female or male rats as these studies were not performed in parallel.

Studies reporting regulation of infarct size and/or LV dysfunction post-MI by E2 are summarized in Table 1.1. Ovary-intact and OVX+veh mice exhibit modest differences in infarct sizes at 12 weeks post-MI: 31% vs. 38%, respectively (Cavasin et al., 2003), but OVX+veh mice exhibit a larger decrease in ejection fraction than ovary-intact mice (41% vs. 53% from 88% in sham mice)(Cavasin et al., 2003). However, one cannot exclude that the non-significant 7% increase in infarct size may have mediated a worsening of LV function. Van Eickels et al., (2003) reported that OVX+high E2 mice exhibit modestly decreased infarct sizes at 6 weeks post-MI compared to OVX+veh mice: 23% vs. 28%. These mice did not exhibit changes in LVEDP and LVPSP vs. sham mice; positive dP/dt is decreased and negative dP/dt is increased similarly in both groups. OVX+high E2 MI mice exhibit larger increases in LV internal dimensions than OVX+veh MI mice but similar decreases in fractional shortening (van Eickels et al., 2003).

In rats, several studies have reported variable effects of E2 on LV dysfunction post-MI, as summarized in Table 1.1. A study by Hügel and colleagues (1999) is not helpful in determining the effects of E2 status on infarct size as, by design, rats with infarct sizes <30% and >50% were excluded to make the comparison of LV function easier. Furthermore, it was not reported to which treatment groups these excluded rats belonged,

so it is impossible to determine whether E2 affected infarct size. They showed that at infarct sizes of 39-40%, ovary-intact, and OVX rats treated with vehicle, or E2 at physiological levels, exhibit similar 29-33% increases in diastolic dimension, similar 10-15 mmHg increases in LVEDP and similar decreases in fractional shortening at 8-9 weeks post-MI. Smith and colleagues (2000) reported that at 10-11 weeks post-MI, OVX rats treated with regular E2 had larger infarct sizes than OVX+veh MI rats, and similar infarct sizes to ovary-intact MI rats. *In vivo* LV function was not measured in this study; using a Langendorf *in vitro* preparation, LVPSP, +dP/dt and -dP/dt were not significantly altered post-MI nor changed by E2 status. Nekooeian & Pang reported that OVX rats treated with E2 at physiological levels exhibit similar infarct sizes compared to OVX+veh rats (31 vs. 34%, respectively) (Nekooeian & Pang, 1998) and OVX rats treated with E2 at physiological levels exhibit significantly smaller increases in LVEDP post-MI than OVX+veh rats (+7 vs. +10.5 mmHg). This may indicate some improvement in LV function by physiological levels of E2 that is independent of infarct size. However, LVPSP and +dP/dt are similarly decreased in both groups (Nekooeian & Pang, 1998).

No study has yet firmly established, in parallel rat treatment groups, whether E2 deficiency and physiological and supra-physiological levels of E2 affect infarct size and/or affect the progression of LV dysfunction in rats post-MI.

Table 1.1. Effects of E2 deficiency and replacement on infarct size and LV function post-MI

	Ovary-intact		OVX + veh		OVX + reg E2		OVX + high E2	
	SH	MI	SH	MI	SH	MI	SH	MI
van Eickels et al., 2003 (m)								
n			16	21			13	14
Infarct size (%)				28±1				23±1$^{\#}$
LVEDP			6±1	8±1			8±1	8±1
LVPSP			98±2	91±1			97±3	92±2
+dP/dt (mmHg/s)			10855 ±372	8184 ±404*			9424 ±351	7804 ±384*
-dP/dt (mmHg/s)			-10312 ±405	-7465 ±441*			-9103 ±348	-7116 ±377*
EDd/BW (mm/g)			0.145 ±0.07	0.175 ±0.07*			0.150 ±0.05	0.200 ±0.08$^{*\#}$
FS (%)			38±4	25±3*			36±4	25±3*
Cavasin et al., 2003 (m)								
n	6	13		10				
Infarct size (%)		31±2		38±3				
EF (%)	88±2	53±2*		41±2$^{\&}$				
Hügel et al., 1999 (r)								
n	11	11	12	11	15	13		
Infarct size (%)		39±2***		39±2***		40±2***		
LVEDP (mmHg)	8±2	23±4*	8±1	18±3*	7±2	20±3*		
LVPSP (mmHg)	137±8	126±5	128±4	125±5	118±4	118±4		
LVIDd (mm)	6.7±0.2	8.9±0.4*	7.7±0.2	9.1±0.2*	6.9±0.1	8.9±0.3*		
FS (%)	50±3	19±2*	47±3	23±2*	48±3	26±23*		
Nekooeian & Pang, 1998 (r)								
n			6	6	6	6		
Infarct size (%)				34±2		31±1		
LVEDP (mmHg)			-1.5±0.8	9±0.7*	ND	5.3±1$^{*\#}$		
LVPSP (mmHg)			123±4	93±5*	ND	83±1*		
+dP/dt (mmHg/s)			4725±85	3208±187*	ND	2900±91*		
Smith et al., 2000 (r)								
	6	6	6	6	6	6		
Infarct size (%)		35±4		27±3		42±2$^{\#}$		
In vitro:								
LVPSP (mmHg)	71±5	73±8	82±4	80±8	79±8	65±9		
+dP/dt (mmHg/s)	1875 ±159	1800 ±190	1875 ±119	2067 ±211	1875 ±128	1550 ±266		
-dP/dt (mmHg/s)	1400 ±125	1267 ±125	1650 ±120	1517 ±164	1560 ±141	1100 ±179		

ND: Values reported as not different from OVX+veh sham rats. ***Excluded rats with infarcts <30% and >50% and thus infarct sizes were similar by design. *p<.0.05 vs. respective sham group; $^{\#}$p<0.05 vs. OVX+veh MI; $^{\&}$p<0.05 vs. ovary-intact MI. m: studies performed in mice. r: studies performed in rats. EF: ejection fraction; LVEDP: LV end diastolic pressure; LVPSP: LV peak systolic pressure; +dP/dt: rate of contraction; -dP/dt: rate of relaxation; EDd: end-diastolic diameter.

1.5. Overview and rationale for the study

Our **first hypothesis:** in female rats, circulating E2 dose-dependently reduces the expression of components of the local RASs in tissues relevant to the cardiovascular system: heart, brain, blood vessels, adrenal, kidney and lung. Therefore, reduction of circulating E2 by removal of the ovaries increases the expression of these components.

The objectives were to determine:

1. whether E2 deficiency, 2 and/or 5 weeks following OVX, increases
   a) ACE activity in plasma
   b) ACE mRNA abundance, activity, density and AT_1R density in kidney
   c) ACE activity, density and AT_1R density in heart, abdominal aorta, adrenal, lung and OVLT, SFO, PVN and MnPO of the brain

2. whether E2 administered sc for 2 and 5 weeks at physiological levels prevents 1a), b) and c) above

3. whether E2 administered sc for 2 and 5 weeks at a high dose reverses the effects of E2 deficiency on 1a), b), and c) above to levels below that of ovary-intact rats

Our **second hypothesis:** the increase in cardiac and/or brain RAS activity caused by E2 deficiency results in worsened LV dysfunction post-MI. The decrease in cardiac and/or brain RAS activity caused by circulating physiological or supra-physiological E2 levels results in less LV dysfunction post-MI.

The objectives were:

1. to measure cardiac function (LVEDP, LVPSP, +/-dP/dt, and LV dimension by echocardiography) in ovary-intact rats, OVX rats treated with E2 at physiological levels and OVX rats treated with E2 at supra-physiological levels
   a) at 3 weeks following 'sham' surgery
   b) at 3 weeks post-MI

2. to measure the activity of the circulatory RAS (plasma renin activity, plasma Ang I and II and plasma ACE activity) in the rats in 1. a) and b) above

3. to measure cardiac ACE and AT_1R density in ovary-intact female rats 3 weeks following 'sham' surgery and 3 weeks post-MI, and compare to previously obtained data in male rats (Tan et al., 2004)

4. to measure the activity of the cardiac RAS (ACE mRNA abundance and activity, Ang I and II concentrations) in the rats in 1. a) and b) above

5. to measure the activity of the brain RAS (hypothalamic ACE mRNA abundance and activity, OVLT, SFO, PVN and MnPO ACE and AT_1R binding densities) in the rats in 1. a) and b) above.

6. to identify any relationships that exist between 1. and 2., 4., & 5. above.

Chapter 2

MATERIALS AND METHODS

2.0. Overview

Two experiments were performed to test our hypotheses. See Section 2.1 for experimental details. Experiment 1 (Figure 2.1) was performed to measure the effects of circulating levels of E2 on components of the local tissue renin-angiotensin systems in the healthy female rat. For this, we used female rats, undergoing one of four experimental treatments: 1) intact rats, with normal, endogenous levels of E2; 2) OVX rats with E2 deficiency (vehicle replacement only) 3) OVX rats with replacement of E2 at physiological levels; and 4) OVX rats with E2 replacement at high levels. After 2 and 5 weeks, levels of ACE and AT_1R were measured in several tissues important to cardiovascular regulation. ACE activity was measured in plasma, ACE mRNA abundance was measured in kidney, and ACE activity, ACE binding density and AT_1R binding density were measured in the heart (RV and LV), cardiovascular brain nuclei (OVLT, SFO, PVN, MnPO), abdominal aorta, kidney, adrenal and lung.

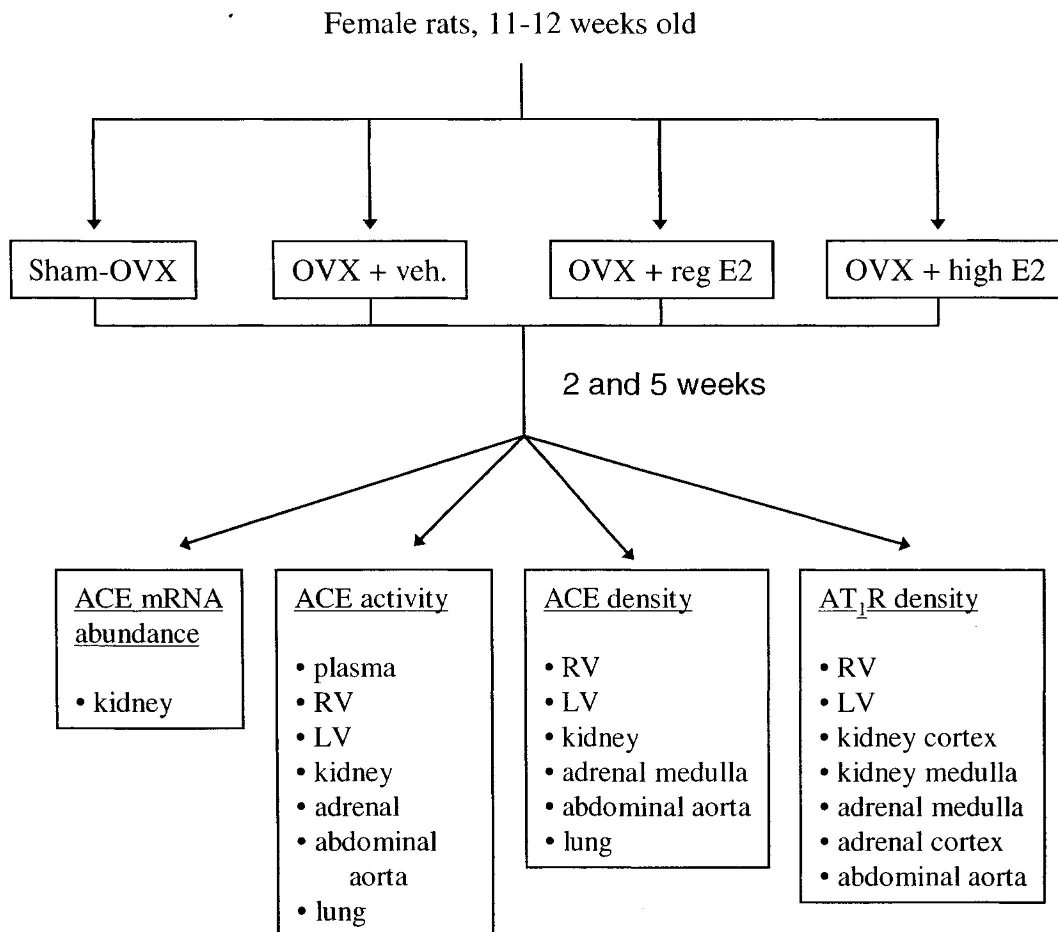


Figure 2.1. Flowchart of Experiment 1 protocol. OVX: ovariectomy; Veh.: Vehicle; reg.: regular; E2: 17β-estradiol; RV: right ventricle; LV: left ventricle.

Experiment 2 measured the effects of circulating levels of E2 in rats 3 weeks following experimental MI, and was performed in three parts, a., b., and c. In Experiment 2a, (Figure 2.2), OVX rats treated with vehicle or high sc E2 for two weeks underwent experimental MI or sham surgery. Three weeks later, LV function was assessed by echocardiography and direct catheterization. ACE mRNA abundance and activity were measured in the RV and infarcted and remote LV, and ACE mRNA abundance and activity were measured in hypothalamic homogenates.

In Experiment 2b (Figure 2.3) OVX rats treated with vehicle or high sc E2 for two weeks underwent experimental MI or sham surgery. Three weeks later, LV function was assessed by echocardiography and direct catheterization. Plasma renin activity, plasma ACE activity and plasma Ang I and II concentrations were measured. Ang I and II concentrations in the infarcted and remote LV were measured, and ACE and AT_1R binding densities were measured in cardiovascular regulatory nuclei of the brain (OVLT, MnPO, PVN and SFO).

In Experiment 2c, (Figure 2.4), ovary-intact female rats underwent experimental MI or sham surgery. Three weeks later, LV function was assessed by echocardiography and direct catheterization. Plasma renin activity, plasma ACE activity, and plasma Ang I and II levels were measured. The brains of these rats were randomly divided into two subsets. In one set, ACE mRNA abundance and activity were measured in hypothalamic homogenates. In the other set, ACE and AT_1R binding densities were measured in cardiovascular regulatory nuclei in the brain (OVLT, MnPO, PVN and SFO). The hearts of these rats were divided into three subsets. One set was used to measure cardiac ACE and AT_1R binding densities. Another set was used to measure ACE mRNA abundance and activity in the RV and remote and infarcted LV. The third was used to measure Ang I and II in the remote and infarcted LV.

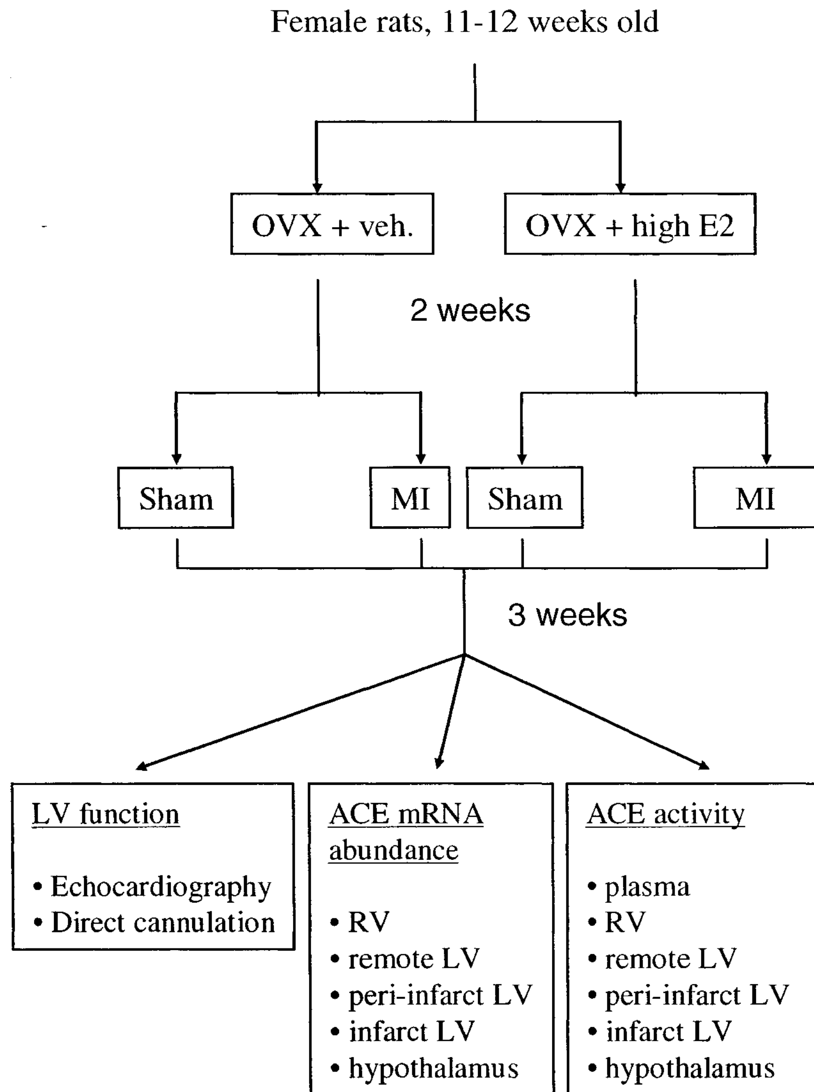


Figure 2.2. Flowchart of Experimental 2a protocol. OVX: ovariectomized; veh.: vehicle; E2: 17β-estradiol; MI: myocardial infarction; RV: right ventricle; LV: left ventricle.

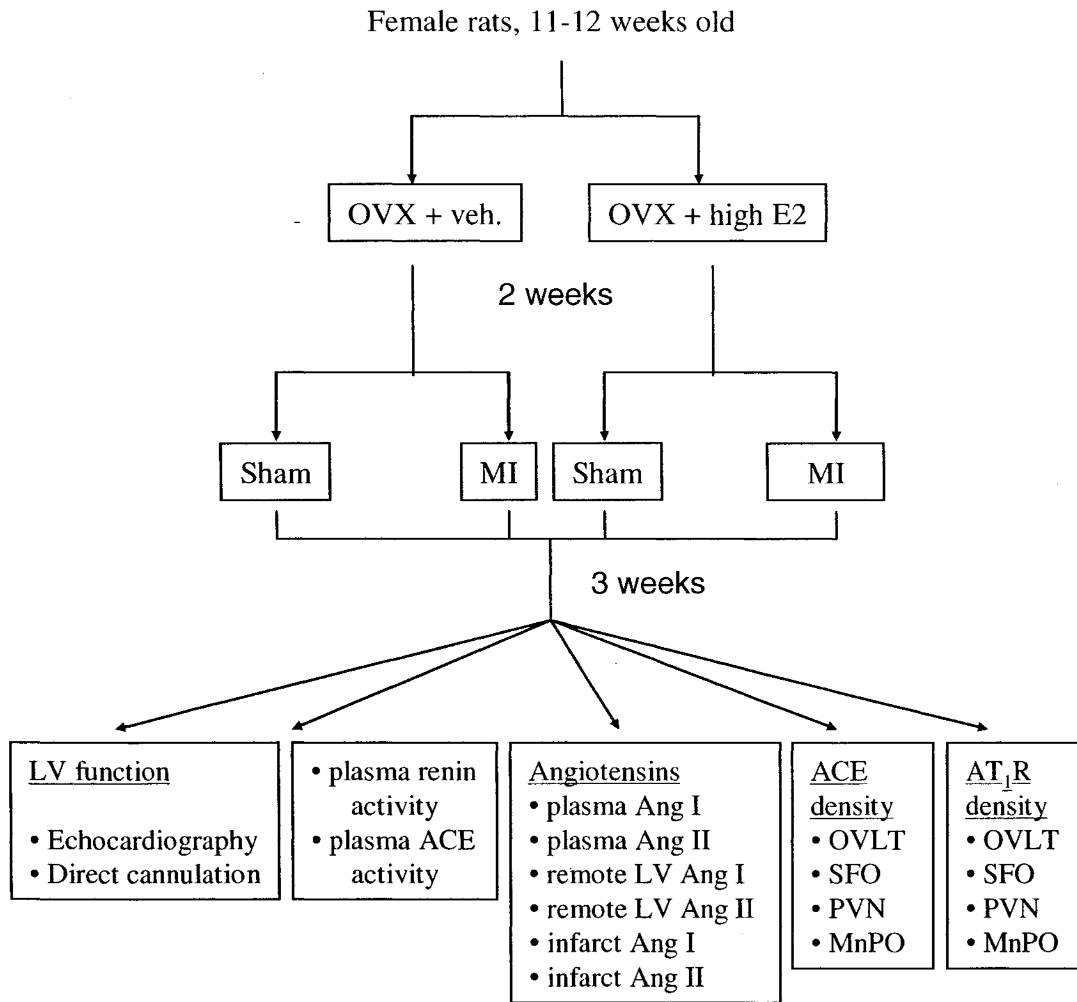


Figure 2.3. Flowchart of Experiment 2b protocol. OVX: ovariectomized; Veh.: vehicle; E2: 17β-estradiol; MI: myocardial infarction; LV: left ventricle; OVLT: organum vasculosum laminae terminalis; SFO: subfornical organ; PVN: paraventricular nucleus; MnPO: median preoptic nucleus

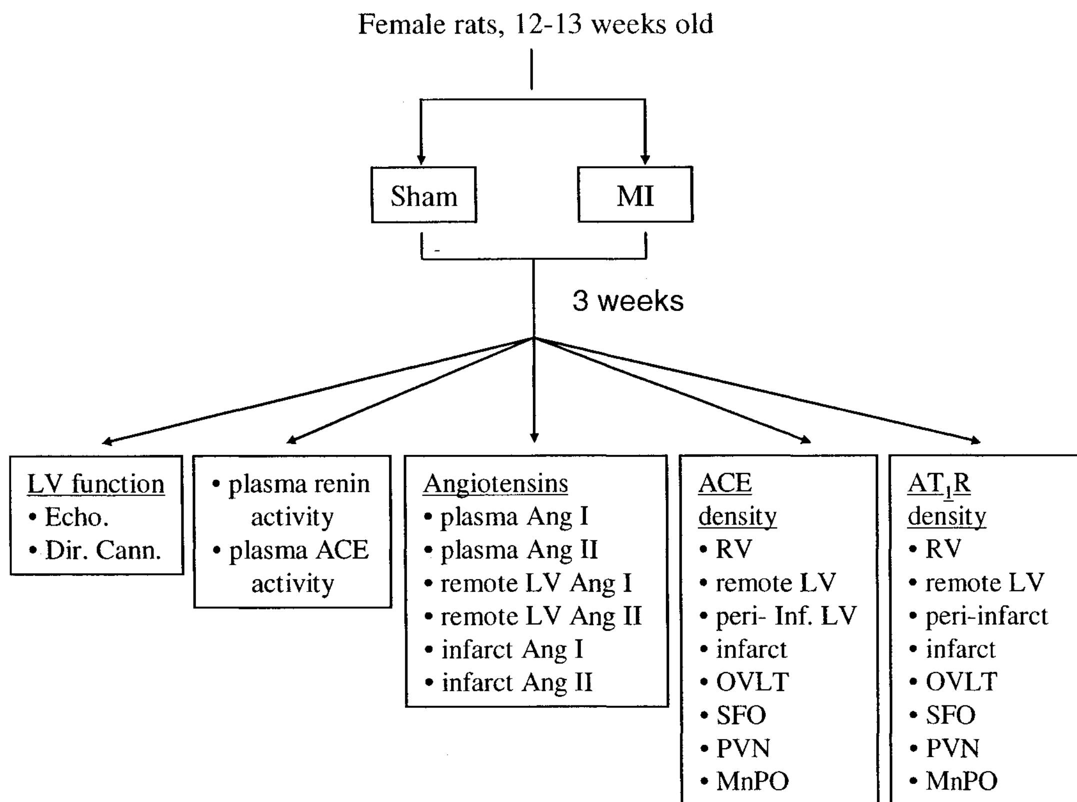


Figure 2.4. Flowchart of Experiment 2c protocol. LV: left ventricle; RV: right ventricle; OVLT: organum vasculosum laminae terminalis; SFO: subfornical organ; PVN: paraventricular nucleus; MnPO: median preoptic nucleus.

2.1. Experimental Methods

2.1.1. Animals

All rats were obtained from Charles River Breeding Laboratories, Montreal, PQ, and were maintained on a 12:12-h light-dark cycle and allowed free access to normal rat chow and water. Rats arrived in the Animal Care facility at least 3 days before experimentation. In Experiment 1 and Experiments 2a and 2b, 10-11 week old female Wistar rats were used. In Experiment 2c, as rats were not ovariectomized, 12-13 week old female Wistar rats were used to ensure age-matching with rats in the other

experiments. All experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care, and were approved by the University of Ottawa Animal Care Committee. All chemicals and reagents were purchased from Sigma (Oakville, Ontario, Canada) except where noted.

2.1.2. Ovariectomy and pellet implantation (Experiments 1 and 2)

Experiment 1. Rats were randomly placed into one of four groups: 1) sham ovariectomy (Sham-OVX, n=12), 2) ovariectomy plus sc vehicle (OVX+veh, n=12); 3) ovariectomy plus sc pellet containing 1.5mg 17β-estradiol (25 μg/day, 60-day release, Innovative Research of America, Sarasota, Fla., USA) (OVX+reg. E2, n=11) and ovariectomy plus sc pellet containing 15 mg 17β-estradiol (250 μg/day, 60-day release) (OVX+high E2, n=12). Pellets containing 1.5 mg E2 with 60-day release results in plasma E2 levels within the normal range during the estrous cycle of the rat (Nekooeian & Pang, 1998; Nekooeian & Pang, 2000). Ten fold that dose (pellets containing 15 mg with 60-day release) was used as 'high' E2 treatment. Bilateral ovariectomies were performed as previously described (Waynforth, 1980). At least one hour prior to surgery, buprenorphine (0.03mg/ml, 0.1ml/rat) was given for pain relief. Under inhaled isofluorane anaesthesia, rats were placed face-down and a 2 cm-long midline incision was made halfway down the back. The skin was carefully separated from the muscle layer, and a 1 cm incision was made in the muscle layer. The fat pad was exteriorized, and the ovary was carefully excised. This process was completed bilaterally. Sham-operated rats underwent the same procedure except the ovaries were exteriorized but not removed. Immediately following ovariectomy, sc pellets were implanted in the dorsal neck area. In Sham-OVX rats, the incision for implantation surgery was made, but no

pellet was implanted. All rats received sc buprenorphine (0.03mg/ml, 0.1ml/rat twice daily x 3 days) for pain relief.

Experiment 2. In Experiments 2a and 2b, rats were ovariectomized as described in the previous paragraph. Rats were randomly assigned to OVX+veh and OVX+high E2 groups, and pellets were implanted in the dorsal neck area, as described above. Rats were followed for 2 weeks following ovariectomy to allow for chronic adaptation to the E2 deficiency and excess.

2.1.3. Coronary artery ligation (Experiment 2 only)

In Experiments 2a and 2b, at 2 weeks post-OVX, OVX+veh and OVX+high E2 rats were randomly assigned to coronary artery ligation (MI) or sham operation. In Experiment 2c, ovary-intact rats were similarly randomized. In MI rats, coronary artery ligation was performed as described previously (Pfeffer et al., 1979). Rats were anesthetized via halothane/oxygen and connected to a respirator. The left thorax was opened at the fourth intercostal space (space between the 4th and 5th ribs). A 6.0 silk suture on an atraumatic needle was passed 2-3 mm deep through the myocardium in the area where the left anterior descending coronary artery originates. The artery was permanently ligated and the chest was then closed in layers with two separate vicryl sutures. Anesthetic treatment was halted and the animals remained ventilated until they started breathing autonomously. Sham-operated rats underwent a similar procedure without ligation of the coronary artery. Buprenorphine (0.03mg/ml, 0.1ml/rat twice daily x 3 days) was used for pain relief in both MI and sham rats. Rats were followed for three (3) weeks following MI/sham surgery, therefore, these experiments assess the effects of E2 status on early LV dysfunction post-MI.

2.1.4.0 LV function measurements

LV function was measured by three complementary methods. Echocardiography measures LV dimensions, and thus measures the degree of MI-induced dilatation of the LV. Using echocardiography, two indices of pump function, ejection fraction and fractional shortening, can be derived from the measured dimensions. However, it does not directly assess the hemodynamic function of the heart (i.e. its ability to pump blood). LV cannulation using saline-filled PE-50 tubing measures the hemodynamic function of the LV but the pressures obtained are influenced by damping, or the impedence of blood movement within the tubing. LV cannulation using the micromanometer-tipped Millar catheter also measures the hemodynamic function of the LV, and avoids the damping that occurs when fluid-filled catheters are used. However, the pressures obtained by the Millar catheter method are somewhat influenced by the use of light anesthesia of the rats during the procedure.

2.1.4.1 LV function by echocardiography (Experiments 2a, 2b, 2c only)

All rats in Experiment 2a, 2b and 2c underwent echocardiography 3-7 days before euthanasia (2-2.5 weeks post-MI). A Philips Sonos 4500 echocardiography system with a 12 MHz transducer at a depth setting of 2-3 cm was used for the recordings. Under light isofluorane anesthesia, the rat was placed on its back and the chest was shaved. The transducer was placed on the left hemithorax, and using the 2-D parasternal long-axis imaging plane as a guide, a left ventricular M-mode tracing just below the tips of the mitral leaflets was obtained and recorded on videotape. Simultaneous M-mode recording of intraventricular septum thickness (IVS), posterior wall thickness (PW), and chamber size was performed for at least 6 cardiac cycles and the mean results used for statistical

analysis. Left ventricular internal dimensions in systole and diastole (LVIDs and LVIDd, respectively), were then used to estimate ejection fraction (EF) for each rat, using the formula: $EF(\%) = [(LVIDd^3 - LVIDs^3)/LVIDd^3] \times 100\%$. Fractional shortening (FS) was calculated using the formula: $FS(\%) = (LVIDd - LVIDs)/ LVIDd) \times 100\%$.

2.1.4.2 LV function by direct PE-50 catheterization (Experiment 2a only)

At 3 weeks following coronary artery ligation or sham surgery, rats in Experiment 2a underwent LV cannulation. One hour before the procedure, all rats were given buprenorphine (0.03mg/ml, 0.1ml/rat sc) for pain relief. Under inhaled halothane/oxygen anesthesia, the right carotid artery was isolated and a polyethylene catheter (PE-50) filled with heparinized saline was threaded through the right carotid artery to the left ventricle. The position of the catheter in the LV was confirmed by the distinct increase in amplitude of blood movement within the tube. Rats were allowed to recover for four to six hours and then, while conscious and unrestrained, were connected to a pressure transducer connected to a personal computer. Thirty minutes later, for several minutes, LVEDP, LVPSP and heart rate (HR) were recorded and analyzed with AcqKnowledge (ACQ 3.2) data acquisition software. Rats were allowed to recover and arterial blood was taken through the PE-50 catheter as described in Section 2.1.7.

2.1.4.3 LV function by direct catheterization with Millar catheter (Experiments 2b and 2c only)

At 3 weeks following coronary artery ligation or sham surgery, rats in Experiment 2b and 2c underwent LV cannulation with a Millar catheter. One hour before the procedure, buprenorphine (0.03mg/ml, 0.1ml/rat) was given for pain relief. Under inhaled isofluorane/oxygen anesthesia, the right carotid artery was isolated and a 2 F

high-fidelity micromanometer catheter (SPR-407, Millar Institute, Houston, Texas) was threaded via the carotid retrogradely into the left ventricle. Entry of the catheter into the LV was confirmed by the characteristic decrease in diastolic pressure that occurs when the catheter crosses the aortic valves. The rat's tail was pinched gently until peak systolic pressures remained stable for 10 seconds. This ensured each rat was under similar mild anesthesia when recordings were taken. The waveforms of pressure changes were recorded for 1-2 minutes via a personal computer equipped with the data acquisition software program AcqKnowledge (ACQ 3.2). The catheter was then carefully removed from the LV, and a PE-50 catheter filled with heparinized saline was inserted into, and left within the carotid artery. Rats were allowed to recover for several hours, and then the PE-50 catheters were used to remove arterial blood, as described in Section 2.1.7.

2.1.5. Plasma and tissue collection

Experiment 1 At two and five weeks following OVX/sham surgery, rats were euthanized by decapitation, and trunk blood was collected into heparin tubes, spun at 3000 RPM, and the plasma isolated for measurement of plasma ACE activity. Because estrogens maintain the size of the uterus, uterine tissue was isolated from surrounding fat and weighed as a measure of E2 status.

The abdominal aorta was isolated from surrounding tissue and fat, cut in two and one-half frozen in 2-methylbutane/dry ice at -40°C and the other half frozen in liquid nitrogen. The heart was removed, weighed, washed in ice-cold saline and cut in half laterally. The apical half was frozen in 2-methylbutane/dry ice and the basal half was separated into right and left ventricle/septum and frozen in liquid nitrogen. The brain was removed and frozen in 2-methylbutane/dry ice, as were the left adrenal, left kidney,

and a piece of lung. The right adrenal and another piece of lung were frozen in liquid nitrogen. The right kidney was cut in half and frozen in liquid nitrogen. Tissues frozen in liquid nitrogen were kept at –80°C until assayed for ACE activity. Tissues frozen in 2-methylbutane/dry ice were kept at –20°C until ACE and AT_1R autoradiography were performed.

<u>Experiment 2</u> *<u>Plasma collection.</u>* In Experiments 2a and 2c, trunk blood was collected into chilled heparin tubes and plasma separated as described above for measurement of plasma ACE activity. In Experiments 2b and 2c, blood was collected from the PE-50 arterial lines from unrestrained, unstressed, rats at 3-6 hours following arterial cannulation. The line of each rat was first allowed to flow until all heparinized saline was ejected. For plasma Ang I and II measurements, 2 ml of blood were collected into a prechilled microcentrifuge tube containing 1,10-phenanthroline and EDTA-Na_2 at final concentrations of 0.8 mM and 1.2 mM in blood, respectively. For plasma renin activity, 1.0 ml of blood was collected into a prechilled tube containing 200 μl of 2.6 mM EDTA-Na_2. The blood was centrifuged for 5 min at 8,000 RPM at 4°C, using an Eppendorf model 5415C microcentrifuge. The plasmas were immediately frozen at -80°C until assay.

<u>Infarct area measurement (Experiments 2a, 2b, 2c).</u> Infarct size is an important determinant of the degree of LV dysfunction post-MI. For this reason, rats with small infarct sizes (<30%), or by visual inspection in the case of rats whose hearts were used for autoradiography, "zero", and "small", have been excluded from the analysis; i.e. only data from rats with infarct sizes >30% has been reported. Thus 3/60 ovary-intact rats, 8/58 OVX+veh rats and 4/58 OVX+high E2 rats were excluded. While it would be

reasonable to include these rats in the analysis within separate "small MI" groups, this was not done because, within measurements of a single parameter, none of these potential groups had numbers equal or greater than three, a requirement for statistical analysis.

The infarct area is defined in this study as the area of clearly visible, thin, white-grey scar spanning the left ventricular free wall. Only transmural infarcts (i.e. those spanning the whole thickness of the LV free wall) were measured. Non-transmural infarcts were considered small. For rats in Experiments 2a and 2b, hearts were immediately removed, washed thoroughly in ice-cold 0.9% NaCl and the atria and large vessels cut away and discarded. The RV was dissected from the left ventricle, weighed, and frozen in liquid nitrogen. The infarct size was measured as previously reported (Leenen et al., 1995). Briefly, four or five incisions were made in the LV so that the tissue could be pressed flat. The circumference of the entire flat LV and the visualized transmural infarct area was outlined on a clear plastic sheet. The area of the whole LV outlined on the plastic sheet was then cut off and weighed and the portion representing the infarct area was cut off from the whole LV piece and weighed separately. The infarct size was expressed as a ratio of the weight of the tracing of the whole LV / the weight of the tracing of the infarct area expressed as a percentage. This method is a somewhat crude way to measure infarct sizes but because each heart was required for biochemical manipulations, this tracing method was essential. It is rapid and does not alter the tissue in a way that precludes further biochemical measurements.

In Experiment 2a, after the measurement of infarct size, the LV was dissected into non-infarcted area remote from the infarct scar (rLV), peri-infarct area, a region of approximately 3 mm wide taken from the area surrounding the infarct scar (p-inf) and the

infarct area, proper, as illustrated in Figure 2.5. These pieces were individually weighed, frozen in liquid nitrogen and stored at -80°C until further processed for ACE mRNA/activity. All pieces of RV and LV were ground separately in liquid nitrogen. Half of the resultant powder was used to isolate RNA to measure ACE mRNA abundance, and half was used to measure ACE activity. Brains were removed, frozen in liquid nitrogen, and stored at -80°C. Later, brains were thawed slightly, but kept cold by cutting on a glass plate over dry ice. The hypothalamus was isolated according to Glowinski & Iversen (1966), as diagrammed in Figure 2.6. Briefly, cut (1) was made to remove the cerebellum and hindbrain (A), and cut (2) was made to remove much of the cerebral cortex (C). With the anterior commissure as a horizontal reference, the cerebral cortex, hippocampus and midbrain tissue dorsal to the hypothalamus was removed. The hypothalamus was ground in liquid nitrogen and half was used to measure ACE mRNA abundance, and half used to measure ACE activity.

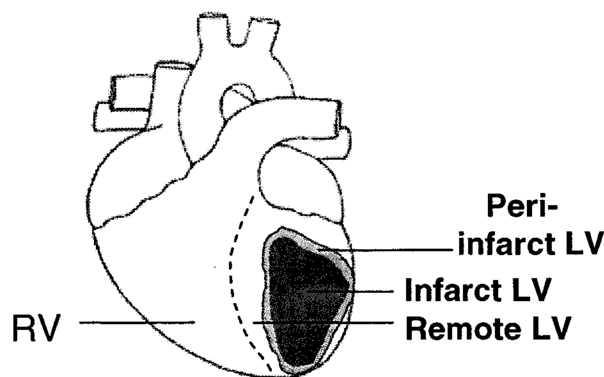


Figure 2.5. The regions of the heart as dissected for Experiment 2. LV: left ventricle; RV: right ventricle

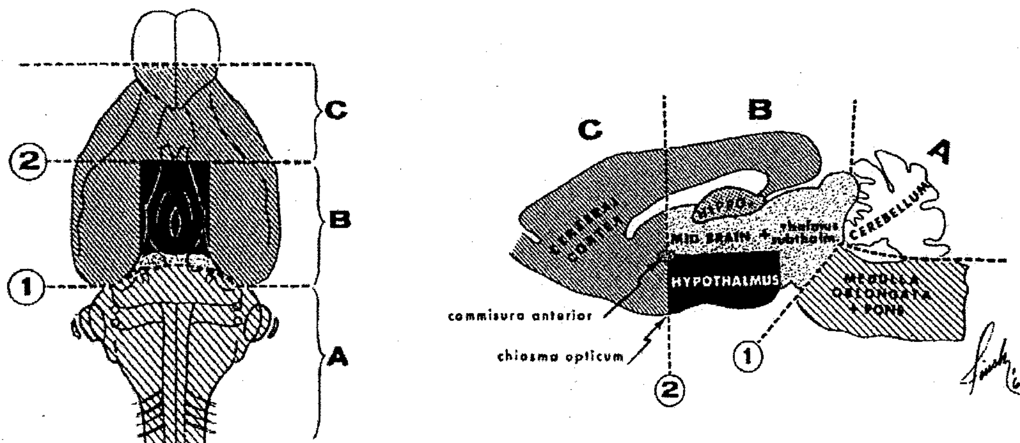


Figure 2.6. Dissection of the hypothalamus. Designation of numbers and letters described on p66. from Glowinski and Iversen, 1966. Used with permission.

In Experiment 2b, LV tissue was processed similarly to that performed in Experiment 2a, but the peri-infarct and infarct area were processed together, to ensure enough tissue for the analysis of Ang peptides. These pieces were individually weighed, frozen in liquid nitrogen and stored at -80°C until further processed for Ang I and II analysis. Brains were removed and frozen in 2-methylbutane cooled with dry ice, and stored at -80°C until ACE and AT_1R autoradiography.

In Experiment 2c, one-third of hearts were processed as in Experiment 2a for ACE activity and mRNA, and one third processed as in Experiment 2b, for Ang peptides.

The remaining hearts were used for ACE and AT_1R in vitro autoradiography, a technique which requires intact cardiac morphology. In this study, infarct size was assessed by visual inspection to be "zero", "small", and "large", then the atria and large vessels were removed, the whole heart was frozen in 2-methylbutane cooled with dry ice, and stored at -80°C. Brains were removed and frozen similarly.

2.1.6 ACE mRNA abundance by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) (Experiments 1, 2a, 2b, 2c)

The quantification of ACE and phosphoglycerate kinase-1 (PGK-1, a glycolytic enzyme as endogenous reference) mRNA abundance by quantitative RT-PCR has been performed previously in our laboratory using a standard PCR machine (Lear et al., 1997; Zhao et al., 2001b). Although the primer sequences used are the same as the previous studies, for these experiments we used real-time RT-PCR, therefore re-optimization was required. A short description of the optimization and use of the technique follows.

ACE and PGK-1 mRNA was measured exactly the same way for each tissue tested. Total RNA was extracted from tissues using a phenol-chloroform reagent (RNAwiz, Ambion, Austin Texas). Since RNA isolated by this technique is often contaminated with genomic DNA that could potentially be amplified by PCR, 15 μg of each RNA sample was treated with a DNAse I reagent (DNA-free, Ambion). RNA was then quantified by absorbance at 260 nm and its integrity verified by agarose gel electrophoresis. One μg of total RNA in RNAse-free water in a total volume of 28 μl was incubated in a thermocycler with 500 ng oligo$(dT)_{12-18}$ primer (Invitrogen, Burlington, ON, Canada) for 15 minutes at 65°C. The temperature was lowered to 4°C and 12 μl of mix containing following constituents were added to each tube: 5X RT

buffer, 32U RNAguard, 0.5mM deoxyribonucleotides, 15U avian myeloblastoma virus (AMV) reverse transcriptase (all from Amersham Biosciences, Baie d'Urfé, PQ, Canada). Samples were then heated at 42°C for 2h, then at 65°C for 15 min.

The cDNA from the RT reaction was amplified in real time using the Roche Lightcycler (Roche Diagnostics, Laval, PQ). Two microlitres of RT reaction product was used in the PCR reaction. The PCR reactions for ACE and PGK-1 were performed in a 20 μl volume with 2 μM of each primer (primer sequences as described in Lear et al., 1997) and 3-3.5 μM $MgCl_2$ using the LightCycler Faststart DNA Master SYBR Green I kit. SYBR green fluoresces upon binding to double-stranded DNA (dsDNA) only, not single-stranded (i.e. denatured) DNA. In real time, after each PCR cycle, the LightCycler quantifies the increase in fluorescence resulting from the amplification of dsDNA by the PCR reaction.

The conditions of the ACE and PGK-1 PCR reactions were as follows: for ACE, after initial denaturation step of 95°C for 10 minutes, cycling was performed (45 cycles): 95°C for 30 sec, 60°C for 30 sec, 72°C for 34 sec and to denature primer-dimers, 89°C for 5 sec. For PGK, after the initial 10 minute denaturation step of 95°C, cycling was performed (40 cycles): 95°C for 0 sec, 62°C for 37 sec, and to denature primer-dimers, 83°C for 5 sec. The extra denaturation step to eliminate primer-dimers is a requirement for quantification with SYBR green and will be discussed in detail shortly.

As shown in Figure 2.7, the PCR reactions for ACE and PGK-1 result in specific, single-product target bands upon agarose gel electrophoresis. This is important to determine as SYBR green quantifies all dsDNA present, whether it is target DNA or non-specific. Any non-specific products would limit the sensitivity of the quantification of

target sequences. For this reason, as noted above, each cycle of the PCR for ACE and PGK-1 contains an extra denaturation step (89°C for 5 seconds for ACE and 83°C for 5 seconds for PGK-1). This step occurs immediately before quantification in each cycle and is required to denature primer-dimers before this quantification occurs. Figure 2.8 shows the melting curve for a typical PCR reaction of ACE. The melting curve is created after the PCR reaction is complete by incrementally increasing the temperature of the sample and quantifying the amount of dsDNA present. This results in a decrease in fluorescence over time due to the denaturation of the dsDNA present in the sample. Sharp melting peaks are present when there are populations of similar dsDNA complexes in the sample as when they are denatured, a sharp decrease in fluorescence occurs. In Figure 2.8, two melting peaks are present: one sharp peak at 92°C, and one less sharp melting peak at 88°C. The melting peak at the higher temperature is the specific target of the reaction: the 834bp ACE product. The other peak likely represents primer-dimers: the non-specific by-products of the reaction that occur when the primer pairs bind to each other randomly. The temperature of 89°C in this extra step is high enough to denature the primer dimers in the sample but not high enough (i.e. not ≥92°C) to denature the target sequence. The only dsDNA remaining to be quantified is the target sequence. A similar process was followed to determine that the temperature required for this step for PGK-1 was 83°C (data not shown).

Using these optimized conditions, the concentration of ACE and PGK-1 of unknown samples was determined by running these samples with standard curve in the same assay. For ACE, the standard curve was made from 0.1, 1, 10 and 100 pg of rat ACE cDNA plasmid. For PGK, the standard curve was from 0.1, 1, 10 and 100 pg of rat

PGK-1 cDNA plasmid. The ACE plasmid was a generous gift from Francois Alhenc-Gelas, INSERM, Paris, France. A representative PGK-1 standard curve is shown in Figure 2.9A. The development of fluorescence in each standard was plotted as a function of cycle number. Within the exponential range of this curve, but above the background noise, a value of fluorescence was arbitrarily set by the Roche LightCycler software, as represented by the dashed line. The cycle number at which the fluorescence reaches this fluorescence value is called the 'crossing over point'. The crossing over points were plotted on a concentration curve (Figure 2.9B) and from this curve, the crossing over points of unknown samples were used to calculate their PGK concentration. A similar procedure was used to measure the amount of ACE target DNA in each RT reaction sample. ACE mRNA abundance was then expressed as a ratio of the concentration of ACE / concentration of PGK-1 x 100%.

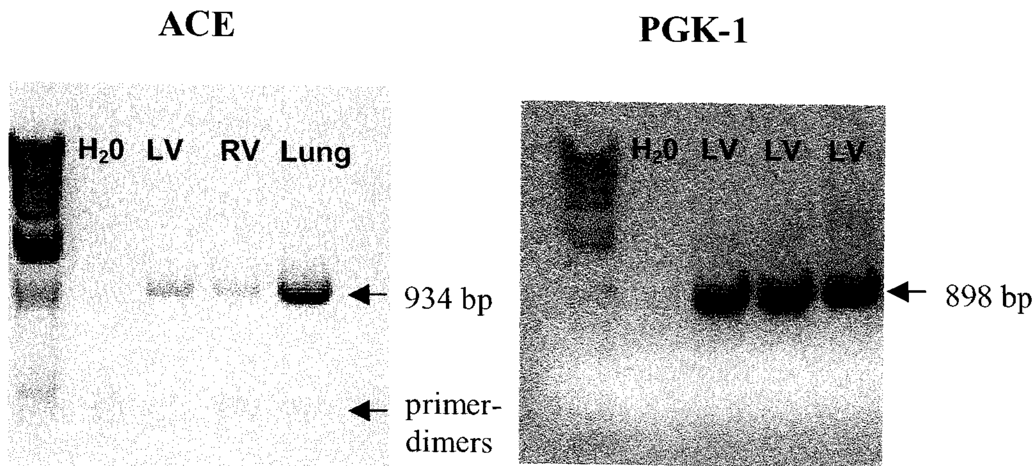


Figure 2.7. Representative agarose gels of PCR products of ACE and PGK real-time RT-PCR. Two microlitres of RT reaction from LV, RV and/or lung from ovary-intact female rats were amplified by real-time PCR according to conditions described in Section 2.1.4. Ten microlitres of the PCR reaction were loaded on 1.5% agarose gels and gels were stained with ethidium bromide.

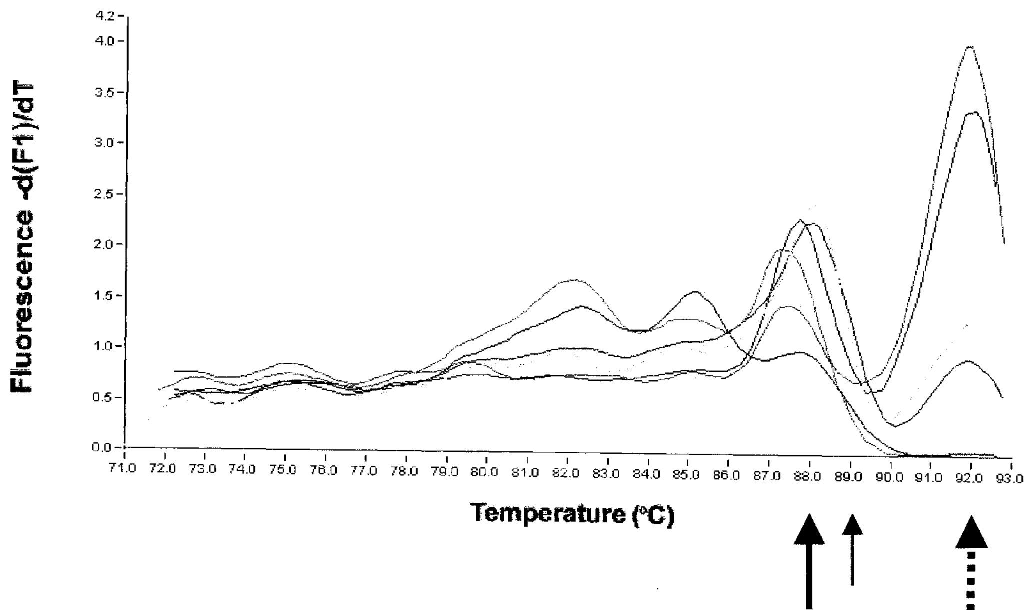
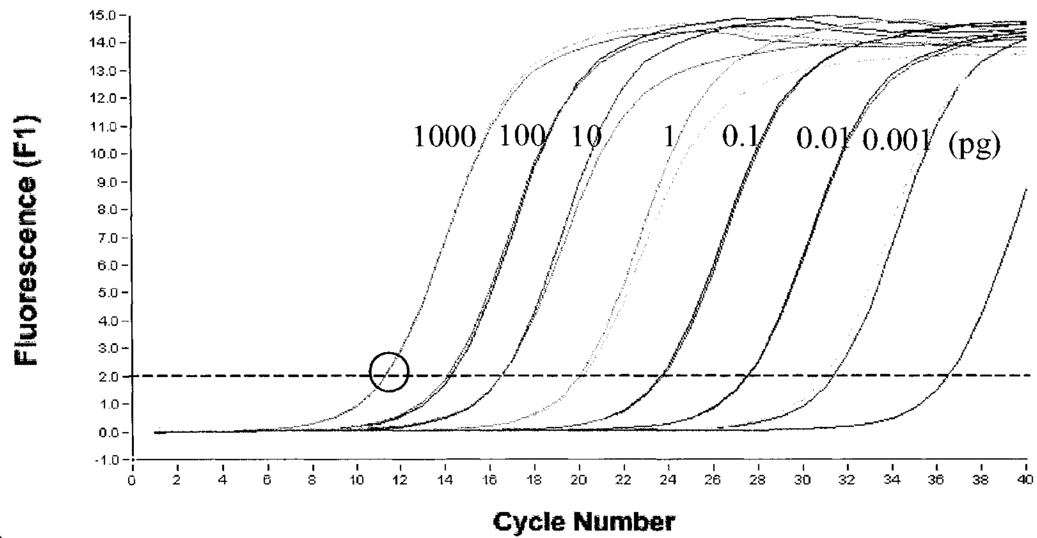


Figure 2.8. Representative melting curve of samples assayed by real-time RT-PCR for ACE. Curves represent the changes in fluorescence with increasing temperature for seven different samples, including water, in purple. The solid black arrow indicates approximate melting temperature of primer-dimers. The dashed black arrow indicates approximate melting temperature of ACE target sequence. The red arrow indicates the temperature used to denature primer-dimers before fluorescence measurement.

A.



B.

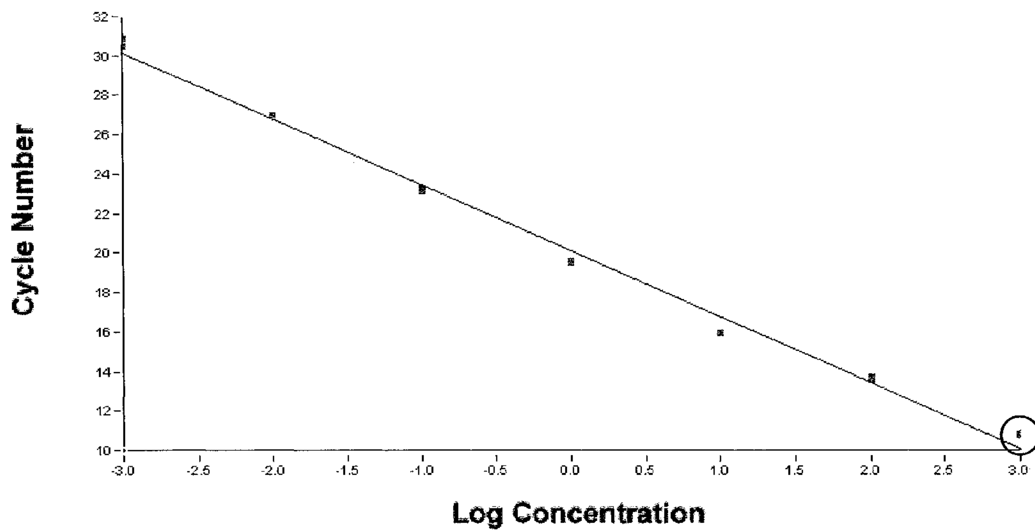


Figure 2.9. Representative standard curve for PGK-1. (A) Development of fluorescence of duplicate standard PGK plasmid cDNA (0-1000pg) over time/cycle number. The red circles indicate the "crossing over" point of the 1000pg standard. (B) Plot of the $\log_{10}$ [concentration of standards] as a function of cycle number at which the 'set point' fluorescence value is reached.

2.1.7 ACE activity by synthetic substrate cleavage and flurometric detection

Tissue processing. Tissue pieces excluding aorta were homogenized with a Polytron homogenizer (Brinkmann Instruments, Canada) in ten volumes of buffer containing 50 mM Tris and 150 mM NaCl, pH 7.4 and centrifuged at 16,000 x g for 15 minutes at 4°C. To minimize tissue loss, aortas were ground in the above Tris-NaCl buffer using an Ettan sample preparation kit (Amersham Biosciences, Baie d'Urfé, PQ, Canada) according to the manufacturer's directions. After centrifugation at 16,000 x g for 15 minutes, the supernatant was collected and kept at –80°C until assay. The total protein content of the samples was determined by the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, IL, USA) or Bradford method (Sigma, Oakville, ON, Canada) with BSA as standard.

ACE activity assay. ACE activity was measured as published previously (Zhao et al., 2000b), with modifications. The assay is based on the ability of ACE to enzymatically cleave the peptide Ang I to Ang II by removing its carboxyl-terminal Histidyl-Leucine. Because Ang I is rapidly degraded by other peptidases in a homogenate, we used the more stable substrate Hippuryl-Histidyl-Leucine (HHL). HHL, like Ang I, is cleaved by ACE to release Histidyl-Leucine (His-Leu, HL). O-pthaldialdehyde, when added, binds to His-Leu, and fluoresces. The amount of HL released by the ACE in the sample is related to a HL standard curve. While HHL is more stable than Ang I, it is still cleaved by other endogenous peptidases into HL. The specificity of the reaction is therefore controlled by the incubation of duplicate sample tubes with captopril, a specific ACE inhibitor. The amount of HL in tubes with captopril is subtracted from the amount of HL in tubes without captopril to calculate ACE activity.

The assays were performed as follows: forty micrograms of total protein or in the case of plasma, 25 μl of a 1:15 dilution in above Tris-NaCl buffer were pre-incubated at 37°C for 20 min in the presence or absence of 100 μM captopril. Following 2 min on ice, 100 μl of 10 mM HHL (dissolved in buffer containing 50 mM HEPES and 300 mM NaCl, pH 8.3) was added to sample tubes as substrate and incubated at 37°C for 1 hour. Standard tubes contained increasing concentrations (1 μM-10 μM) of HL standard instead of substrate HHL. The reaction was terminated by the addition of 1.45 ml 0.28 M NaOH, and immediately 100 μl of (10 mg/ml) o-pthaldialdehyde in 100% methanol was added and the samples incubated at room temperature for 10 min. Then 200 μl of 3 M HCl was added and samples were incubated at room temperature, protected from light, for 30 min. Following this incubation, the samples were centrifuged at 1900 x g at 4°C and 325 μl of the supernatant were added to an opaque 96-well plate (Microfluor 2, VWR; West Chester, PA, USA). The concentration of HL in standard and sample tubes was determined by measuring the fluorescence of the samples at wavelengths 360 nm (excitation) and 480 nm (emission), using a Fluostar Galaxy fluorometer (BMG Labtechnologies, Durham, NC, USA). ACE activity is expressed as nmol of HL released per minute of incubation at 37°C per gram of total protein in the sample. The intra- and inter-assay coefficients of variance for this assay are 3 and 8%, respectively.

2.1.8. AT_1R binding density by in vitro autoradiography

AT_1 receptor ligand binding was performed as described recently (Tan et al., 2004). Cryostat serial 20 μm sections of tissue were mounted onto Superfrost Plus microscope slides (VWR; West Chester PA, USA), dried overnight, and stored at –20°C. Sections were preincubated in buffer (10 mM sodium phosphate, 120 mM NaCl, 5 mM

disodium EDTA, pH 7.4) containing 0.2% w/v BSA for 15 min at 20°C. Sections were then incubated in this buffer containing 0.5 mg/ml bacitracin, and 0.3 μCi/ml of ^{125}I-labeled Sar^{1}Ile^8-Ang II (Washington State University Peptide Radioiodination Service Centre, Pullman, WA; specific activity: 2176 Ci/mmol) and 10^{-5}M PD-123319, an AT_2 receptor antagonist for 1 h. Nonspecific binding was determined in parallel incubations with 1 μM unlabeled Ang II. After four successive 1 min washes in ice-cold buffer without BSA, sections were air-dried in the fumehood and exposed to Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY, USA). Methylacrylate ^{125}I standards (Washington State University Peptide Radioiodination Service Centre) were included during the exposure. The film was processed in a Kodak X-OMAT automatic developer. Using a computer-assisted image analysis system, AIS/C (Imaging Research; St. Catherines, ON, Canada), relative optical density within each tissue area was quantified by comparison with the ^{125}I standards. Specific binding density was determined by subtraction of non-specific binding (about 2-5%) from total binding density and was expressed as fmol/mg wet weight of tissue. The densitometry quantification was performed without knowledge of experimental groups. Brain nuclei were defined according to the rat brain atlas of Paxinos and Watson (1986).

2.1.9 ACE binding density by in vitro autoradiography

ACE autoradiography was performed as described (Tan et al., 2004), similarly to AT_1 receptor autoradiography with several exceptions. The ACE inhibitor lisinopril derivative 351A was iodinated in house using the protocol of Chai et al., (1987) and was used at a concentration of 0.3 μCi/ml (30 pM). Non-specific binding was determined by

incubating with 100 mM EDTA, which completely eliminated the binding of radiolabel. ACE binding density was expressed as fmol/g wet weight of tissue.

2.1.10 Plasma renin activity by radioimmunoassay

Plasma renin activity (PRA) was measured by radioimmunoassay of Ang I following its in vitro generation according to previously described methods (Leenen et al., 2001). Briefly, 60 μl plasma was placed in a tube containing 10 μl each of the inhibitors (BAL: 1.7% 2,3-dimercaptopropanol and 3.3% benzyl benzoate in peanut oil; and 6.8% 8-hydroxyquinoline in water), and 225 μl 0.2 M Tris buffer (containing 0.5% BSA and 2 mM EDTA) was added. Duplicate 50 μl aliquots were incubated at 37° or 4°C for 30 min. A standard curve was set up using 50 μl Ang I in concentrations from 5 to 500 pg. Anti-Ang I antiserum, (diluted to give 45-50% zero binding) and ^{125}I-labelled Ang I (total counts adjusted to 5000 cpm) were then added to all tubes. The tubes were incubated at 4°C for 16-24 h. A 2.5% charcoal (dextran-coated) solution was diluted 1:4 and 1.0 ml was added to all tubes, which were mixed and then centrifuged at 1875 x g for 30 min. The supernatants were decanted to another set of tubes and then counted. PRA was calculated from the difference between the 37° and 4°C incubations and expressed as ng Ang I generated/min/ml plasma. The intra- and inter-assay coefficients of variance for this assay are 3% and 13%, respectively.

2.1.11 Plasma Ang I and II concentrations by HPLC separation followed by radioimmunoassay

Plasma Ang I and II concentrations were determined with a method previously established in our laboratory (Ruzicka et al., 1995). Plasmas were applied to Sep-pak C18 cartridges pre-conditioned with 15 ml 100% methanol followed by 10 ml deionized

water, the columns were washed with 10 ml 0.1% trifluoroacetic acid (TFA), and the angiotensin peptides eluted with 2 ml methanol:water:trifluoroacetic acid (80:19.9:0.1). The eluates were evaporated to dryness using a Savant Speed Vac. Plasma and LV angiotensins were assessed by radioimmunoassay after separation by HPLC (Ruzicka et al., 1995; Leenen et al., 1999) on a CSC-Select-ODS2 C18 column, 15 x 0.46 cm with a 5 μm particle size. Gradient elution was used, starting with a 100%/40% vol/vol methanol in 10 mM acetate buffer at time 0, changing to 100%/80% vol/vol methanol in 10 mM acetate buffer at 38 minutes (slope -4), flow rate 1.0 ml/min (models 501 and 510 HPLC pumps, Waters; controlled by Maxima 820 workstation software, Millipore). Eluate fractions were collected every min, with a Spectrum Spectra/Chrom CF-1 Fraction Collector, then evaporated to dryness in a Savant SpeedVac vacuum concentrator. The tubes containing the dried eluates were divided into two groups containing the peaks of Ang II and I, at approximately 8-20 minutes and 21-31 minutes, respectively and separate radioimmunoassays for Ang II or Ang I was performed on the appropriate fractions. Ang I and II concentrations were expressed as pg/ml for plasma and pg/g tissue. The sensitivity of the detection of Ang I is 0.5 pg and for Ang II is 0.2 pg (Ruzicka et al., 1995). The angiotensin II antibody has 100% cross-reactivity with Ang II, 55% with Ang III, and 0.1% with Ang I. The inter-assay coefficient of variance for Ang II is 13% and Ang I is 14%; the intra-assay coefficient of variance for Ang II is 8% and 5% for Ang I (Leenen et al., 1999a).

2.1.12 LV Ang I and II concentrations by HPLC followed by radioimmunoassay

Heart tissues were boiled in 5 ml of 1 M acetic acid for 15 min, followed by homogenization with a Polytron for 25s and centrifugation at 5000 x g for 30 min.

Similar to the determination of plasma Ang I and II, the supernatants were applied to pre-conditioned Sep-Pak cartridges and the angiotensins determined according to section 2.1.13.

2.1.13 Statistical analysis

In Experiment 1, data are presented as means ± SEM of raw values or of percentage changes compared to Sham-OVX values. Statistical analysis was performed with SigmaStat software (SPSS, Chicago, IL). All comparisons between groups were determined by a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test where applicable. In Experiment 2, values are represented as means ± SEM of raw data or of mean percentage changes vs. ovary-intact, sham values. Comparisons were made using either one- or two-way analyses of variance (ANOVA), followed by the Student-Newman-Keuls test where applicable. Relationships between variables were analyzed using Pearson's Correlation. Differences in mortality rates were determined by Chi-squared test. The level of statistical significance was set at $p<0.05$.

Chapter 3

RESULTS

3.0. Overview

The results section is divided into two parts according to the two different experiments. The results of Experiment 1 are presented first, and summarized; the results of Experiment 2 are then presented and summarized.

3.1.0. Experiment 1: Regulation of ACE and AT_1R by 17β-estradiol in female rats

Experiment 1 was performed to determine to what extent in the healthy rat, the presence, absence, or excess of circulating E2 affects the levels of ACE and AT_1R. We measured ACE in plasma and ACE and AT_1R in several tissues within which the local RAS has direct relevance to the regulation of the cardiovascular system. Each tissue will be discussed separately and then a brief overview of the results will follow.

3.1.1. Body, uterus and heart weights

Two and 5 weeks after OVX, body weights were significantly increased (Table 3.1). After two weeks, rats treated with regular and high E2 had significantly lower body weights compared to Sham-OVX rats. After 5 weeks, this difference was no longer present for OVX+reg E2 rats but remained for OVX+high E2 rats.

Two and 5 weeks after OVX heart wet weight (RV and LV combined) was significantly increased (Table 3.1). However, this difference did not persist when corrected for body weight. On the other hand, OVX rats treated with E2 (regular or high dose) exhibited lower absolute heart weights, but had larger heart weights when corrected for body weight.

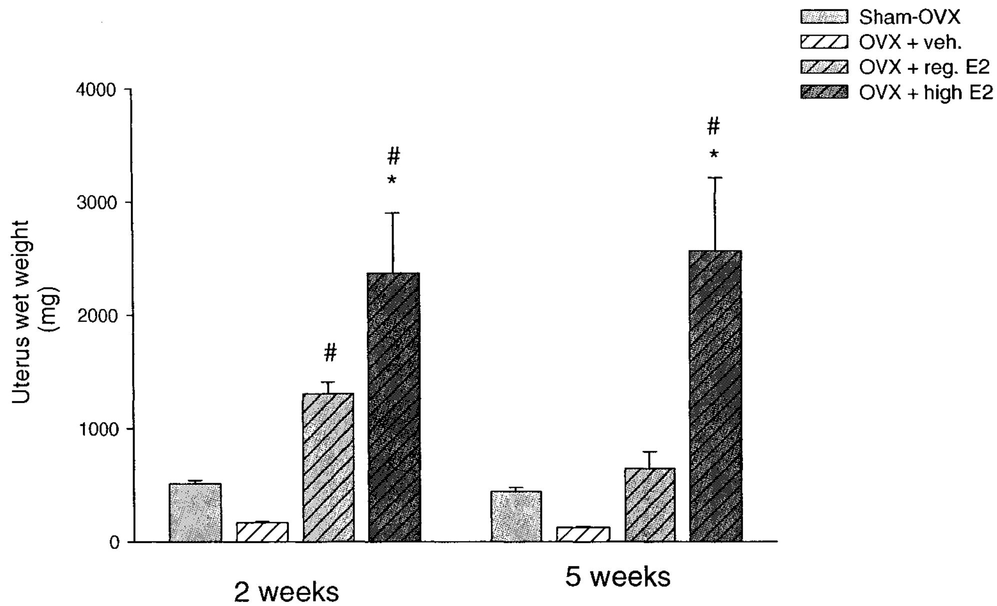
Two and 5 weeks after OVX, uterus wet weights were decreased (Figure 3.1 A&B). E2 dose-dependently increased both uterus wet weight and wet weight corrected for body weight. After 2 weeks of treatment uterus weights were significantly higher in OVX+reg E2 rats than Sham-OVX rats, an effect that did not remain after 5 weeks (Figure 3.1 A&B). The observed pattern of difference in uterus wet weight is consistent with levels of E2 expected by each respective treatment, except for the somewhat higher-than-normal uterus weights in OVX+reg E2 rats at 2 weeks post-OVX.

Table 3.1. Body and heart weights of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation

	Weeks	**Sham-OVX**	**OVX+ Veh.**	**OVX+ reg. E2**	**OVX+ high E2**
Body Weight	2	235 ± 3	276 ± 6*	210 ± 4$^{*\#}$	211 ± 3$^{*\#}$
(g)	5	272 ± 7	339 ± 8*	255 ± 6$^{\#}$	221 ± 10$^{*\#}$
Heart Weight	2	651 ± 12	786 ± 11*	624 ± 15$^{\#}$	605 ± 35$^{\#}$
(mg)	5	734 ± 20	857 ± 17*	742 ± 21$^{\#}$	664 ± 20$^{\#}$
Heart weight/BW	2	278 ± 4	275 ± 2	298 ± 7	288 ± 13
(mg/100g)	5	270 ± 3	253 ± 6	302 ± 10$^{*\#}$	298 ± 8$^{*\#}$

Values are means ± SEM; n=5-6 rats/group; * $p<0.05$ vs. Sham-OVX, $^{\#}p<0.05$ vs. OVX + Veh. at same time-point, by 1-way ANOVA. OVX: ovariectomized; Veh.: vehicle. reg.: regular. E2: 17β-estradiol. BW: body weight.

A. Uterus wet weights



B. Uterus wet weight indexed to body weight

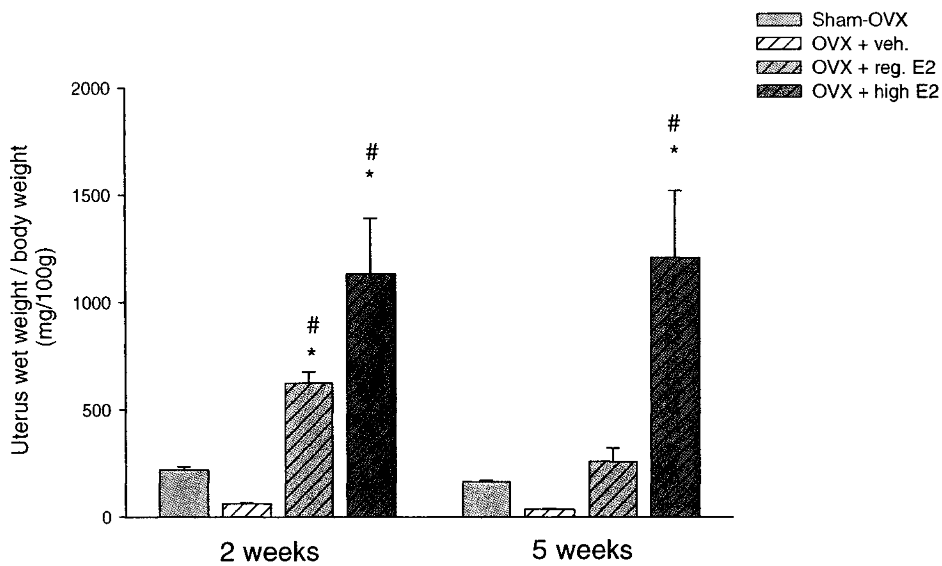


Figure 3.1. Uterus weights of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation. Values expressed as mean ± SEM. n=5-6/group. *$p<0.05$ vs. Sham-OVX. #$p<0.05$ vs. OVX+veh., by 1-way ANOVA. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

3.1.2. ACE activities and binding densities and AT_1R binding densities in the RV and LV

Figure 3.2 shows actual representative autoradiographs, Table 3.2 shows activities and densities, and Figure 3.3 shows percent changes in densities and activities.

RV. After 2 weeks of treatment, RV ACE activity was not altered by E2 status (Table 3.2, Figure 3.3). Five weeks after OVX, RV ACE activity tended to increase and RV ACE and AT_1R densities were increased by 60-70% (Table 3.2, Figure 3.2, Figure 3.3). These OVX-induced increases were consistently prevented by regular E2 treatment and reversed to 25-30% decreases by high E2 treatment.

LV. After 2 weeks of treatment, LV ACE activity was not altered by E2 status (Table 3.2, Figure 3.3). Five weeks after OVX, LV ACE activity was increased by ~40% and LV ACE and AT_1R densities were increased by ~65% (Table 3.2, Figure 3.2, Figure 3.3). As in the RV, these increases were consistently prevented by regular E2 treatment and reversed to decreases by high E2 treatment.

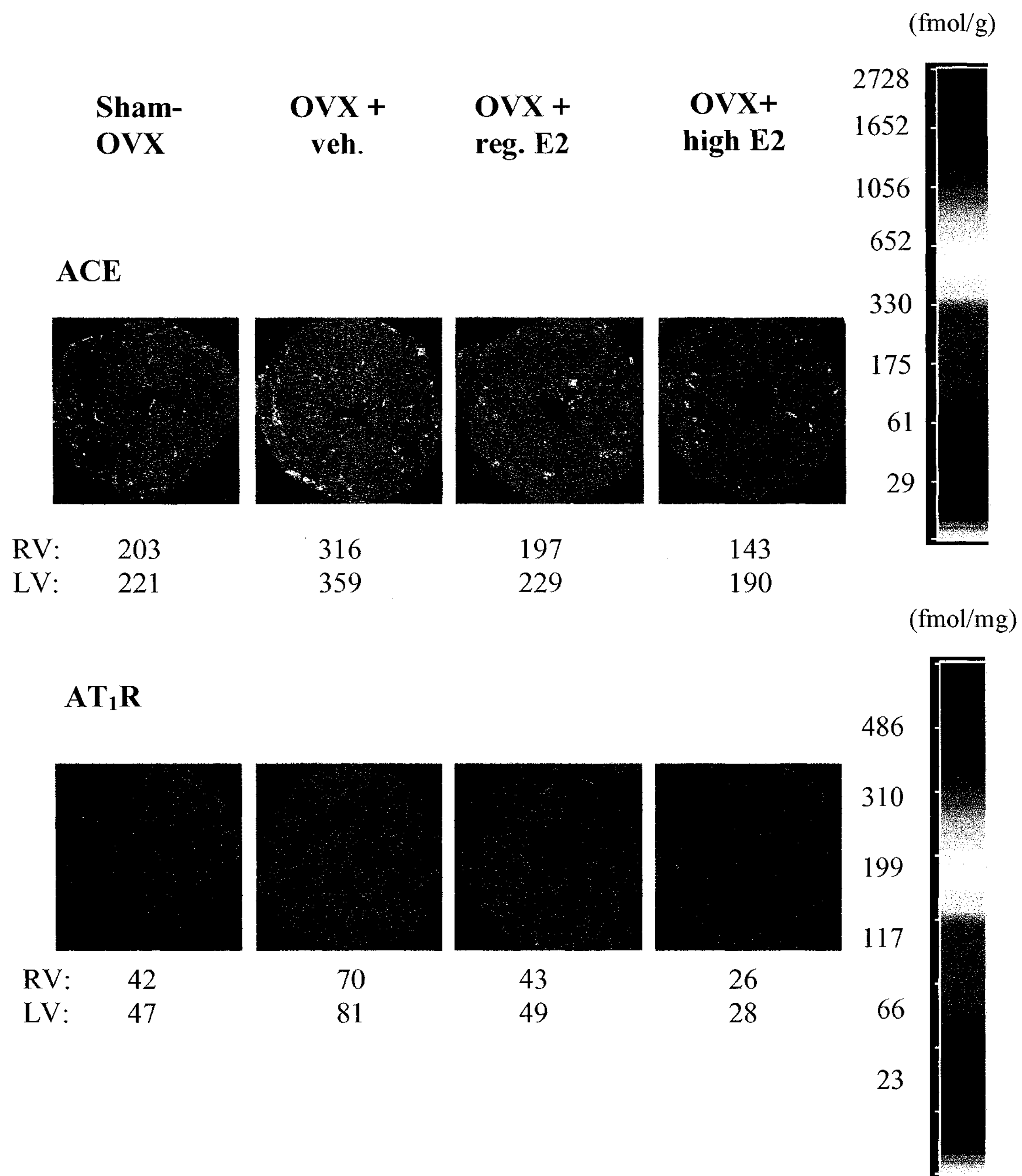


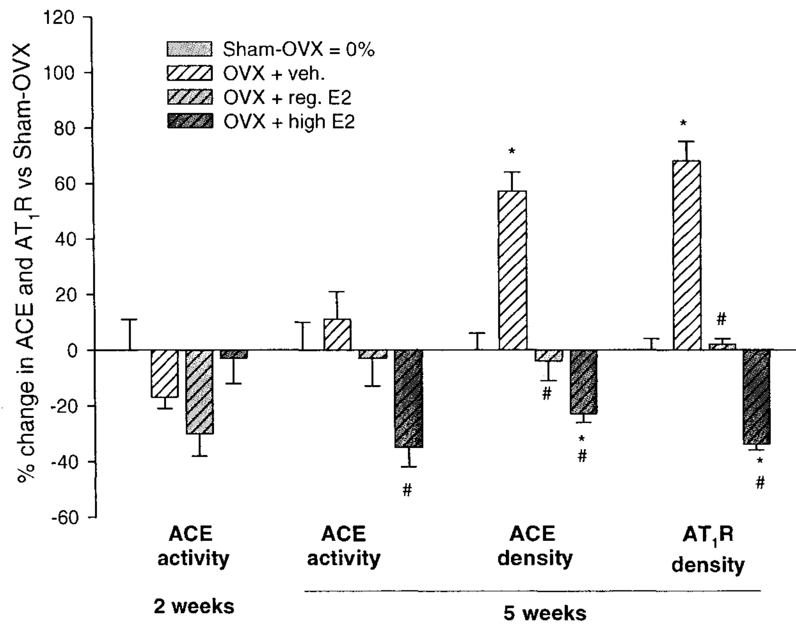
Figure 3.2. Representative autoradiographs of ACE and AT_1R binding densities in hearts of female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

Table 3.2. ACE activity and binding densities and AT_1R binding densities in the RV and LV of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation

	Sham-OVX	OVX + veh.	OVX + reg. E2	OVX + high E2
Right ventricle				
2 weeks				
ACE activity	47 ± 5	39 ± 2	33 ± 3	46 ± 4
5 weeks				
ACE activity	38 ± 4	43 ± 4	37 ± 4	$25 \pm 3^{\#}$
ACE density	192 ± 11	$302 \pm 14^{*}$	$185 \pm 13^{\#}$	$148 \pm 6^{*\#}$
AT_1R density	41 ± 2	$69 \pm 3^{*}$	$42 \pm 1^{\#}$	$27 \pm 1^{*\#}$
Left ventricle				
2 weeks				
ACE activity	37 ± 3	42 ± 3	39 ± 5	46 ± 5
5 weeks				
ACE activity	31 ± 4	$43 \pm 5^{*}$	$30 \pm 4^{\#}$	$25 \pm 3^{\#}$
ACE density	210 ± 13	$355 \pm 16^{*}$	$246 \pm 20^{\#}$	$185 \pm 11^{*\#}$
AT_1R density	52 ± 4	$89 \pm 6^{*}$	$55 \pm 4^{\#}$	$32 \pm 3^{*\#}$

Values expressed as mean ± SEM. ACE activity: nmol of His-Leu/min/g protein. ACE binding density: fmol of ^{125}I-351A bound/g wet tissue. AT_1R binding density: fmol of ^{125}I-AngII bound/mg wet tissue. n=5-6/group. $^{*}p<0.05$ vs Sham-OVX. $^{\#}p<0.05$ vs OVX + veh., by 1-way ANOVA. OVX: ovariectomized. Veh: vehicle. E2: 17β-estradiol.

A. Right Ventricle



B. Left ventricle

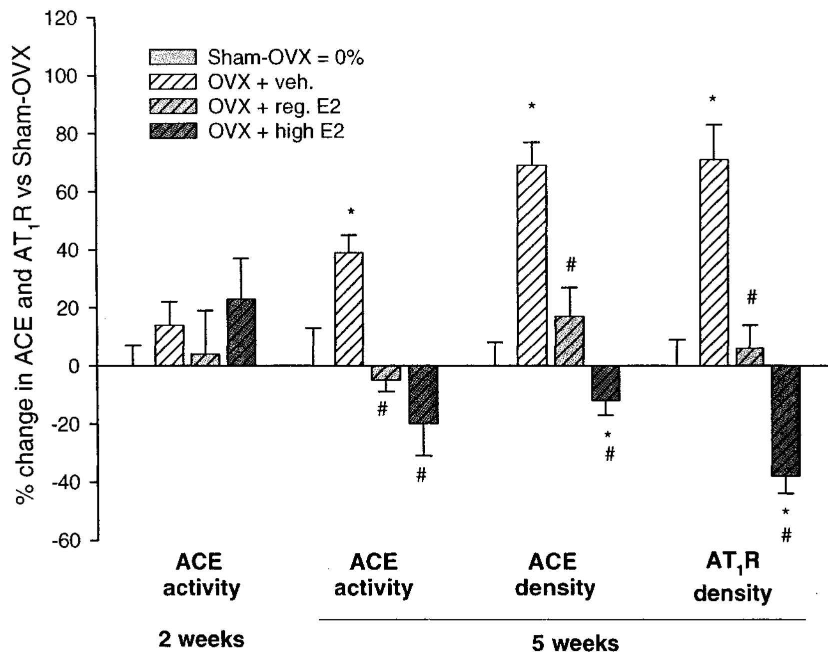


Figure 3.3. Changes in ACE activity and binding densities and AT_1R binding densities in the left and right ventricles of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation. Values are mean ± SEM of percentage change compared to Sham-OVX group. n=5-6/group. *p<0.05 vs. Sham-OVX. #p<0.05 vs. OVX + Veh, by 1-way ANOVA. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

3.1.3. ACE and AT_1R binding densities in the brain

Figure 3.4 shows actual autoradiographs, Table 3.3 shows actual densities, and Figure 3.5 shows percent changes in densities. Five weeks after OVX, ACE densities were significantly increased in the SFO, PVN and MnPO by 20-30%. ACE densities in the OVLT were not altered by OVX (Table 3.3, Figure 3.4, Figure 3.5). Regular E2 treatment prevented all OVX-induced increases in ACE and high E2 treatment reversed the changes to significant 10-15% decreases in the OVLT, SFO and MnPO. Five weeks after OVX, AT_1R densities were increased by 20-30% in all four brain nuclei - an effect prevented in all cases by regular E2 treatment. AT_1R densities in the SFO and PVN were reversed to 12-20% decreases and densities in the OVLT tended to decrease.

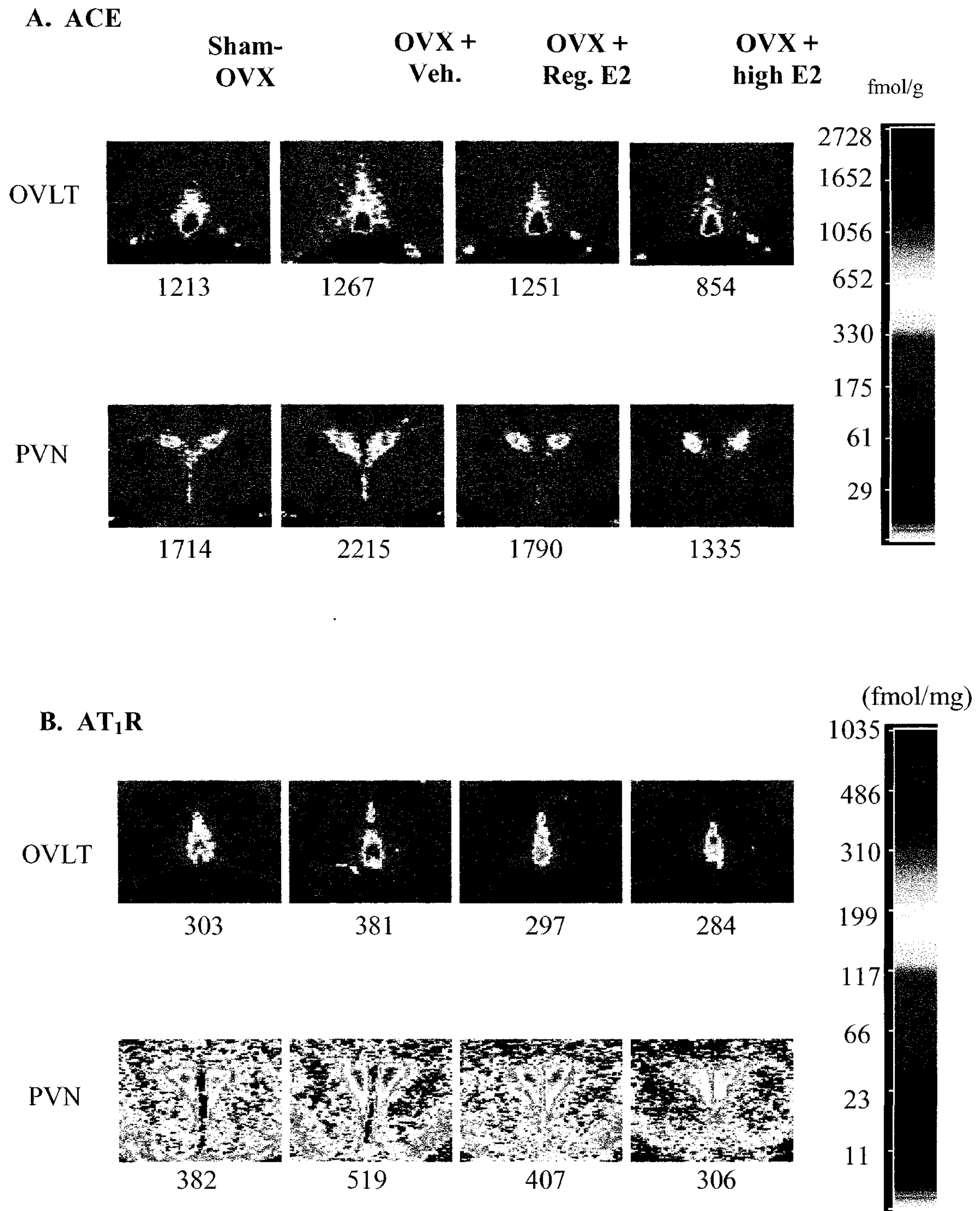


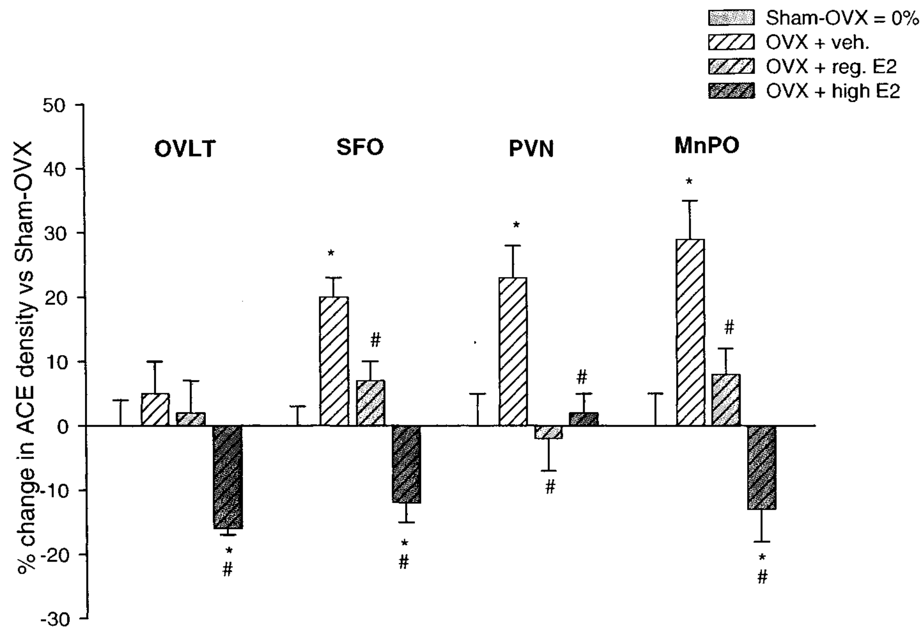
Figure 3.4. Representative autoradiographs of ACE and AT_1R binding densities in brain nuclei of female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation. OVLT: organum vasculosum laminae terminalis. SFO: subfornical organ. PVN: paraventricular nucleus. MnPO: median preoptic nucleus. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

Table 3.3. ACE and AT_1R binding densities in brain nuclei in female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation

	Sham OVX	OVX + veh.	OVX + reg. E2	OVX + high E2
ACE				
OVLT	1182 ± 71	1246 ± 61	1204 ± 55	875 ± 16$^{\#}$
SFO	3162 ± 108	3794 ± 92*	3385 ± 116$^{\#}$	2473 ± 87$^{\#}$
PVN	1796 ± 74	2208 ± 93*	1752 ± 81$^{\#}$	1378 ± 52$^{\#}$
MnPO	764 ± 42	985 ± 46*	821 ± 29	665 ± 37
AT_1R				
OVLT	314 ± 12	375 ± 6*	310 ± 9$^{\#}$	292 ± 7$^{\#}$
SFO	561 ± 23	684 ± 19*	580 ± 26$^{\#}$	436 ± 15$^{*\#}$
PVN	389 ± 10	512 ± 11*	397 ± 13$^{\#}$	315 ± 9$^{*\#}$
MnPO	302 ± 8	374 ± 6*	312 ± 7$^{\#}$	304 ±11$^{\#}$

Values are mean ± SEM of bound ^{125}I-351A in fmol/g wet tissue or ^{125}I-AngII in fmol/mg wet tissue. *$p<0.05$ vs. Sham-OVX. $^{\#}p<0.05$ vs. OVX + veh. Comparisons by 1-way ANOVA. n=5-6/group. SFO: subfornical organ. OVLT: organum vasculosum laminae terminalis. PVN: paraventricular nucleus. MnPO: median preoptic nucleus. Veh: vehicle. E2: 17β-estradiol. Reg.: regular. OVX: ovariectomized.

A. ACE



B. AT_1R

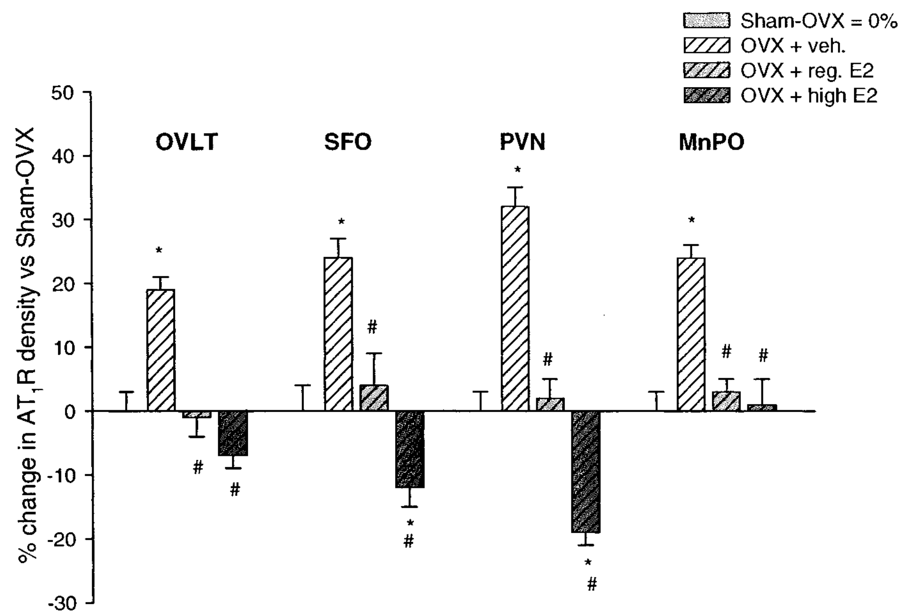


Figure 3.5. Changes in ACE and AT_1R binding densities in brain nuclei of female rats 5 weeks following Sham/OVX surgery and E2 pellet implantation. Values are expressed as mean ± SEM of percentage change compared to Sham-OVX group. *$p<0.05$ vs. Sham-OVX. $^{\#}p<0.05$ vs. OVX + veh., 1-way ANOVA. OVLT: organum vasculosum laminae terminalis. SFO: subfornical organ. PVN: paraventricular nucleus. MnPO: Median preoptic nucleus. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

3.1.4. ACE mRNA abundance, activities, binding densities and AT_1R binding densities in kidney, abdominal aorta and adrenal

Figures 3.6 and 3.7 show actual autoradiographs. Table 3.4 shows actual ACE mRNA abundances, ACE activities, and ACE and AT_1R densities. Figures 3.8 and 3.9 show percent changes in ACE mRNA abundances, ACE activities, and ACE and AT_1R densities.

Kidney. ACE mRNA abundance was measured only in the kidney because after ACE activity and ACE and AT_1R autoradiography, there was still enough tissue remaining to perform an additional analysis. Two weeks after OVX, kidney ACE mRNA abundance tended to increase whereas ACE activity was significantly increased by 65% (Table 3.4, Figure 3.8). Kidney ACE mRNA abundance was significantly decreased in OVX rats treated with regular or high E2. The increase in ACE activity was prevented by regular E2 treatment and reversed to a significant decrease by high E2 treatment. Five weeks after OVX, ACE mRNA and activity tended to increase ($p=0.09$ and 0.07, respectively) and ACE density was significantly increased in the proximal convoluted tubules (Table 3.4, Figure 3.6A, Figure 3.8). The increases in kidney ACE were prevented by regular E2 treatment; high E2 treatment had an effect no greater than that of regular E2. Five weeks after OVX, AT_1R density was increased in the kidney medulla and cortex by 55-60% (Table 3.4, Figure 3.7A, Figure 3.8). These increases were prevented by regular E2 replacement and reversed to 30-35% decreases by high E2.

Abdominal aorta. After 2 weeks of treatment, ACE activity in the abdominal aorta was unchanged according to E2 status (Table 3.4, Figure 3.9A). Five weeks

after OVX, ACE activity was significantly increased and ACE density tended to increase (Table 3.4, Figure 3.9A). Regular E2 prevented the increase in ACE activity but high E2 had no additional effect. ACE density was significantly decreased by high E2. Five weeks after OVX, aortic AT_1R density was increased by 60% (Table 3.4; Figure 3.9A), an effect prevented by regular E2 and reversed to a decrease by high E2.

Adrenal. After 2 and 5 weeks of treatment, ACE activity was unaltered according to E2 status in homogenates of the whole adrenal (Table 3.4, Figure 3.6B). Autoradiography is more informative in this organ, as ACE and AT_1R densities can be measured within the adrenal medulla and cortex separately. Five weeks after OVX, ACE binding density was increased by 65% in the adrenal medulla (Table 3.4, Figure 3.7B, and Figure 3.9B). This increase was prevented by regular E2 replacement and tended to reverse with high E2 treatment. Five weeks after OVX, AT_1R densities were significantly increased in the adrenal medulla (by 90%) and cortex (by 15%) (Table 3.4, Figure 3.7B, and Figure 3.9B). These OVX-induced increases were prevented by regular E2 treatment and reversed to significant 25-30% decreases by high E2 treatment.

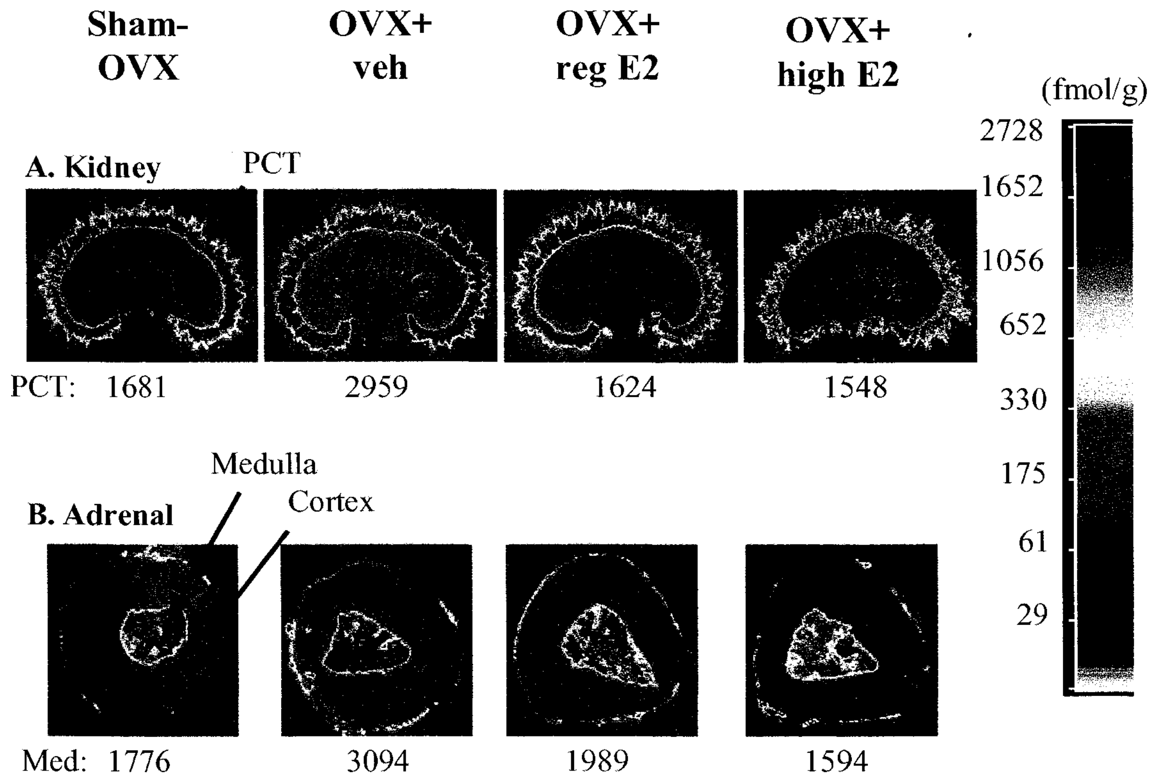


Figure 3.6. Representative autoradiographs of ACE binding densities in kidney and adrenal 5 weeks following Sham/OVX surgery and E2 pellet implantation. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle; PCT: proximal convoluted tubules. Med: medulla.

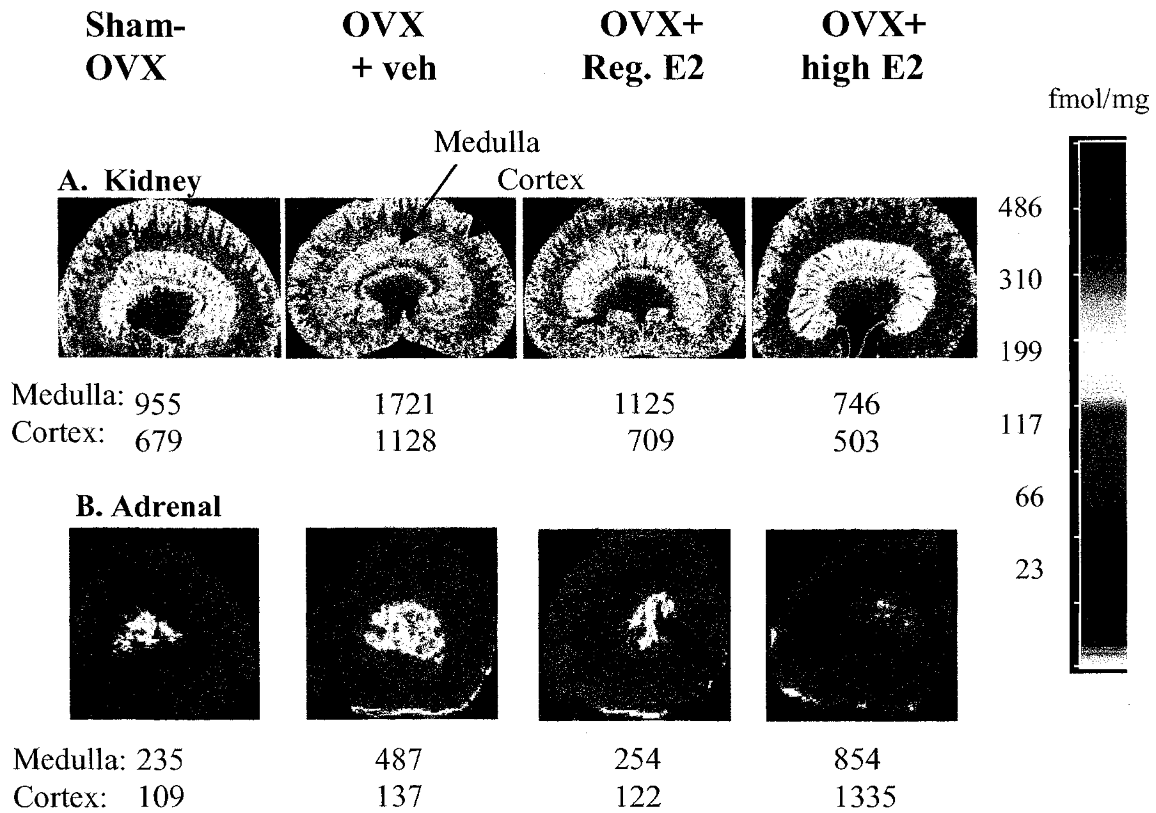


Figure 3.7. Representative autoradiographs of AT_1R binding density in kidney and adrenal 5 weeks following Sham/OVX surgery and E2 pellet implantation. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle

Table 3.4. ACE mRNA abundance, activity and binding densities and AT_1R binding densities in the kidney, abdominal aorta, and adrenal of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation

	Sham OVX	OVX + veh.	OVX + reg. E2	OVX + high E2
Kidney				
2 weeks				
ACE mRNA abund.	31 ± 2	37 ± 2	21 ± 3*#	20 ± 3*#
ACE activity	26 ± 6	44 ± 5*	17 ± 3#	8 ± 2*#
5 weeks				
ACE mRNA abund.	29 ± 3	40 ± 4	32 ± 2	24 ± 3#
ACE activity	43 ± 4	61 ± 9	30 ± 7#	28 ± 7#
ACE density (PCT)	1720 ± 61	2975 ± 86*	1673 ± 52#	1561 ± 49#
AT_1R density (medulla)	994 ± 41	1650 ± 79*	1083 ± 50#	736 ± 30#
AT_1R density (cortex)	703 ± 36	1125 ± 49*	745 ± 28#	499 ± 28#
Abdominal Aorta				
2 weeks				
ACE activity	467 ± 47	568 ± 67	592 ± 81	547 ± 69
5 weeks				
ACE activity	519 ± 51	798 ± 99*	514 ± 65#	533 ± 42#
ACE density	1182 ± 71	1347 ± 76	1204 ± 55	875 ± 49*#
AT_1R density	198 ± 12	315 ± 16*	207 ± 9#	134 ± 12*#
Adrenal				
2 weeks				
ACE activity (whole)	30 ± 6	28 ± 3	27 ± 3	23 ± 3
5 weeks				
ACE activity (whole)	17 ± 2	15 ± 2	15 ± 4	16 ± 2
ACE density (medulla)	1844 ± 97	3056 ± 182*	2189 ± 116#	1687±109*#
AT_1R density (medulla)	246 ± 17	473 ± 19*	262 ± 15#	167 ± 9#*
AT_1R density (cortex)	114 ± 4	132 ± 6*	118 ± 5#	99 ± 5*#

All values are mean ± SEM. ACE mRNA: a ratio of ACE/PGK mRNA abundance x 100%. ACE activity: nmol of His-Leu/min/g protein. ACE binding density: fmol of ^{125}I-351A bound/g wet tissue. AT_1R binding density: fmol of ^{125}I-Ang II bound/mg wet tissue. *p<0.05 vs. Sham-OVX. #p<0.05 vs. OVX + veh., by 1 way ANOVA. n=5-6/group. PCT: proximal convoluted tubules. OVX: ovariectomized. E2:17β-estradiol. Veh: vehicle. Abund.: Abundance.

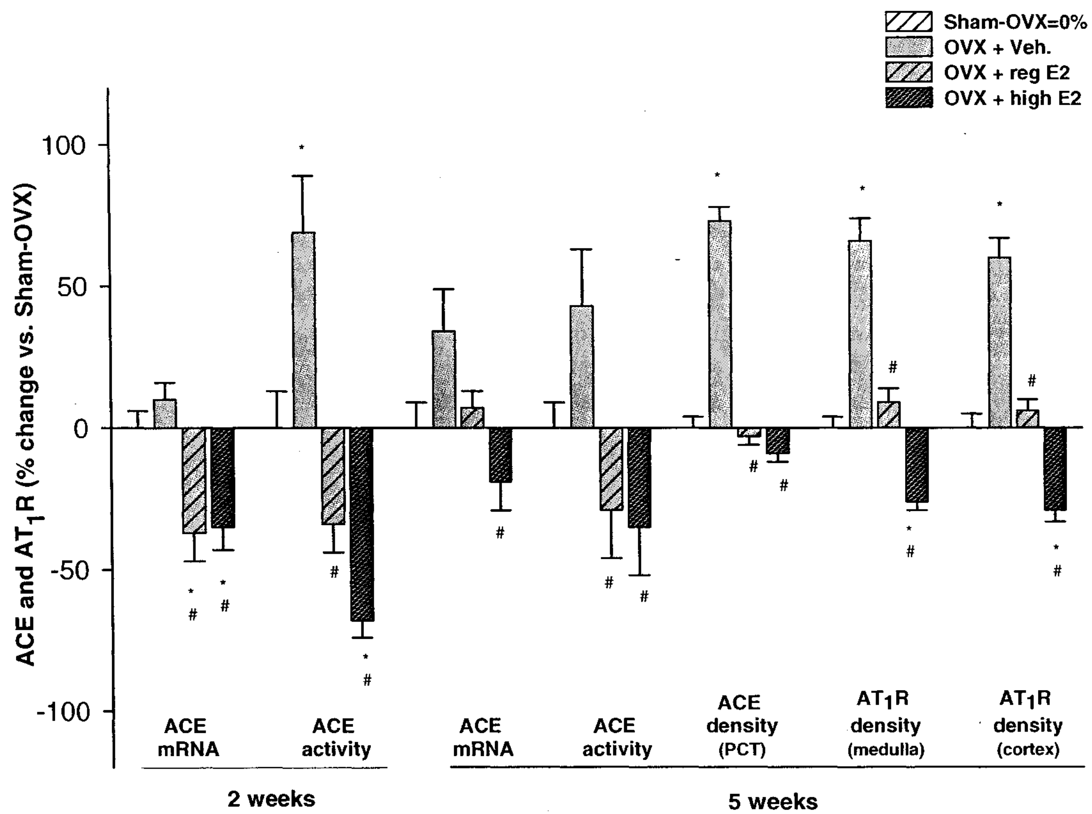
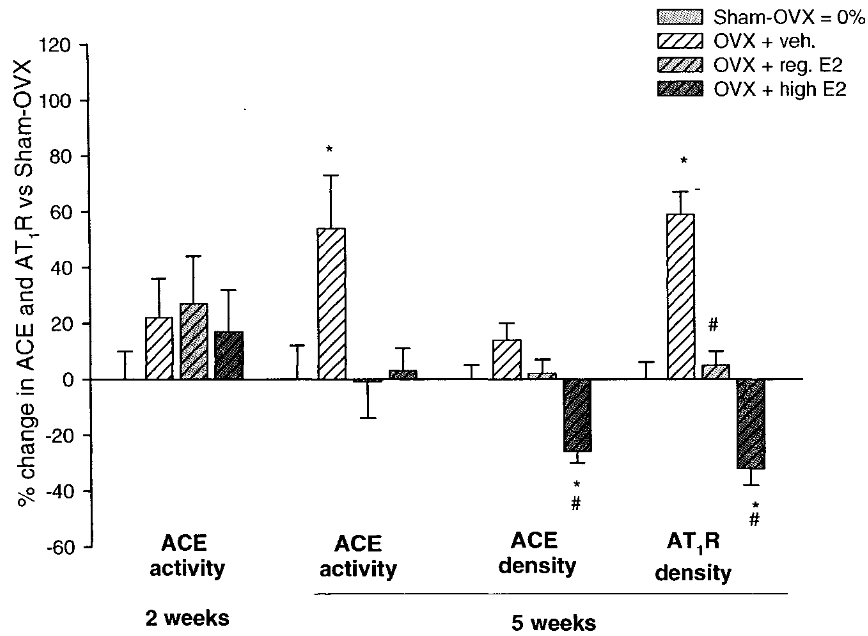


Figure 3.8. Changes in ACE mRNA abundance, activity and binding densities, and AT_1R binding densities in kidney of female rats 2 and 5 weeks following Sham/OVX surgery and E2 implantation. Values are mean ± SEM of percent changes compared to Sham-OVX. n=5-6/group. *$p<0.05$ vs. Sham-OVX. #$p<0.05$ vs. OVX + Veh. by 1-way ANOVA. OVX: ovariectomy. E2: 17β-estradiol. Veh: vehicle.

A. Abdominal Aorta



B. Adrenal

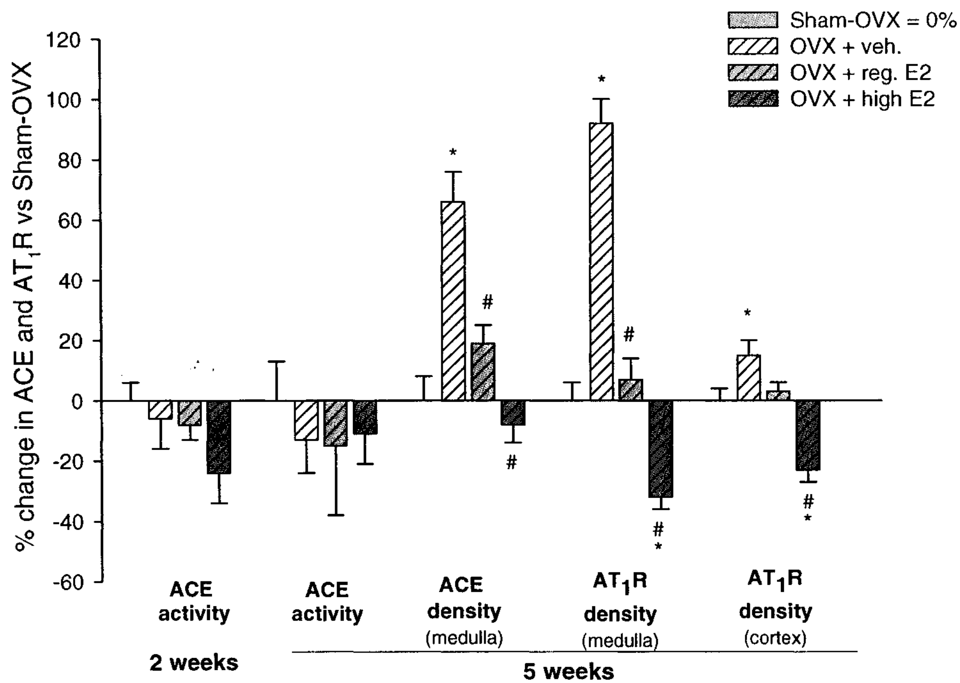


Figure 3.9. Changes in ACE activity and binding densities and AT_1R binding densities in abdominal aorta and adrenal 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation. Values are expressed as mean ± SEM of percent changes compared to Sham-OVX group. n=5-6/group. *p<0.05 vs. Sham-OVX, #p<0.05 vs. OVX + veh., by 1-way ANOVA. OVX: ovariectomized. E2:17β-estradiol. Veh: vehicle.

3.1.5. Plasma ACE activity and lung ACE activity and binding density

Changes in plasma and lung ACE are discussed together as they have an important connection: the majority of plasma ACE is cleaved through a regulated process from the vascular (mostly pulmonary) endothelium.

Lung. Two weeks after OVX, lung ACE activity was significantly decreased (Table 3.5, Figure 3.10A). This decrease was prevented by regular E2 treatment and reversed to a significant increase by high E2 treatment. Five weeks after OVX, the pattern of ACE activity in the lung was reversed: ACE activity was increased by OVX (p=0.056), and ACE binding densities were nearly doubled post-OVX (Table 3.5, Figure 3.10A). These increases were prevented by treatment with regular E2 and reversed to decreases by treatment with high E2.

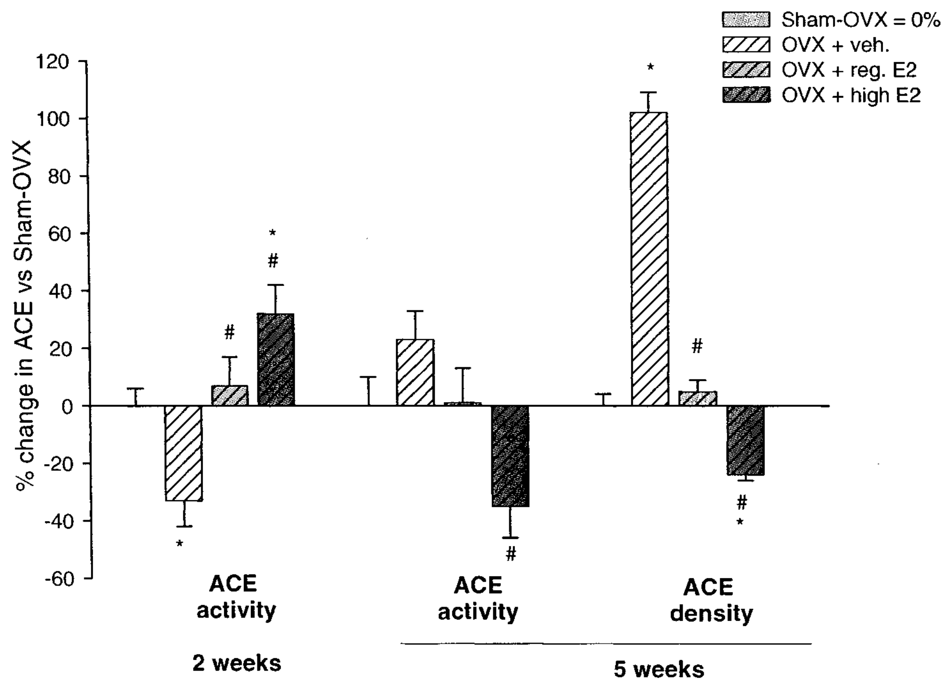
Plasma. Plasma ACE activities were not changed by OVX alone at 2 and 5 weeks after OVX (Table 3.5, Figure 3.10). However, treatment of OVX rats with either regular or high E2 for 2 weeks resulted in a significant decrease in plasma ACE activity. This effect was transient as the decrease was no longer significantly different compared to Sham-OVX or OVX+veh groups after 5 weeks.

Table 3.5. ACE activity in plasma, and ACE activity and binding densities in lung of female rats 2 and 5 weeks following Sham/OVX surgery and pellet implantation

	Sham OVX	OVX + veh.	OVX + reg. E2	OVX + high E2
Lung				
2 weeks				
ACE activity	1925 ± 107	1303 ± 173*	1896 ± 231#	2537 ± 212#*
5 weeks				
ACE activity	2553 ± 255	3133 ± 268	2585 ± 310	1660 ± 283#
ACE density	1604 ± 39	3238 ± 104*	1686 ± 57#	1227 ± 32*#
Plasma				
2 weeks				
ACE activity	15.3 ± 1.7	15.2 ± 2.3	9.5 ± 1.0*#	10.2 ± 1.4*#
5 weeks				
ACE activity	14.2 ± 1.2	15.7 ± 1.1	12.1 ± 1.8	12.7 ± 1.4

All values are mean ± SEM. n=5-6/group. ACE activity values are expressed in nmol of His-Leu/min/g protein. ACE binding density values are expressed as fmol of ^{125}I-351A bound/g wet tissue. *p<0.05 vs. Sham-OVX. #p<0.05 vs. OVX + veh. Comparisons by 1-way ANOVA. E2: 17β-estradiol. Veh: vehicle. OVX: ovariectomized.

A. Lung



B. Plasma

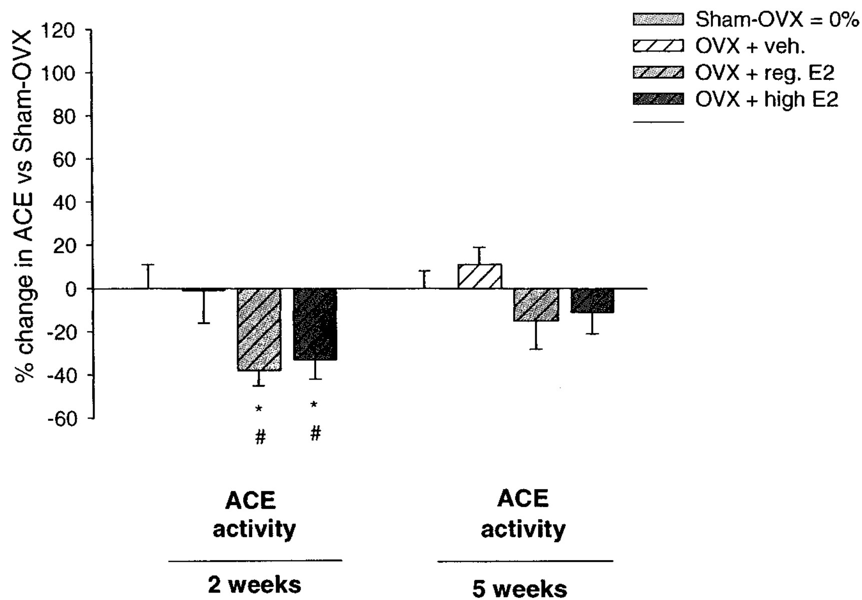


Figure 3.10. Changes in ACE activity in plasma and ACE activity and binding densities in lung of female rats 2 and 5 weeks following Sham/OVX surgery and E2 pellet implantation. Values are mean ± SEM of percent changes compared to Sham-OVX. n= 5-6/group. $^{*}p<0.05$ vs. Sham-OVX. $^{\#}p<0.05$ vs. OVX+veh. by 1-way ANOVA. OVX: ovariectomy. E2: 17β-estradiol. Veh.: vehicle.

3.1.6. Summary

Two weeks after OVX, rats treated with regular and high E2 exhibited transient ~35% decreases in plasma ACE activity. Plasma ACE activity was similar in all groups after 5 weeks. While 2 weeks of E2 deficiency had few effects, five weeks of E2 deficiency upregulated ACE and AT_1R by ~15-90% in the heart, brain, kidney, abdominal aorta, and adrenal. These increases were all prevented by replacement of E2 at physiological levels and for the most part reversed to 15-30% decreases by high E2. The regulation in the lung was different from all other tissues. Two weeks after OVX, ACE was decreased in the lung - an effect also prevented by replacement of E2 at physiological levels and reversed to an increase by high E2. After 5 weeks, the effects of E2 were exactly opposite, similar to all other tissues.

3.2. Experiment 2: Regulation of circulatory, cardiac, and brain renin-angiotensin systems by 17β-estradiol in female rats with MI-induced LV dysfunction

This set of experiments was performed primarily to determine whether the deficiency versus excess of 17β-estradiol has a significant effect on the development of LV dysfunction by 3 weeks post-MI, and whether E2 status significantly alters the circulatory, cardiac and brain RASs 3 weeks post-MI. It is important to note that the data from the ovary-intact rats were obtained in a different sub-experiment than the OVX+veh and OVX+high E2 rats. The LV function data (echocardiography and LV catheterization) is therefore analyzed separately, as these *in vivo* techniques are highly sensitive to inter-experiment variability. Although care must be taken in the overall interpretation of the results, tissue weights and all biochemical data from all three groups of rats have been analyzed together, as the observed inter-assay variabilities of each method are low (<15%).

3.2.1. Survival rates 3 weeks post-MI

In Experiments 2a, 2b and 2c, 100% of all sham-operated rats survived (intact: 16/16, OVX+veh: 16/16, OVX+high E2: 16/16). Mortality of MI rats occurred within 24 hours of coronary artery ligation. In Experiments 2a and 2b, survival was 35/58 (60%) for OVX+veh MI and 25/58 (43%) for OVX+high E2 MI rats. In Experiment 2c, ovary-intact MI rats had a survival rate of 30/60 (50%). Differences in survival rates were not statistically significant ($p = 0.174$ by Chi-Squared test).

3.2.2. Body, weights, uterus weights, infarct sizes and heart weights 3 weeks post-MI

<u>*Body weights.*</u> Body weights related to E2 status. At time of MI/sham surgery, OVX rats treated with vehicle, both sham and MI, had increased body weights compared to ovary-intact sham and MI rats (Table 3.6). OVX rats treated with high E2 had

decreased body weights compared to ovary-intact rats. There was no effect of MI alone on the final body weights.

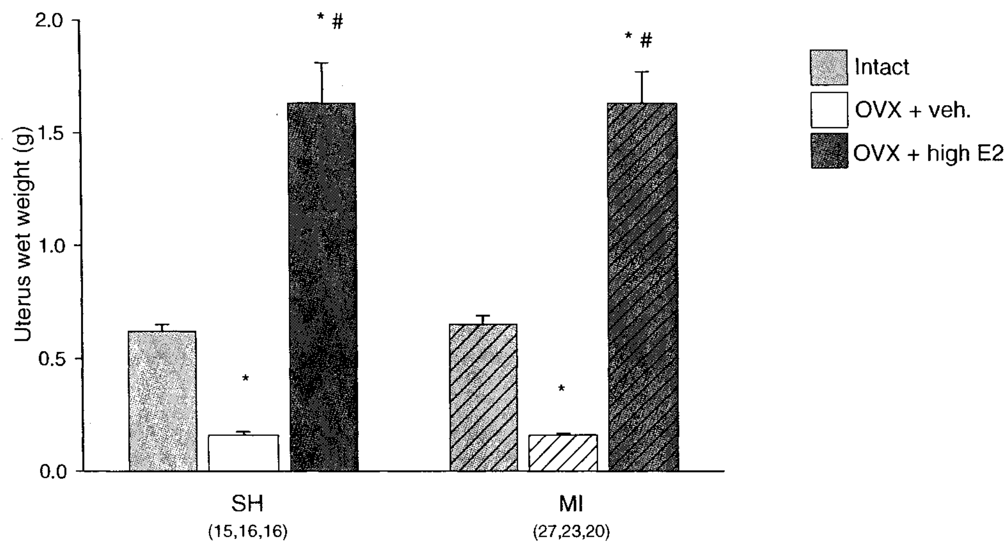
Table 3.6. Body weights of female rats 3 weeks post-MI

	Intact		OVX + veh		OVX + high E2	
	Sham	**MI**	**Sham**	**MI**	**Sham**	**MI**
n	16	27	16	23	16	21
BW at OVX	-	-	237±3	245±3	239±4	245±4
BW at MI	271±4	267±3	303±5$^{\#}$	288±8$^{\#}$	219±6$^{\#\&}$	218±4$^{\#\&}$
BW at echo	282±6	285±3	347±6$^{\#}$	345±5$^{\#}$	218±5$^{\#\&}$	229±9$^{\#\&}$
Final BW (g)	294±4	292±2	359±7$^{\#}$	357±5$^{\#}$	221±4$^{\#\&}$	227±4$^{\#\&}$

Values are expressed as mean ± SEM. $^{\#}p<0.05$ vs. respective intact group. $^{\&}p<0.05$ vs. respective OVX+veh group. Comparison made by 2-way ANOVA. BW: body weight. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

Uterus weights. There was no effect of MI on uterus weights; however, the expected change in uterus weights due to E2 status was present (Figure 3.11). OVX rats treated with vehicle had uterus wet weights and wet weights indexed for body weight that were about one-third those from ovary-intact rats. OVX rats treated with high E2 had uterine weights approximately 3 fold those of ovary-intact rats. This indicates that the E2 replacement had been performed as required for the objectives of this experiment.

A. Uterus wet weight



B. Uterus wet weight/ body weight

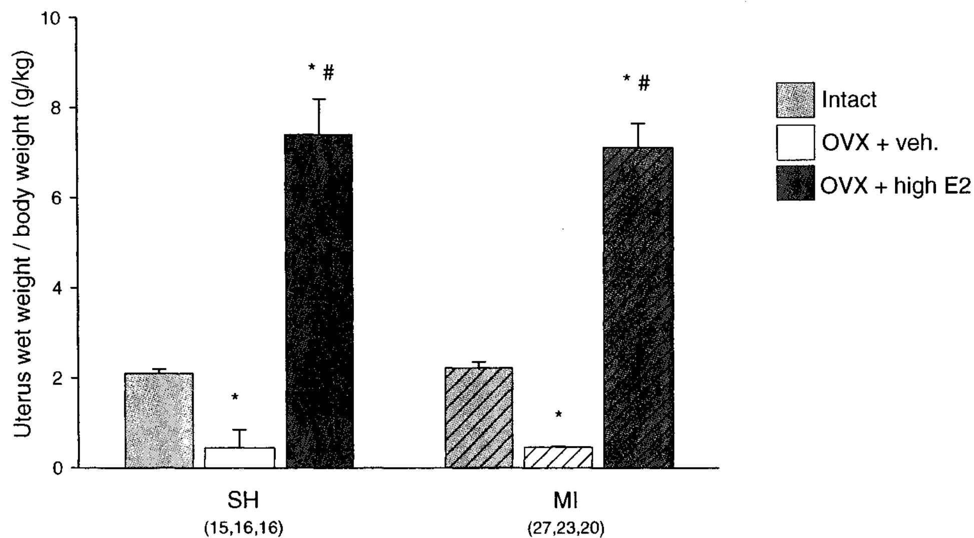


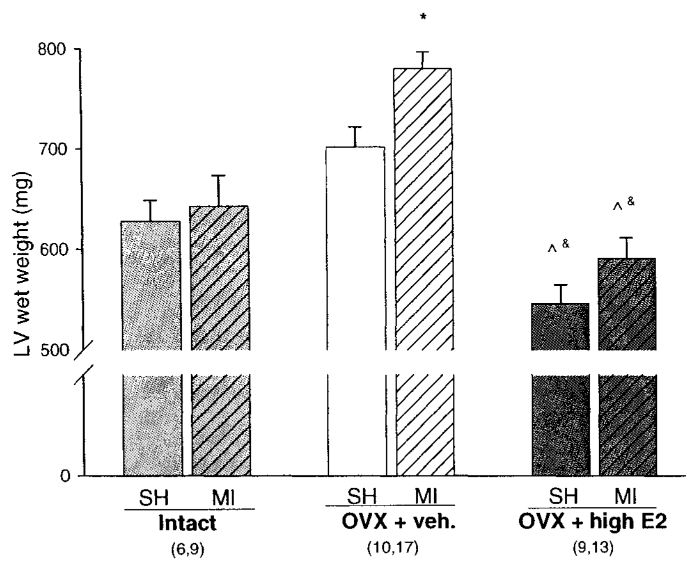
Figure 3.11: Uterus weights of female rats 3 weeks post-MI. Values are mean ± SEM. Numbers in parenthesis represent n/group. *$p<0.05$ vs. respective intact group. #$p<0.05$ vs. respective OVX + E2 group. OVX: ovariectomized. Veh: vehicle. E2: 17β-estradiol. Hatched bars represent myocardial infarction.

Infarct size. Infarct sizes were first calculated separately for each individual parameter studied in these experiments, using only the infarct sizes from rats with data for that parameter. In all cases, infarct sizes were similar by 1 way-ANOVA for ovary-intact, OVX+veh, and OVX+high E2 groups. This data is shown in tables for each individual parameter. To enhance the power of the comparison, we also analyzed infarct size data from rats in Experiment 2a, 2b and 2c combined. The effect of E2 status on infarct size was determined two ways: 1) with only discernable infarct sizes included (>0%) and 2) with only large infarct sizes included (>30%). In both cases, no significant difference was found in infarct sizes between the three E2 status groups (intact, OVX+veh, OVX+high E2, respectively). For 1): 41±2, 39±2, and 45±3% of LV (p=0.168). For 2): 42±2, 42±2, and 47±3% of LV (p=0.248), both by 1-way ANOVA.

LV and RV hypertrophy. In sham rats, LV wet weight was significantly lower in OVX+high E2 rats compared to ovary-intact rats and OVX+veh rats (Figure 3.12A). This trend was reversed when the LV weights were indexed to body weight (Figure 3.12B). In MI rats, similar effects of E2 status are present as in sham rats. Only OVX+veh rats exhibited MI-induced hypertrophy – their LV weight indexed for body weight was 13% larger in MI rats compared to sham (compared to only 1% and 4% larger in ovary-intact and OVX+high E2 rats, respectively; Figure 3.12B). In male rats, the LV is not consistently hypertrophied post-MI, as it includes both the non-infarcted and infarct scar regions of the ventricle, the latter of which is thin and light. The RV on the other hand is often hypertrophied post-MI and is a good indicator of the degree of post-MI remodeling. In this experiment, in sham rats, OVX rats treated with high E2 had significantly lower RV wet weights than ovary-intact and OVX+veh rats (Figure 3.13A) but this was not significant when RV weight was indexed for body weight (Figure 3.13B). Following MI,

only ovary-intact rats exhibited a significant increase in RV weight/body weight (63%, compared to non-significant 23% and 11% increases for OVX+veh and OVX+high E2 respectively; Figure 3.13B).

A. LV wet weight



B. LV wet weight/ body weight

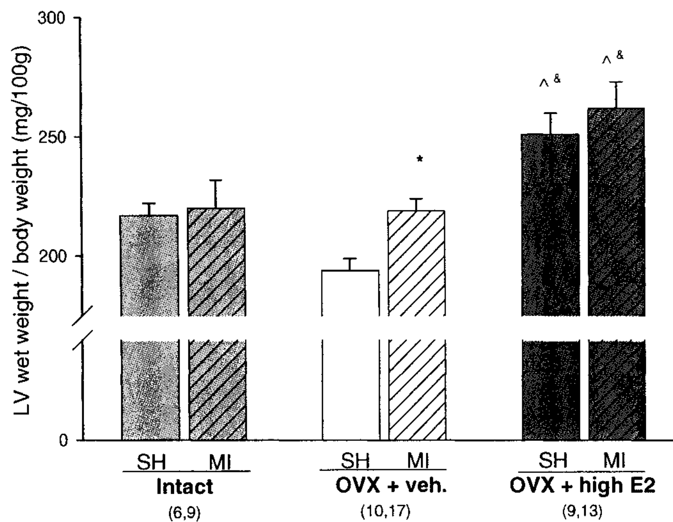
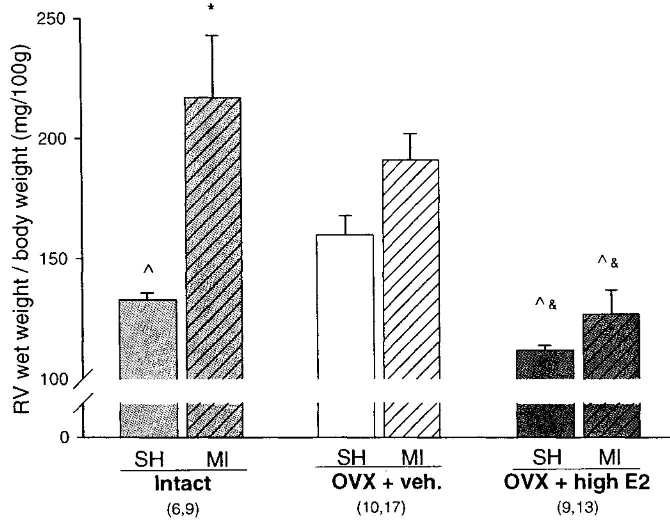


Figure 3.12. LV wet weight and LV wet weight corrected for body weight. OVX: ovariectomized; Veh: vehicle; E2: 17β-estradiol. LV: left ventricle. *$p<0.05$ vs. respective sham group. ^$p<0.05$ vs. respective OVX+veh group. $^{\&}p<0.05$ vs. respective intact group. Hatched bars represent myocardial infarction.

A. RV wet weight



B. RV wet weight/ body weight

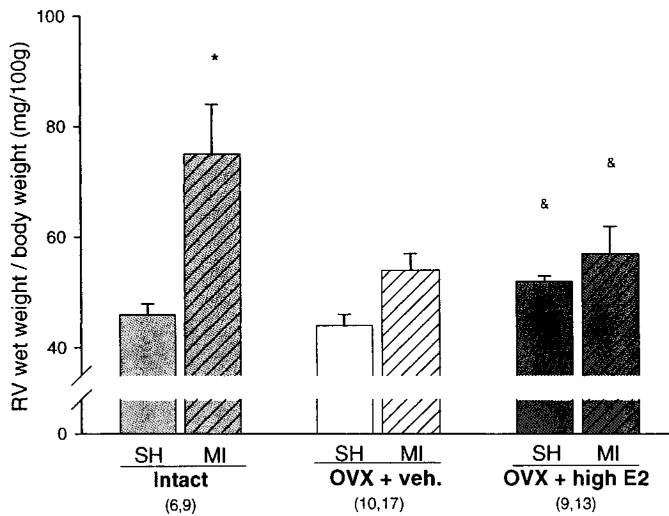


Figure 3.13. RV wet weights and wet weights corrected for body weight. OVX: ovariectomized; veh: vehicle; E2: 17β-estradiol; RV: right ventricle. *$p<0.05$ vs. respective sham group. ^$p<0.05$ vs. respective OVX+veh group. &$p<0.05$ vs. respective intact group. Hatched bars represent myocardial infarction.

3.2.3. LV dimensions and function by echocardiography

Echocardiographic measurements from ovary-intact rats were taken in a different experiment from the OVX+veh and OVX+high E2 rats (Experiment 2c versus 2a and 2b). Therefore, statistical comparisons of the data from ovary-intact rats have been made separately from the comparisons of the data from OVX+veh and OVX+high E2 rats.

In sham rats, intraventricular septum (IVS) thickness and posterior wall (PW) thickness were similar in OVX+veh and OVX+high E2 rats (Table 3.7). LV internal dimension (LVID) in diastole and systole were decreased by 17 and 20%, respectively, in OVX+high E2 rats compared to OVX+veh rats. When corrected for body weight, the decrease in LVIDd was reversed to an increase, and the decrease in LVIDs was no longer significant. The differences in LVID between sham groups are likely a result of the E2 status-mediated weight differences in these rats (Table 3.6) and not necessarily direct effects of E2 status on cardiac dimensions.

IVS and the PW thicknesses in diastole and systole were not different in either of the E2 status groups 3 weeks post-MI (Table 3.7). When the MI and sham rats from each E2 status group were combined to increase the power of the observation, the IVSd, PWd and PWs of intact rats were significantly larger than those from OVX+high E2 and OVX+veh rats. These data were analyzed in separate experiments and thus conclusions based on these differences must be made with caution.

In MI rats, significant increases in the LVID in diastole were present (45%, 18%, 37% in intact, OVX+veh and OVX+high E2 rats, respectively, compared to sham groups). LVID was also increased in systole in all three groups (by 100%, 70%, 105%, respectively). Despite differences in the degree of LVID increase, fractional shortening

was decreased by 36-39% and ejection fraction was decreased by 23-25%, similarly in all groups.

Table 3.7. Cardiac function and dimension measured by echocardiography in female rats 3 weeks following MI/Sham surgery.

	Intact		OVX + veh.		OVX + high E2	
	Sham	**MI**	**Sham**	**MI**	**Sham**	**MI**
n	13	23	16	21	12	16
Infarct size - (% of LV)		$^{\$}41±2		43±2		47±3
IVSd (mm)	2.2±0.2	2.5±0.1	1.9±0.1	2.1±0.1	2.0±0.1	1.8±0.1
IVSs (mm)	3.5±0.1	3.4±0.1	3.3±0.2	3.1±0.2	3.4±0.2	2.8±0.2
PWd (mm)	2.3±0.2	2.3±0.1	1.9±0.1	1.9±0.2	2.0±0.2	1.7±0.2
PWs (mm)	3.3±0.2	3.5±0.1	3.1±0.2	3.0±0.1	3.1±0.2	2.7±0.1
LVIDd (mm)	5.5±0.3	8.0±0.3*	6.5±0.2	7.7±0.3*	5.4±0.2$^{\#}$	7.4±0.3*
LVIDd/BW (mm/100g)	1.9±0.1	2.8±0.1$^{*\wedge}$	1.9±0.1	2.2±0.1$^{*\wedge}$	2.5±0.1$^{\&}$	3.3±0.2$^{*\wedge}$
LVIDs (mm)	2.7±0.3	5.4±0.4*	3.0±0.1	5.2±0.5*	2.4±0.3$^{\#}$	4.9±0.4*
LVIDs/BW (mm/100g)	1.0±0.1	1.9±0.1*	0.9±0.1*	1.5±0.1$^{\wedge*}$	1.1±0.1	2.2±0.2*
FS (%)	53±3	33±3*	53±3	34±3*	57±3	35±3*
EF (%)	88±2	67±4*	89±1	67±4*	90±3	69±5*

Values are expressed as mean ± SEM. *p<0.05 vs. respective sham group. #p<0.05 vs. OVX+veh., sham. &p<0.05 vs. other sham groups. ^p<0.05 vs. other MI groups. $MI size measured from a sub-set of 14/23 rats from this group; all others had 'large' or 'medium' infarct sizes by visual inspection. IVS: interventricular septal thickness. PW: posterior wall thickness. LVID: left ventricular internal dimension. EF: ejection fraction. s: systole. d: diastole. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

3.2.4. LV function by direct catheterization 3 weeks post-MI

LV function was also measured on the day of euthanasia by direct catheterization with PE-50 tubing (Experiment 2a) or using a Millar catheter (Experiments 2b and 2c). The Millar catheter method measures negative and positive dP/dt and thus these parameters were measured in Experiment 2b and 2c only. Similar to the echocardiographic data, determination of LVEDP, LVPSP, +/- dP/dt in ovary-intact rats was performed in a separate experiment (2c) from the OVX+veh and OVX+high E2 rats (2b).

LVEDP, LVPSP and +/- dP/dt were not different between sham OVX+veh and OVX+high E2 rats. When measured by PE-50 catheter, heart rates were significantly lower in OVX+high E2 versus OVX+veh rats, in both sham and MI rats. The trend was similar when measured by Millar catheter, but did not reach statistical significance ($p=0.07$).

At 3 weeks post-MI, LVEDP was similarly increased in all three groups of rats compared to their respective shams (Table 3.8). LVPSP was similarly decreased in all three groups of rats: 11% in intact rats, 10-13% in OVX+veh rats, and 14% in OVX+high E2 rats (Table 3.8). Positive dP/dt was decreased 16-20% and negative dP/dt was increased 23-30% at 3 weeks post-MI, ultimately to similar levels in all three MI groups. Heart rate was not significantly affected by MI.

Table 3.8. Left ventricular function measured by direct catheterization in female rats 3 weeks post-MI

	Intact		OVX + veh		OVX + high E2	
	Sham	MI	Sham	MI	Sham	MI
PE-50 in conscious rats						
n	np	np	8	8	5	4
infarct size (% of LV)				44±3		52±6
LVEDP (mmHg)	np	np	3±1	14±4*	3±1	13±3*
LVPSP (mmHg)	np	np	159±5	139±6*	155±5	134±11*
Heart Rate (bpm)	np	np	428±18	415±10	403±13	367±28
Millar in rats under mild anesthesia						
N	8	20	3	7	3	6
Infarct size (% of LV)		$43±3		41±2		41±3
LVEDP (mmHg)	3±2	16±3*	5±3	12±2*	5±1	13±2*
LVPSP (mmHg)	129±4	117±3*	147±12	132±5	151±7	130±4*
+dP/dt (mmHg/h)	8284 ±322	6609 ±234*	8782 ±531	7309 ±267*	8311 ±299	7014 ±213*
-dP/dt (mmHg/h)	-7663 ±410	-5683 ±256*	-8428 ±664	-6313 ±324*	-8057 ±456	-5661 ±294*
Heart Rate (bpm)	387±10	374±7	372±18	348±16	327±23	317±17

(PE-50 Heart Rate: OVX + veh groups vs. OVX + high E2 groups, $p<0.05$)

Values are expressed as mean ± SEM. *$p<0.05$ vs. respective sham group. Comparisons made by 2-way ANOVA. $MI size measured from 13/20 rats from this group, all others had 'large' or 'medium' infarct sizes by visual inspection. np: not performed. bpm: beats per minute. LVEDP: left ventricular end diastolic pressure. LVPSP: left ventricular peak systolic pressure. +dP/dt: positive first derivative of pressure. -dP/dt: negative first derivative of pressure. OVX: ovariectomized. E2: 17β-estradiol. veh: vehicle.

3.2.5. Regulation of circulatory RAS by E2 in female rats 3 weeks post-MI

In sham rats, OVX decreased PRA (ns), plasma Ang I (significantly), and plasma Ang II (ns) but not ACE activity or the Ang II/I ratio (Table 3.9). Treatment with high E2 decreased PRA by nearly 3 fold and Ang I by 3.3 fold. Treatment with high E2 decreased ACE activity by 16% (ns) compared to ovary-intact sham rats and 25% (significantly) compared to OVX+veh sham rats and decreased Ang II by 60% compared to ovary-intact rats (significantly) and by 38% (ns) compared to OVX+veh rats. The ratio of plasma Ang II/Ang I, a measure of 'effective' ACE activity was unchanged by E2 status.

At 3 weeks post-MI, PRA, plasma Ang I, plasma ACE activity, plasma Ang II and the ratio of Ang II/I were unchanged in ovary-intact and OVX+veh MI rats versus their respective sham groups. At 3 weeks post-MI in OVX+high E2 MI rats, PRA was increased by 140%, Ang I was increased by 110% and plasma Ang II concentration was increased by 120%, whereas ACE activity and the ratio of plasma Ang II/Ang I were unchanged compared to their shams. However, absolute levels of circulatory RAS components in OVX +high E2 MI rats were no different from the levels in ovary-intact MI rats. In ovary-intact MI rats, PRA and Ang I were higher than those exhibited in OVX+veh MI rats. Of note, plasma Ang II levels did not differ across MI groups.

Table 3.9. Circulatory renin-angiotensin system activity in female rats 3 weeks post-MI.

	Intact		OVX + veh.		OVX+high E2	
	Sham	MI	Sham	MI	Sham	MI
n	6	15$^{\$}$	5	9	6	7
Infarct size (% of LV)		43±2		43±3		48±4
PRA (ng Ang I/min/ml)	5.0±0.5	4.8±0.5	3.8±0.9	2.9±0.4^	1.7±0.4$^{\#}$	4.0±0.8*
log$_{10}$(PRA)	0.68 ±0.05	0.64 ±0.05	0.52 ±0.14$^{\&}$	0.42 ±0.06^	0.14 ±0.13$^{\#}$	0.57 ±0.07*
n	6	11$^{\$}$	4	9	5	6
Infarct size (% of LV)		42±3		42±3		48±4
Ang I (pg/ml)	378±77	299±68	136±21$^{\#}$	129±25^	114±19$^{\#}$	234±52*
log$_{10}$(Ang I)	2.56 ±0.09	2.32 ±0.13	2.12 ±0.07$^{\#}$	2.02 ±0.11^	2.03 ±0.08$^{\#}$	2.33 ±0.08*
n	16	24$^{\$}$	10	17	9	13
Infarct size (% of LV)-		42±2		42±1		47±4
ACE activity (nmol/ml)	16.1 ±0.9	17.5 ±0.9	17.8 ±2.0	19.0 ±1.8	13.6 ±1.8$^{@}$	12.5 ±0.7$^{@}$
n	6	12$^{\$}$	5	10	6	7
Infarct size (% of LV)		43±3		43±3		48±4
Ang II (pg/ml)	15.9 ±2.8	11.4 ±2.7	9.8 ±0.4	11.2 ±3.1	6.7 ±1.7	14.7 ±2.9
Log$_{10}$(Ang II)	1.16 ±0.09	0.94 ±0.09	0.99 ±0.02	0.93 ±0.11	0.71 ±0.16$^{\#}$	1.11 ±0.09*
n	6	11$^{\$}$	4	9	5	6
Ang II/Ang I	0.05 ±0.01	0.05 ±0.01	0.08 ±0.01	0.07 ±0.01	0.06 ±0.01	0.06 ±0.02

Values are expressed as mean±SEM. *$p<0.05$ vs. respective sham group. $^{\#}p<0.05$ vs. Intact, sham. $^{\&}p<0.05$ vs. OVX+high E2, sham. ^$p<0.05$ vs. Intact MI. $^{@}p<0.05$ vs. OVX+veh, sham. Comparisons made by 2-way ANOVA. $^{\$}$infarct size calculated from 10/15 rats for PRA, 7/11 rats for Ang I and Ang II/I ratio, 17/24 for ACE activity and 8/12 for Ang II; the remaining had infarct sizes of 'med-large' by visual inspection. OVX: ovariectomized. Veh: vehicle. E2:17β-estradiol.

3.2.6. ACE mRNA abundance and activity, and ACE and AT_1R density in the RV and LV of female rats 3 weeks post-MI

ACE mRNA abundance and activity were measured in the RV and LV of all six groups of rats. ACE and AT_1R binding densities were only measured in the RV and LV of sham and MI ovary-intact rats. Figure 3.14 shows representative autoradiographs, Figure 2.13 shows ACE mRNA abundance and activity in the RV and LV, and Figure 3.15 shows ACE and AT_1R densities.

RV. In sham rats, OVX alone increased RV ACE mRNA abundance by 30% and tended to increase RV ACE activity (Figure 3.15 A&B). The increase in ACE mRNA abundance was prevented by high E2 treatment, and ACE activity tended to decrease with high E2 treatment. ACE activities were significantly lower in the RV in Experiment 2 compared to those in Experiment 1 (Table 3.2). The reason for this is not readily apparent but may be the result of technical problems with sample preparation for these tissues only, as repeated assay of these samples yielded similar low results.

In ovary-intact MI rats, RV ACE mRNA abundance, ACE activity and ACE density increased significantly (55, 110, 55%, respectively) compared to their respective sham levels (Figure 3.14, Figure 3.15, Figure 3.16A). In OVX+veh MI rats and OVX+high E2 MI rats, RV ACE mRNA abundance tended to increase, and ACE activity was significantly increased (80% and 180%, for OVX+veh and OVX+high E2, respectively) versus their respective shams. The absolute values of RV ACE mRNA abundance and activity were similar in all three MI groups.

In ovary-intact rats, RV AT_1R density tended to increase post-MI (Figure 3.14, Figure 3.16B).

LV. In sham rats, OVX increased LV ACE mRNA by 100% and activity by 35%, and both effects were prevented by treatment with high E2 (Figure 3.15 A&B). In ovary-intact MI rats, ACE mRNA abundance and activity in the remote LV tended to increase and ACE density was increased by 230% compared to their respective shams (Figure 3.14, Figure 3.15, Figure 3.16A). These parameters were also significantly increased by several fold in the peri-infarct and infarct areas of ovary-intact MI rats compared to their respective sham levels. In OVX+veh MI rats, ACE mRNA abundance and activity tended to increase in the remote LV and increased significantly in the peri-infarct and infarct areas compared to their respective sham levels. In OVX+high E2 MI rats ACE mRNA abundance tended to increase in the rLV and ACE activity was increased by 170% in the rLV compared to the LVs of their respective sham rats and these parameters were also increased in these rats in the peri-infarct and infarct areas by several fold. The percent increases in ACE activity in the three LV areas of OVX+high E2 MI rats were significantly larger compared to the same areas of the other MI groups (Table 3.10). In the remote LV and in the peri-infarct areas, the absolute ACE mRNA abundances were higher in OVX+veh and OVX+high E2 MI rats than ovary-intact MI rats whereas ACE activity was higher in OVX+high E2 MI rats compared to the other two MI groups. In contrast, in the infarct scar, the absolute ACE mRNA abundances and ACE activities were not significantly different between groups.

In ovary-intact MI rats, AT_1R binding densities were increased by 130, 300 and 520%, in the remote, peri-infarct and infarct areas, respectively, as compared to their respective sham levels (Figure 3.14, 3.16B).

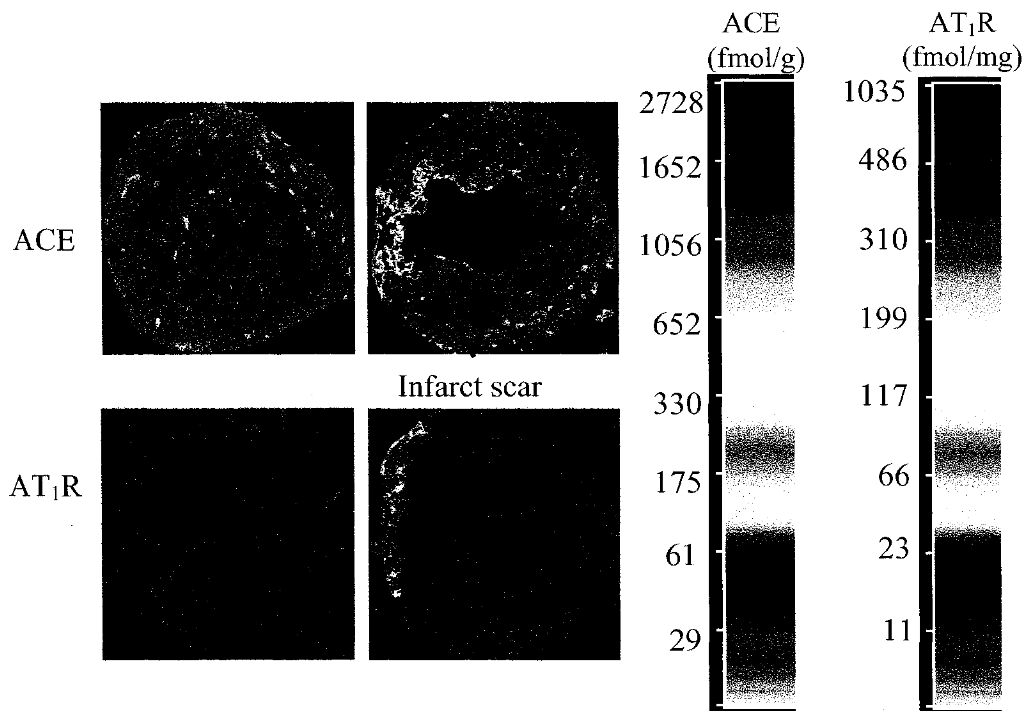
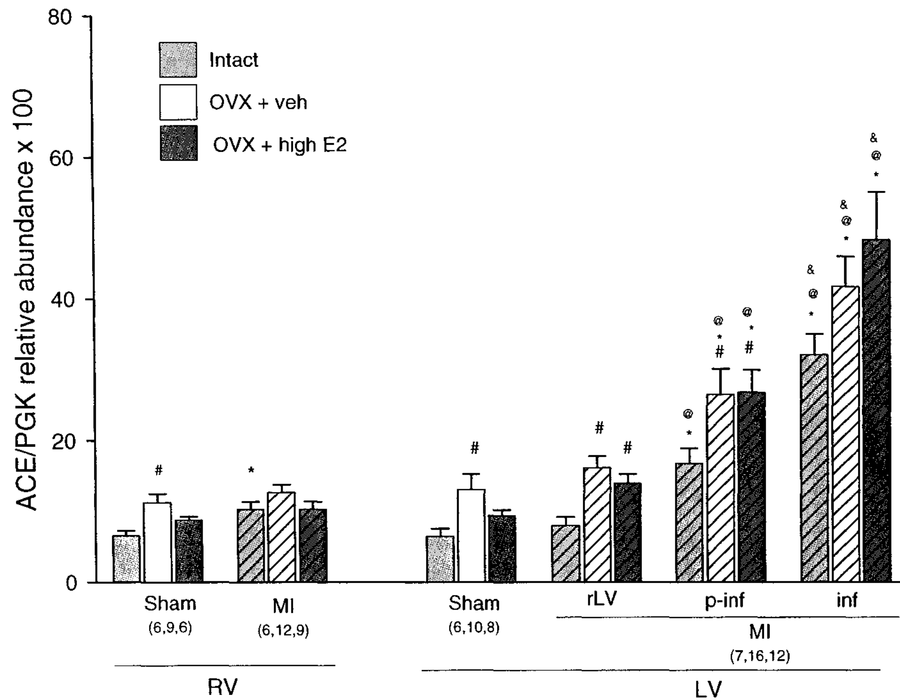


Figure 3.14. Representative autoradiographs of ACE and AT_1R binding densities in hearts of ovary-intact female rats 3 weeks post-MI.

A. ACE mRNA abundance



B. ACE activity

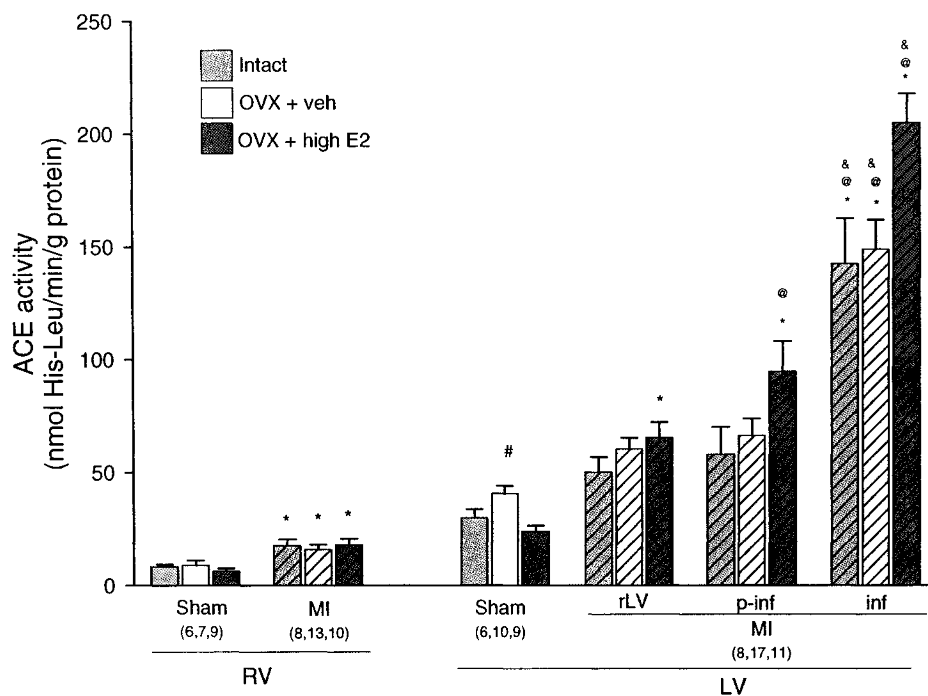
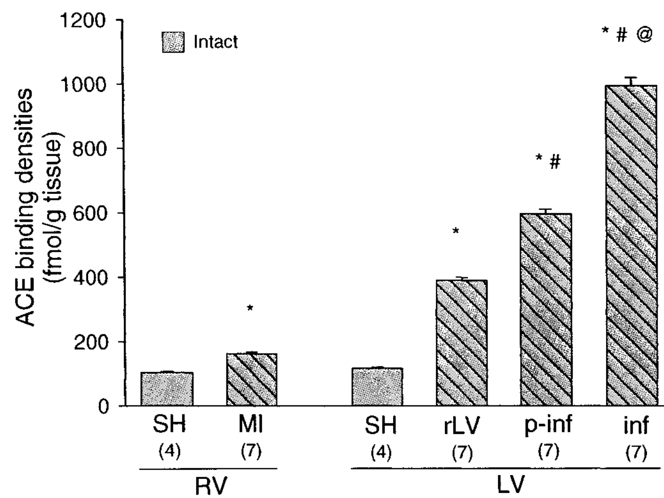


Figure 3.15. ACE mRNA abundance and activity in the left and right ventricles of female rats 3 weeks post-MI. Values expressed as mean ± SEM. Numbers in parentheses represent n/group. Numbers lower in some groups due to sample loss. *$p<0.05$ vs. resp. sham group. $^{\#}p<0.05$ vs. other RV or LV sham groups. $^{@}p<0.05$ vs. resp. rLV group. $^{\&}p<0.05$ vs. resp. p-inf group. rLV: remote left ventricle; p-inf: peri-infarct left ventricle. Hatched bars represent myocardial infarction

A. ACE densities



B. AT_1R densities

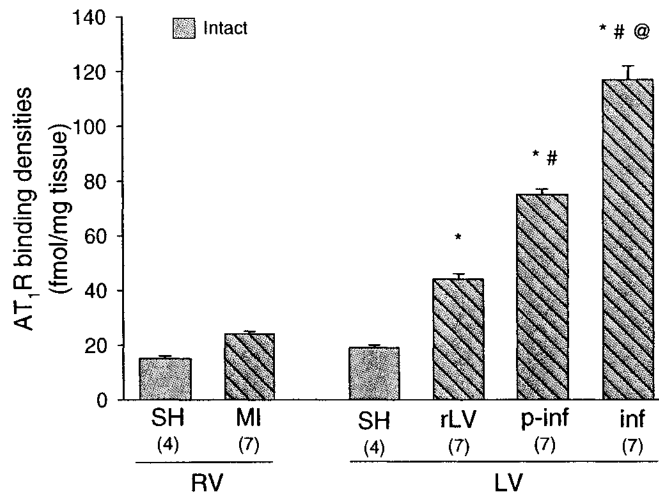


Figure 3.16. ACE and AT_1R densities in hearts of ovary-intact female rats 3 weeks following MI/Sham surgery as measured by in vitro autoradiography. Values expressed as mean ± SEM. Numbers in parentheses represent n/group. $^{*}p<0.05$ vs. representative sham area. $^{\#}p<0.05$ vs. rLV, 1 way ANOVA. $^{@}p<0.05$ vs. p-inf LV. LV: left ventricle. RV: right ventricle. rLV: remote left ventricle. p-inf: peri-infarct left ventricle. Inf: infarcted left ventricle Hatched bars represent myocardial infarction.

Table 3.10. Percent increases in LV ACE activity 3 weeks post-MI according to E2 status

	ovary-intact	OVX+veh	OVX+high E2
Sham	0	0	0
rLV	68 ± 22	49 ± 12	173 ± 28 $^{\#*\wedge}$
peri-infarct	94 ± 44	60 ± 19	295 ± 56 $^{\#*\wedge}$
infarct	377 ± 102 $^{\wedge}$	275 ± 35 $^{\wedge}$	747 ± 56 $^{\#*\wedge}$

Results expressed as percent increase ± SEM compared to respective sham LV levels. $^{\wedge}p<0.05$ vs. respective sham LV. $^{\#}p<0.01$ vs. respective OVX+veh. group. $^{*}p<0.01$ vs. respective ovary-intact group.

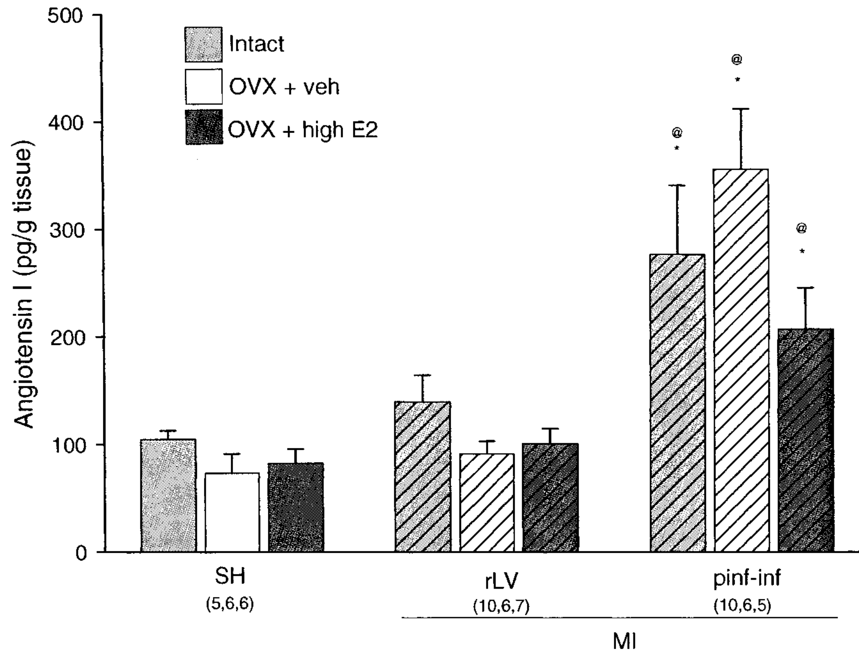
3.2.7. LV Ang I and II concentrations in female rats 3 weeks post-MI

In sham rats, E2 status did not alter LV Ang I (Figure 3.17) or II (Figure 3.18) concentrations. At 3 weeks post-MI, in all three E2 status groups, Ang I levels were not significantly increased in the rLV, but were increased by approximately 100-200% in the combined peri-infarct and infarct areas, to similar levels in all three groups. At 3 weeks post-MI, Ang II concentrations were unchanged in the rLV of all three E2 treatment groups. In ovary-intact MI rats, Ang II levels in the combined pinf-inf area were unchanged compared to the respective sham values. In contrast, in OVX+veh MI rats, Ang II in the pinf-inf area was increased by 240% and in OVX+high E2 MI rats, it was increased by 140%, both compared to their respective sham group. The absolute levels of Ang I in the pinf-inf area were similar across MI groups, whereas Ang II levels in the pinf-inf area were highest in OVX+veh and OVX+high E2 MI rats.

In sham rats, the LV Ang II/I ratio was significantly increased by 130-140% in OVX+veh and OVX+high E2 rats compared to ovary-intact rats (Figure 3.19). At 3 weeks post-MI, this ratio in the rLV was not altered in any MI group compared to their respective sham. In the combined pinf-inf areas, only OVX+high E2 MI rats had elevated 'effective' ACE activity compared to their respective shams; the ratio was also higher in

these rats compared to the other MI rats. Although the increase is statistically significant, the low number (n=3) in the OVX+high E2, MI group (due to sample loss) requires cautious analysis.

A. LV Angiotensin I



B. Log_{10} (LV Angiotensin I)

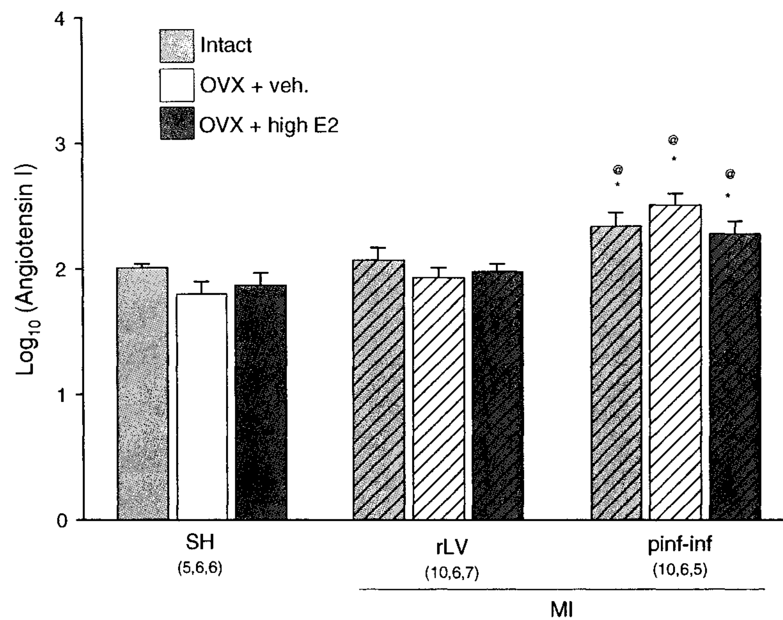
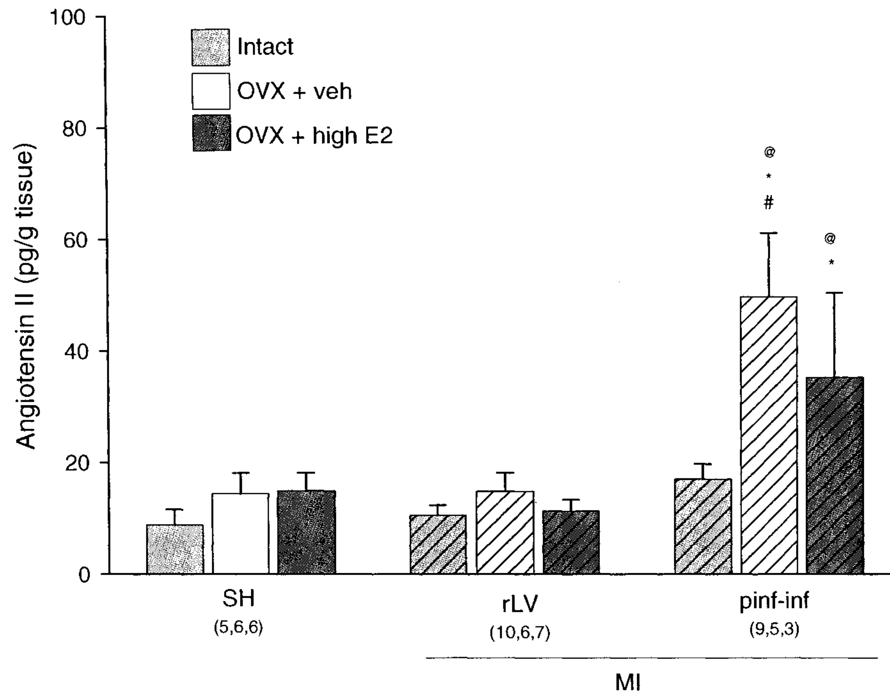


Figure 3.17. Angiotensin I concentrations in the left ventricle of female rats 3 weeks post-MI. Values are expressed as mean ± SEM of raw (A) or log-transformed (B) data. Numbers in parentheses are n/group; pinf-inf group for OVX+E2 has 2 fewer numbers due to sample loss. *$p<0.05$ vs. respective sham group. $^{@}p<0.05$ vs. respective rLV group. rLV: remote left ventricle. pinf-inf: area of left ventricle including the infarct and peri-infarct zones. Veh: vehicle. E2:17β-estradiol. OVX: ovariectomized. Hatched bars represent myocardial infarction.

A. LV Angiotensin II



B. Log_{10}(LV Angiotensin II)

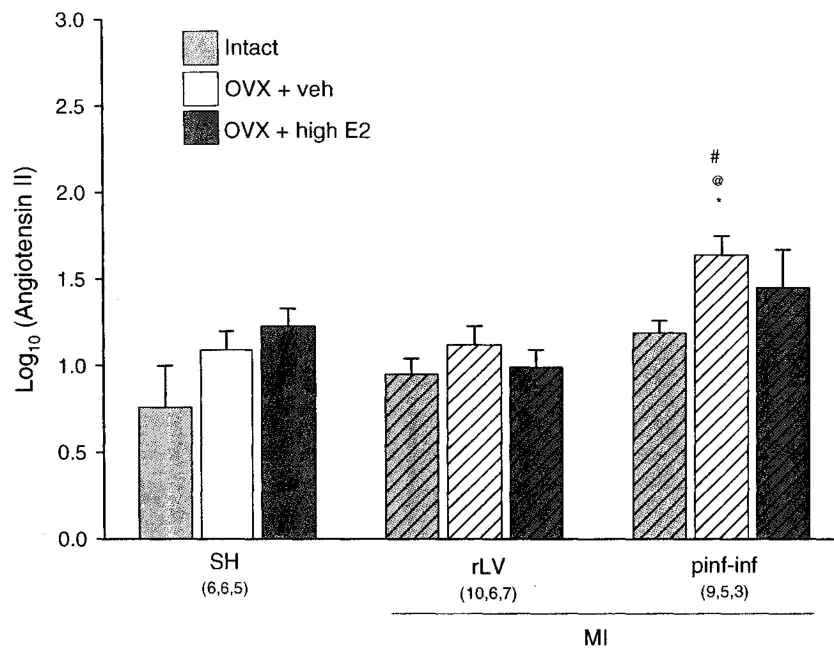


Figure 3.18. Angiotensin II concentrations in the left ventricle of female rats 3 weeks post-MI. Values are expressed as mean ± SEM of raw (A) or log-transformed (B) data. Numbers in parentheses are n/group; pinf-inf has smaller numbers in some cases due to sample loss. *$p<0.05$ vs. respective sham group. @$p<0.05$ vs. respective rLV group. #$p<0.05$ vs. intact, pinf-inf. rLV: remote left ventricle. pinf-inf: area of left ventricle including the infarct and peri-infarct zones. SH: sham. Veh: vehicle. E2:17β-estradiol. OVX: ovariectomized. Hatched bars represent myocardial infarction.

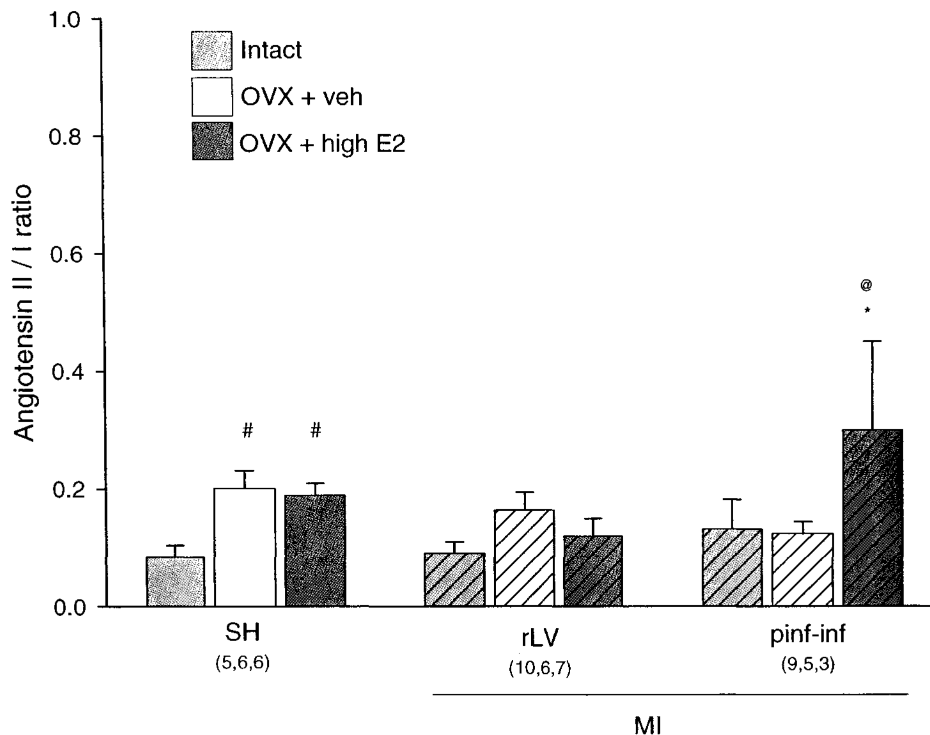
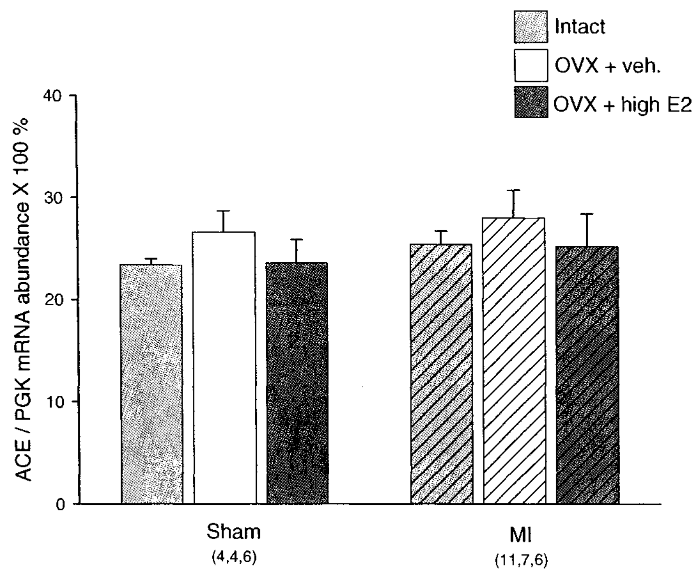


Figure 3.19. Ratio of angiotensin II / angiotensin I concentration in left ventricle of female rats 3 weeks post-MI: indirect measurement of ACE activity. Values are expressed as mean ± SEM. Numbers in parentheses are n/group; pinf-inf area numbers are lower due to sample loss. $^{*}p<0.05$ vs. respective sham group. $^{\#}p<0.05$ vs. Intact, sham group. $^{@}p<0.05$ vs. respective rLV group, all 2-way ANOVA. rLV: remote left ventricle. pinf-inf: tissue area containing the infarct and peri-infarct zones. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle. Hatched bars represent myocardial infarction.

3.2.8. Hypothalamic ACE mRNA abundance and activity in female rats 3 weeks post-MI

To determine the effect of E2 status and MI on brain ACE expression, ACE mRNA abundance and activity were measured in hypothalamic homogenates. At 3 weeks post-MI, ACE mRNA abundance and ACE activity were unchanged according to E2 status or MI (Figure 3.20 A&B).

A. ACE mRNA



B. ACE activity

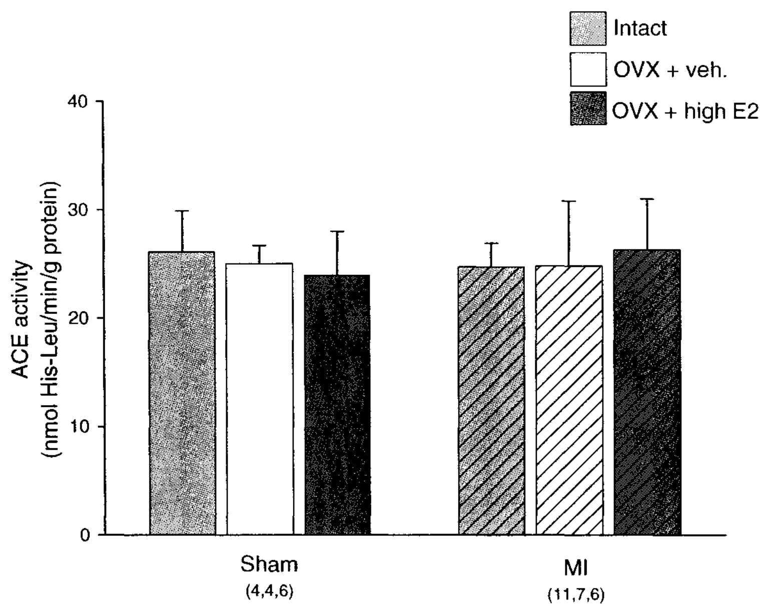


Figure 3.20. ACE mRNA abundance and activities in hypothalamic homogenates of female rats 3 weeks post-MI. Values are expressed as mean ± SEM. Numbers in parenthesis are n/group. OVX: ovariectomized. Veh: vehicle. E2: 17β-estradiol. Hatched bars represent myocardial infarction.

3.2.9. ACE and AT_1R binding densities in brain nuclei of female rats 3 weeks post-MI

The lack of change in ACE mRNA abundance and activity in hypothalamic homogenates could represent a lack of regulation of ACE by E2 and MI. However, changes as small as 25% in hypothalamic ACE mRNA abundance and activity have been detected previously using similar methodology (Zhao et al., 2001). In the hypothalamus, ACE and AT_1R are expressed in highly discrete regions, including the SFO, OVLT, PVN and MnPO. To address the possibility that the treatments may result in small changes in discrete nuclei, *in vitro* autoradiography was used to measure ACE and AT_1R densities. This technique is highly sensitive as it measures the density of these proteins on the plasma membrane of individual nuclei using radioactively labeled ligands. Figure 3.21 shows representative autoradiographs, Table 3.11 shows densitometry data, and Figure 3.22 shows percent changes in ACE and AT_1R by this method.

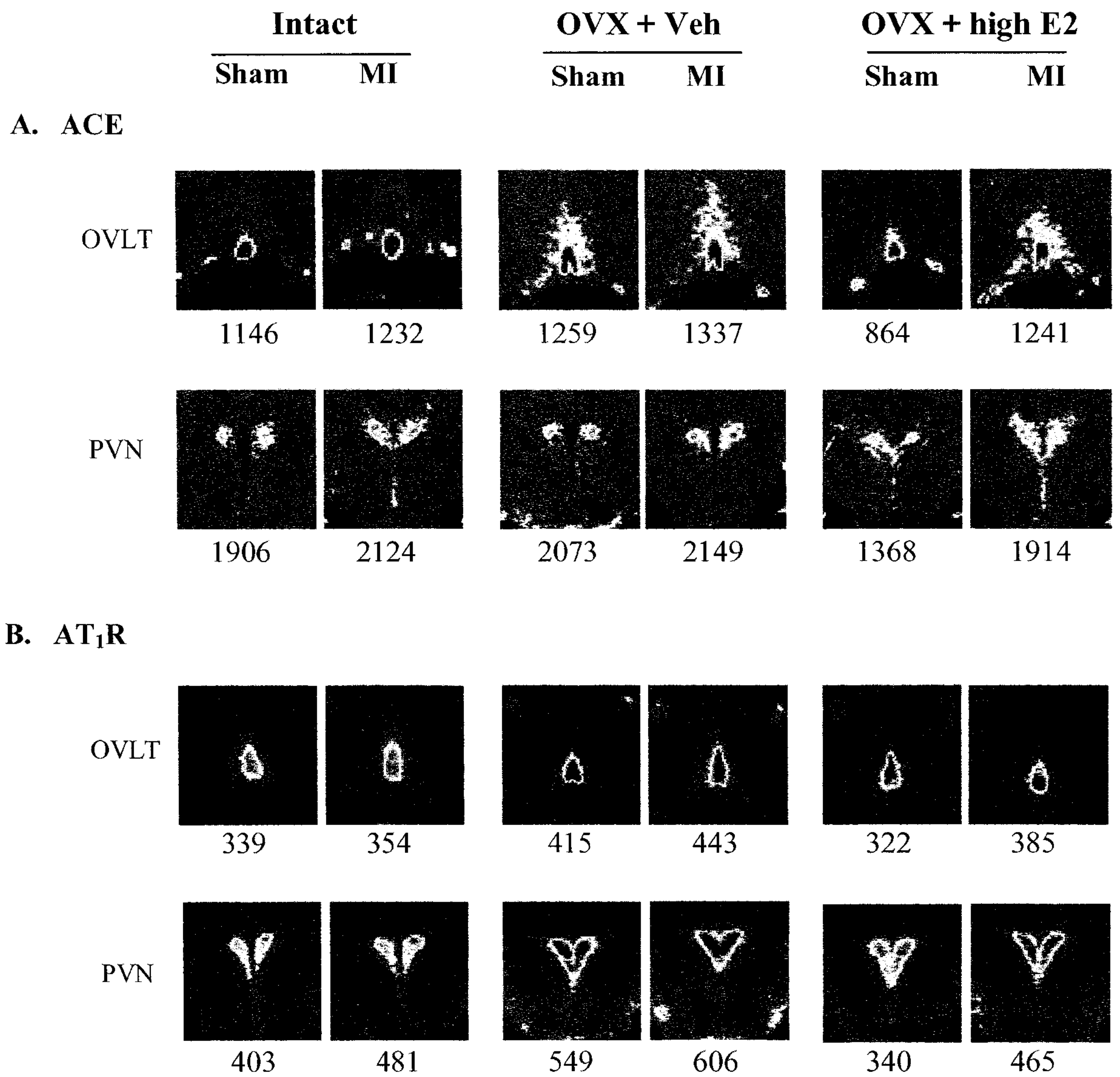
ACE density. In sham rats, OVX+veh rats exhibited modest (<15%) increases in ACE densities in SFO and MnPO compared to ovary-intact rats (Table 3.11, Figure 3.22A). The increases were reversed to significant decreases by high E2 treatment, and high E2 also decreased ACE densities in the OVLT and PVN.

At 3 weeks post-MI, in ovary-intact MI rats, ACE binding densities were modestly (<10%) increased in the PVN and MnPO compared to their respective sham rats. In OVX+veh MI rats, only minor, non-significant increases in ACE densities were apparent in any nuclei compared to their shams. In OVX+high E2 MI rats, ACE densities were increased in all four nuclei compared to their respective sham rats. Across MI groups, ACE densities were highest in OVX+veh rats for three or four nuclei, similar in ovary-

intact and OVX+high E2 rats for the OVLT and SFO, and significantly lower in OVX+high E2 rats versus ovary-intact rats in the MnPO and PVN.

AT_1R density. In sham rats, OVX+veh rats exhibited significant, but modest (<20%), increases in AT_1R densities in all four nuclei (Table 3.11, Figure 3.21). The increase was somewhat larger in the PVN (30%). These increases were reversed or tended to reverse to decreases by high E2 treatment.

In ovary-intact MI rats, AT_1R densities were modestly (<15%) increased in the SFO and PVN compared to their respective sham rats. In OVX+veh MI rats, AT_1R densities in the PVN and MnPO were modestly increased compared to their respective sham rats, about 10% over and above the increase in density due to OVX alone. In OVX+high E2 MI rats, AT_1R densities were significantly increased or tended to increase compared to their respective shams. Across MI groups, AT_1R densities were highest in OVX+veh rats in all nuclei. OVX+veh and OVX+high E2 rats had similar AT_1R densities in the OVLT, MnPO and PVN. In the SFO, OVX+high E2 rats had lower AT_1R density than ovary-intact rats.



3.21. Representative autoradiographs of ACE and AT_1R binding densities in brain nuclei of female rats 3 weeks post-MI. OVLT: organum vasculosum laminae terminalis. SFO: subfornical organ. PVN: paraventricular nucleus. MnPO: median preoptic nucleus. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle. ^{125}I standard densities as in Figure 3.14.

Table 3.11. ACE and AT_1R binding densities in brain nuclei of female rats 3 weeks post-MI

	Intact		OVX + veh.		OVX + high E2	
	Sham	**MI**	**Sham**	**MI**	**Sham**	**MI**
n	8	12	4	9	4	7
MI size (% of LV)	-	$37±3^{\$}$		41±2		43±4
ACE						
OVLT	1177±36	1212±35	1295±34	$1361±34^{+}$	$872±10^{\#}$	$1220±52^{*}$
SFO	3245±43	3359±47	$3624±92^{\#}$	$3793±84^{+}$	$2413±32^{\#}$	$3317±115^{*}$
PVN	1922±46	$2098±50^{*}$	2116±81	2146±76	$1335±25^{\#}$	$1865±53^{*+}$
MnPO	845±16	$935±28^{*+}$	$954±23^{\#}$	$1024±23^{+}$	$666±26^{\#}$	$777±28^{*+}$
AT_1R						
OVLT	350±10	364±8	$407±9^{\#}$	$431±12^{+}$	$310±9^{\#}$	$379±8^{*}$
SFO	623±11	$658±12^{*+}$	$725±13^{\#}$	$733±9^{+}$	$443±8^{\#}$	$623±9^{*+}$
PVN	412±8	$461±12^{*}$	$535±10^{\#}$	$593±14^{*+}$	$333±6^{\#}$	$478±7^{*}$
MnPO	343±11	370±10	$387±9^{\#}$	$429±12^{*+}$	323±9	354±11

Values expressed as mean ± SEM. *$p<0.05$ vs. respective sham group. $^{\#}p<0.05$ vs. other sham groups. $^{+}p<0.05$ vs. other MI groups, by 2-way ANOVA. ACE binding densities: fmol of ^{125}I-351A bound/g wet tissue. AT_1R binding densities are in ^{125}I-Ang II bound/mg wet tissue. $^{\$}$infarct size calculated from 5/12 rats; the remaining had infarct sizes of 'medium' or 'large' by visual inspection. OVLT: organum vasculosum laminae terminalis. SFO: subfornical organ. PVN: paraventricular nucleus. MnPO: median preoptic nucleus. OVX: ovariectomized. E2: 17β-estradiol. Veh: vehicle.

3.2.10. Relationship between ACE and AT_1R binding densities in brain nuclei and infarct size in female rats 3 weeks post-MI

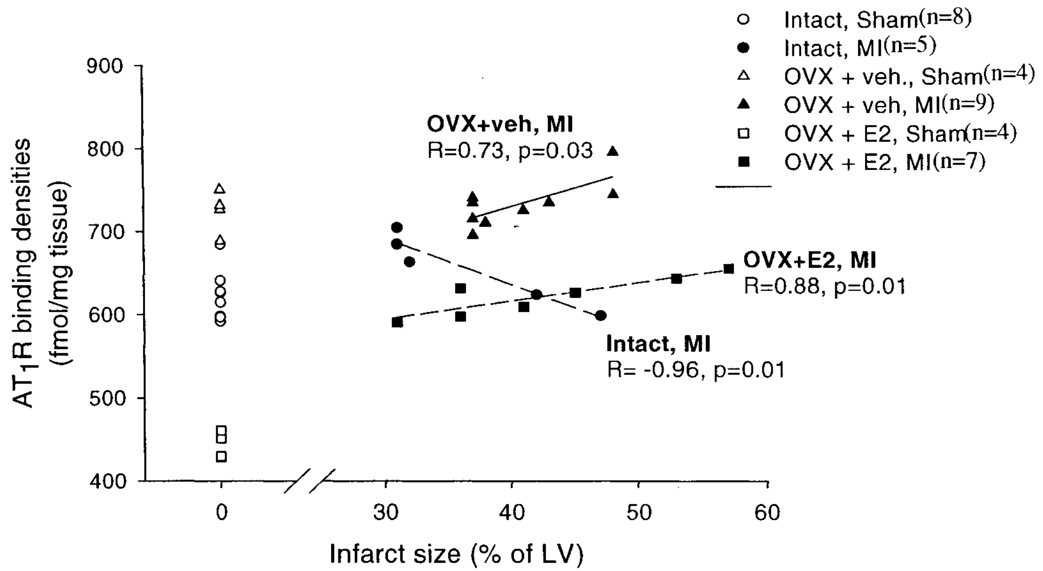
To determine whether a relationship exists between brain ACE and AT_1R binding densities, infarct size, and E2 status, densities were plotted against infarct size for the three MI groups (Table 3.12, Figure 3.22). ACE densities did not correlate well with infarct size except for a negative correlation in the PVN of OVX+veh rats. AT_1R in the MnPO correlated positively with infarct size in only the OVX+high E2 rats. In the SFO, all groups exhibited a significant relationship between AT_1R binding density and infarct size (Figure 3.22A), but interestingly, both OVX+veh. and OVX+high E2 rats exhibited an <u>increase</u> in AT_1R binding densities with infarct size but ovary-intact rats exhibited a <u>decrease</u>. In the PVN, a positive relationship existed for OVX+veh rats, no significant relationship was present for OVX+high E2 rats and a negative relationship existed for ovary-intact rats (Figure 2.22B).

Table 3.12. Relationships between ACE and AT_1R densities in brain nuclei relative to infarct size 3 weeks post-MI

	Correlation coefficient	p-value
SFO		
ACE		
Intact	r = 0.61	p = 0.28
OVX+veh	r = -0.07	p = 0.86
OVX+high E2	r = 0.41	p = 0.36
AT_1R (shown in Figure 3.23)		
Intact	**r = -0.96**	**p = 0.01**
OVX+veh	**r = 0.73**	**p = 0.03**
OVX+high E2	**r = 0.88**	**p = 0.01**
PVN		
ACE		
Intact	r = -0.84	p = 0.08
OVX+veh	**r = -0.75**	**p = 0.051**
OVX+high E2	r = -0.63	p = 0.09
AT_1R (shown in Figure 3.23)		
Intact	**r = -0.89**	**p = 0.04**
OVX+veh	**r = 0.70**	**p = 0.04**
OVX+high E2	r = 0.63	p = 0.13
OVLT		
ACE		
Intact	r = -0.49	p = 0.41
OVX+veh	r = -0.12	p = 0.76
OVX+high E2	r = -0.33	p = 0.46
AT_1R		
Intact	r = -0.55	p = 0.34
OVX+veh	r = 0.66	p = 0.06
OVX+high E2	r = 0.58	p = 0.17
MnPO		
ACE		
Intact	r = -0.71	p = 0.18
OVX+veh	r = -0.02	p = 0.96
OVX+high E2	r = -0.40	p = 0.37
AT_1R		
Intact	r = -0.71	p = 0.18
OVX+veh	r = 0.60	p = 0.09
OVX+high E2	**r = 0.78**	**p = 0.038**

OVX: ovariectomized. Veh: vehicle. E2: 17β-estradiol. Intact: intact ovaries. All relationships assessed with Pearson's Correlation. n = 5-7/group.

A. SFO



B. PVN

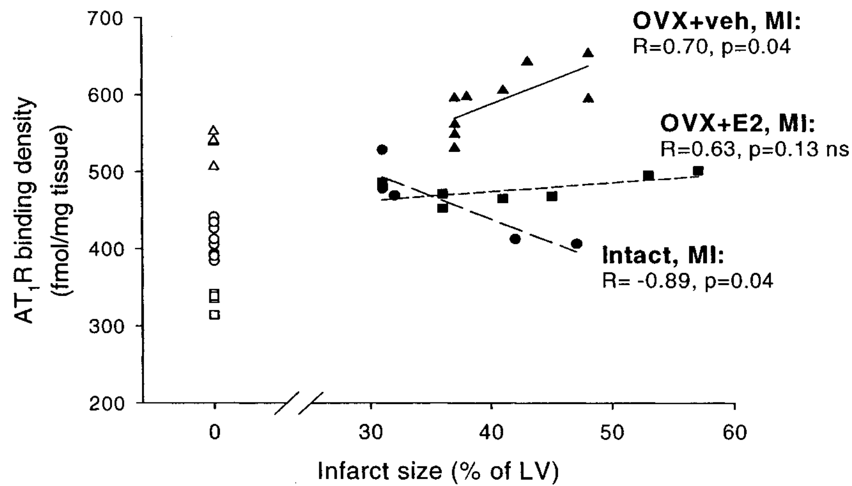


Figure 3.22. Relationship between AT_1R binding densities in the SFO and PVN and infarct size in female rats 3 weeks post-MI. OVX: ovariectomy; E2: 17β-estradiol; Veh: vehicle; MI: myocardial infarction; intact: intact ovaries; ns: non-significant.

3.2.11. Overall summary of Experiment 2a, 2b and 2c

Mortality, hypertrophy and LV function. These parameters occurred to similar degrees at 3 weeks post-MI in female rats irrespective of E2 status. Heart rate was significantly lower in OVX rats treated with high E2 compared to the other groups. RV hypertrophy occurred to a greater degree in ovary-intact rats post-MI versus OVX rats treated with vehicle or high E2.

Circulatory RAS. Five weeks after OVX, PRA tended to decrease, plasma Ang I was significantly decreased, plasma Ang II tended to decrease and plasma ACE activity and the ratio of Ang II/I were unchanged. Treatment with high E2 decreased PRA by nearly 3 fold, and Ang I by 3.3 fold; ACE activity tended to decrease compared to ovary-intact sham rats and was significantly decreased by 25% compared to OVX+veh sham rats. Ang II was significantly decreased by 60% compared to ovary-intact rats and tended to decrease compared to OVX+veh rats. In ovary-intact and OVX+veh MI rats, circulat RAS components were not changed compared to their respective shams. In OVX+high E2 MI rats, all components except ACE activity were increased significantly post-MI compared to their respective shams. However, plasma Ang II levels were not significantly different across MI rats.

Cardiac RAS. In sham rats, OVX alone increased RV and LV ACE mRNA abundance and ACE activity. These effects were prevented, but not reversed to decreases, by high E2 treatment, results similar to those obtained in Experiment 1. This increase in ACE did not translate into an increase in Ang II present in the LV, and did not relate well to Ang II/I ratio. At 3 weeks post-MI, all rats in this study regardless of E2 status exhibited large increases in ACE mRNA abundance and ACE activity in the areas immediately surrounding and within the infarct scar. Ovary-intact MI rats also exhibited

large increases in LV ACE and AT_1R binding densities. The percentage increase in infarct area ACE activity was highest in OVX+high E2 MI rats, but absolute levels of infarct ACE were similar in all MI rats. Ang I was increased similarly in the infarct scar of all three groups of MI rats, whereas Ang II was increased only in OVX+veh and OVX+high E2 MI rats. The absolute levels of LV Ang II were highest in the infarct scar of OVX+veh and OVX+high E2 MI rats.

Brain RAS. Neither E2 status nor MI affected ACE mRNA abundance or activity in hypothalamic homogenates. In sham rats, OVX increased ACE density in the SFO and MnPO, and AT_1R binding densities in all four nuclei. Each of these increases were reversed to decreases in OVX+high E2 sham rats, results similar to those obtained in Experiment 1. In ovary-intact MI rats, ACE density was increased in PVN and MnPO and AT_1R densities were increased in PVN and SFO compared to their respective sham rats. In OVX+veh MI rats, ACE density tended to increase further post-MI, whereas AT_1R density was significantly increased in the PVN and MnPO compared to their respective sham rats. In OVX+high E2 MI rats, both ACE and AT_1R densities in all nuclei were increased compared to sham. Across MI rats, ACE and AT_1R densities were higher in nearly all cases in OVX+veh rats. In two cases, ACE and AT_1R were significantly higher in ovary-intact female rats than OVX+high E2 (ACE in PVN and AT_1R in SFO), but in all other cases they were similar.

While ACE binding densities in brain nuclei did not correlate well with infarct size, AT_1R binding densities in the SFO and PVN were significantly correlated with infarct size in an E2 status-dependent manner. AT_1R densities from ovary-intact rats were negatively correlated with infarct size, whereas densities from OVX+veh rats were

positively correlated with infarct size and densities from OVX+high E2 rats positively correlated with infarct size in the SFO only.

Chapter 4:

DISCUSSION

4.0. Overview

The present experiments provide several new findings regarding the regulation of the local tissue RASs by E2 in otherwise healthy rats and in rats 3 weeks following myocardial infarction. First, in healthy rats, 5 weeks of E2 deficiency increased ACE and/or AT_1R in all tissues examined: the heart, brain, kidney, abdominal aorta, adrenal and lung and, in contrast, tended to decrease the activity of the circulatory RAS. The OVX-induced increases in ACE and AT_1R in tissues were prevented by replacement of E2 at physiological levels and for the most part reversed to decreases by replacement of E2 at high levels. High E2 decreased several components of the circulatory RAS, including Ang II.

At 3 weeks post-MI, in ovary-intact rats, ACE and AT_1R were increased in the RV and LV and in several cardiovascular regulatory nuclei of the brain compared to their respective sham rats. In OVX+veh MI rats, ACE was increased in the RV and LV and AT_1R was increased in the PVN and MnPO. In OVX+high E2 MI rats, ACE was increased in the RV and LV, ACE and AT_1R were increased in several brain nuclei compared to their respective shams. The percentage increases in LV ACE and brain ACE and AT_1R post-MI were highest in OVX+high E2 rats, and only these rats exhibited an increase in plasma Ang II post-MI compared to their shams. However, the absolute values of ACE in the RV and LV and Ang II in plasma post-MI were similar between MI groups. In the brain, OVX+veh MI rats exhibited modestly (20%) higher ACE and AT_1R densities in all nuclei compared to the other two MI groups.

4.1. Regulation of abdominal aorta, adrenal and kidney tissue RASs by E2 status

Abdominal aorta. Five weeks after OVX, abdominal aorta ACE was increased by 60%. This increase was prevented by regular E2 treatment and reversed to a decrease by

high E2. This is consistent with previously published studies. Fourteen weeks after OVX, ACE activity in rat aorta was increased by 26%. This increase was prevented by replacement of E2 at physiological levels (Tanaka et al., 1997). Treatment of OVX rats with high E2 for 3 weeks, versus vehicle, decreased aortic ACE activity by ~30% (Gallagher et al., 1999). It is not clear from the current studies whether an increase in vascular ACE activity results in changes in local vascular Ang peptide concentration. However, in VSMCs, transfection of ACE cDNA increases DNA and RNA synthesis; this effect was blocked by an AT_1R antagonist, suggesting that an increase in ACE expression leads to an increase in local Ang II and to a physiological response (Morishita et al., 1993).

Five weeks after OVX, abdominal aorta AT_1R was increased by 60%. This increase was prevented by replacement of E2 at physiological levels and reversed to a decrease by high E2. These findings are consistent with previous studies. Five weeks after OVX, AT_1R mRNA abundance and binding density in rat aorta were increased by 45-55%. These increases were prevented by E2 replacement at physiological levels (Nickenig et al., 1998a). Similarly, *in vitro*, in VSMCs from ovary-intact rats, E2 treatment for 24 hours decreased AT_1R mRNA (Nickenig et al., 2000). The changes in these vascular RAS components have been previously shown to have effects relevant to cardiovascular system regulation. For example, the changes in AT_1R as noted above (increase with OVX and prevention by treatment with E2) were associated with parallel changes in Ang II-induced constriction of aortic rings (Nickenig et al., 1998a). Together, these studies suggest that E2 deficiency/replacement may alter the sensitivity of vessels to Ang II and thereby regulate vascular tone. Indeed, OVX rats treated with high E2 for 3 weeks exhibit attenuated pressor responses to Ang II (Brosnihan et al., 1997b).

Adrenal. Five weeks after OVX, ACE in the adrenal medulla was increased by 60%. This increase was prevented by regular E2 treatment and tended to reverse to a decrease by high E2 treatment. This is the first study to report regulation of adrenal ACE by E2, but did not assess whether the regulation of adrenal ACE results in changes in the local concentration of Ang I and II. In male ACE knockout mice, adrenal Ang II levels are ~25% that of wild-type (Campbell et al., 2004), indicating that a virtual absence of ACE activity in the adrenal decreases the production of local Ang II. It also indicates that ACE is a major, but not the sole enzyme involved in the production of Ang II in the adrenal. Since data from heterozygous knockout mice were not reported, it is unclear if a dose-dependent decrease in adrenal ACE activity results in a parallel decrease in adrenal Ang II, and therefore whether E2 treatment may downregulate adrenal Ang II levels.

Five weeks after OVX, AT_1R was increased by 90% in the adrenal medulla and by 15% in the adrenal cortex. These increases were prevented by regular E2 treatment and reversed to 20-30% decreases by high E2 treatment. Hinojosa-Laborde and colleagues (2004) demonstrated that 8 weeks after OVX, whole-adrenal AT_1R protein concentration was increased by 38%. This increase was prevented by replacement of E2 at physiological levels. Similarly, treatment of OVX dogs with E2 for 2 weeks decreased AT_1R density by 35% in whole-adrenal membrane fractions (Owonikoko et al., 2004). Treatment of OVX rats with moderate dose of sc E2 for 1, 2, and 4 weeks decreases AT_1R densities in adrenal cortical membrane fractions by 24-35% (Carrière et al., 1986; Wu et al., 2003a; Wu et al., 2003b). The relevance of this regulation of adrenal RAS components by E2 has been demonstrated *in vivo*. Roesch and colleagues (2000) showed that OVX-induced increases in adrenal AT_1R protein levels were associated with parallel changes in Ang II-induced secretion of aldosterone (Roesch et al., 2000; Wu et al., 2003).

High-dose sc EB treatment decreased catecholamine release from the isolated adrenal medulla from OVX rats (de Miguel et al., 1989). This effect may be a result of changes in local adrenal ACE and/or AT_1R expression, as the AT_1R and the AT_2R have been implicated in regulation of catecholamine secretion from isolated medullary fragments (Belloni et al., 1998). The present data is therefore consistent with the notion that adrenal catecholamine and aldosterone release may be altered by E2 status.

Kidney. Two and 5 weeks after OVX, kidney ACE was increased by 70-75%. These increases were prevented by replacement of E2 at physiological levels and reversed to decreases by high E2 at 2 but not 5 weeks post-OVX. Gallagher and colleagues (1999) reported a 30% decrease in kidney ACE mRNA and activity in OVX rats treated with high E2 for 3 weeks. Virtual elimination of ACE activity in the kidney nearly abolishes the production of Ang II: ACE knockout mice exhibited kidney Ang II levels that were only 1% of wild-type (Campbell et al., 2004). On the other hand, in male rats, chronic high E2 treatment increased kidney Ang I levels by 2.3 fold, consistent with a decrease in ACE activity, but did not significantly affect Ang II levels (Campbell, 1997).

Five weeks after OVX, AT_1R in the kidney cortex and medulla were increased by ~60%. These increases were prevented by regular E2 treatment and reversed to decreases by high E2 treatment. Consistent with these findings, Harrison-Bernard and colleagues (2003) demonstrated that in Dahl S rats, 10 days after OVX, AT_1R protein levels were increased by 2 fold. The increase was reversed to a ~50% decrease by E2 replacement at physiological levels. Baiardi and colleagues (2005) reported OVX-induced increases in AT_1R density, but not mRNA abundance, in kidney glomeruli and inner medulla in Wistar-Kyoto rats but not in Wistar-Hanover rats. The increase in binding is consistent with the current findings in Wistar rats. However, in contrast with the current findings,

E2 replacement at supra-physiological levels increased kidney AT_1R density but not AT_1R mRNA abundance in most areas over and above the OVX-induced increases. The only exception was in the vasa recta, where AT_1R was significantly decreased by high E2 (Baiardi et al., 2005). Several methodological differences may account for the differential regulation of AT_1R by E2 in these studies. First, the regulation may be rat strain-specific: the present studies used a Wistar strain different from Wistar-Hanover and Wistar-Kyoto. Second, whereas the rats in the present experiments underwent E2 pellet implantation at the same time as OVX, Baiardi and colleagues (2005) allowed a recovery of two weeks after OVX before starting E2 replacement. This may have allowed physiological compensations to OVX to occur, such as changes in ER levels, which may have altered the regulation of kidney AT_1R by E2. Thirdly, in contrast to the current study, Baiardi et al., (2005) used non-saturating concentrations of label to measure AT_1R density. In the absence of a change in mRNA abundance, it is therefore possible that the changes in AT_1R binding may reflect changes in affinity of the ligand to the receptor rather than changes in receptor number.

The increase in ACE and a concomitant increase in AT_1R in the vasculature, adrenal and kidney may indicate that a steady-state increase in tissue Ang II may not be necessary to observe a RAS-mediated effect. Each of the tissues studied may be more responsive to Ang II due to the increases in AT_1R.

4.2. Regulation of circulatory RAS by E2 status and MI

Sham rats. Five weeks after OVX, PRA tended to decrease, plasma Ang I levels were significantly decreased by nearly 3 fold, and plasma Ang II levels tended to decrease. Plasma ACE activity tended to increase and the ratio of plasma Ang II/I was unchanged. High E2 treatment of OVX rats reduced most RAS components further to

levels lower than OVX+veh rats and ovary-intact rats. The present study is the first to measure changes in plasma Ang I and Ang II due to OVX alone. Consistent with the current study, Scammell and colleagues (1981) demonstrated that treatment of OVX rats with high E2 for 12 weeks decreased PRA by 50% (Scammell et al., 1981). Tanaka and colleagues (1997) found that 14 weeks after OVX, plasma ACE activity was increased by 45%; this increase was prevented by treatment with E2 (0.2μg/day). OVX rats treated with regular or high E2 for 3 weeks exhibited a 30% decrease in plasma ACE activity (Brosnihan et al., 1997b; Gallagher et al., 1999). In the current study, plasma ACE activity only tended to increase 5 weeks after OVX. This discrepancy may reflect a change in plasma ACE activity related to the time of follow-up post-OVX. Two studies reported changes in Ang II that are consistent with the present findings: treatment of OVX rats with E2 at physiological levels for 3 weeks did not change plasma Ang II concentrations (Brosnihan et al., 1997b) but treatment with high EB for 2 weeks decreased Ang II by 25% (Carrière et al., 1986). Together, these studies suggest that in contrast to the regulation of local tissue RASs, OVX tends to decrease the activity of the circulatory RAS, and high E2 treatment decreases it further.

The mechanism by which high E2 downregulates renin is not known. Long-term treatment with E2 may decrease renal renin production and/or secretion leading to decreased plasma levels and thereby lower Ang I and II. This remains to be demonstrated. The mechanism of regulation of plasma ACE by E2 is also not characterized. In the normal rat, plasma ACE is exclusively derived from its regulated cleavage-secretion from the vascular (mostly pulmonary) endothelium. At 2 weeks post-OVX, the effects of E2 status on lung ACE activity are largely opposite to those that occurred in plasma: a decrease due to OVX, prevention by regular E2 and reversal to increases by high E2. The

decrease in plasma ACE by E2 at 2 weeks post-OVX may occur via inhibition of its basal cleavage-secretion from the lung or other vascular endothelia. ACE cleavage-secretion is regulated by 'ACE secretase', an as yet uncharacterized metalloprotease. E2 may dose-dependently inhibit the secretion of ACE leading to decreased plasma ACE levels.

Rats 3 weeks post-MI. In ovary-intact rats at 3 weeks post-MI, PRA, plasma Ang I, plasma ACE activity, plasma Ang II and the ratio of plasma Ang II/I were not changed compared to respective sham levels. These parameters were also unchanged post-MI in OVX+veh rats. In contrast, in OVX+high E2 MI rats, PRA, Ang I, and Ang II were significantly increased compared to the low baseline levels in their respective sham rats, and absolute levels of PRA and Ang I were significantly higher than in OVX+veh MI rats. However, Ang II levels were not significantly different across MI rats. In male rats, at this approximate time-point post-MI, plasma Agt was decreased by ~50% (Wang et al., 2004), whereas PRA, plasma Ang I, and plasma ACE activity were not significantly different from sham (Leenen et al., 1999a; Sun et al., 2001; Lindpaintner et al., 1993; Duncan et al., 1997). Ang II may be increased slightly at this time-point (Leenen et al., 1999a; Duncan et al., 1997). Thus in ovary-intact and OVX+veh rats, the changes in the circulatory RAS at 3 weeks post-MI are quite similar to those that occur in male rats - i.e. are minimal. In OVX+high E2 rats, however, the suppression in these components in the sham rat is overcome post-MI, such that the absolute levels of Ang II are the same as the MI rats of the other groups. How this relative activation of the circulatory RAS occurs in OVX+high E2 rats post-MI remains to be determined.

4.3. Regulation of cardiac ACE and AT_1R by E2 status and MI

Sham rats. Five weeks after OVX, LV ACE was increased by ~70%. This increase was prevented by regular E2 and reversed to a decrease by high E2. Five months

after OVX, LV ACE mRNA abundance was increased in mice by 20% (Freshour et al., 2002). E2 replacement was not performed. The increase in ACE post-OVX was not associated with changes in steady-state concentrations of LV Ang I and Ang II. There are several possible explanations for this apparent discrepancy. First, the production of LV Ang II may be enhanced in these rats but increased metabolism may limit the accumulation of steady-state Ang II (Ganten et al., 1983). Second, Agt concentration and/or renin activity, and not ACE, may be rate limiting in the production of Ang II in the LV. In transgenic rats overexpressing human ACE specifically in cardiac myocytes, LV ACE activity was increased 50-fold and yet LV Ang II levels were not increased (Tian et al., 2004). Similarly, in Dahl S rats fed high salt, LV ACE mRNA abundance and activity were increased 2-4 fold but LV Ang II levels were not increased. These rats have low baseline LV Ang I levels, and the increase in ACE activity may therefore not increase the steady-state level of Ang II (Zhao et al., 2000). An increase in ACE expression in the normal LV therefore may not lead to an increase in LV Ang II. On the other hand, ACE knockout mice exhibit cardiac Ang II concentrations 1% of wild-type (Campbell et al., 2004). This suggests that a virtual absence of LV ACE activity eliminates LV Ang II production. Similar to the kidney and adrenal, data from heterozygous knockout mice were not reported, and thus it cannot be determined whether a graded decrease in LV ACE activity leads to a parallel graded decrease in LV Ang II.

Five weeks after OVX, LV AT_1R was increased by 60%. This increase was reversed to a 35% decrease by high E2. In senescent rats, OVX increases LV AT_1R protein by 15% compared to ovary-intact controls, and high E2 reverses the increase to a decrease (Xu et al., 2003). The implications of the regulation of AT_1R by E2 status are not certain. Overexpresssion of LV AT_1R at very high levels (200-450 fold) has been

shown to be highly detrimental to the otherwise normal mouse heart (Paradis et al., 2000), causing cardiac hypertrophy and decreased survival, both of which improve by treatment with AT_1R antagonists. However, a relatively lower cardiac-specific AT_1R overexpression (4-8 fold) was not associated with a change in cardiac phenotype in transgenic rats (Hoffmann et al., 2001). These rats did exhibit more severe hypertrophy in response to systemic Ang II infusions and pressure- and volume-overload than wild-type rats (Hoffmann et al., 2001). Together, these studies suggest that in the normal myocardium an increase in ACE activity does not necessarily lead to increases in steady-state Ang II, but a simultaneous increase in AT_1R density may result in increased sensitivity to Ang II that is present. Thus, in OVX+veh rats, the steady-state levels of LV Ang II need not be elevated to mediate a significant physiological effect. Indeed, in aged female rats, the OVX-induced increase in LV AT_1R was associated with changes consistent with LV remodeling: an increase in β-myosin heavy chain expression, increased collagen I/III ratio, and decreased matrix metalloproteinase-2 activity. These changes were reversed with E2 replacement at high levels (Xu et al., 2003).

Treatment of OVX rats with high E2 decreased LV ACE activity compared to OVX+veh rats but did not change LV Ang I and II levels. In addition, LV Ang II/I ratios were increased in these rats. There are two possible explanations why ACE activity is decreased and Ang II/I ratio is increased. First, treatment of OVX rats with high E2 may lead to upregulation of a non-ACE Ang II-forming enzyme. Second, high E2 treatment may inhibit Ang II metabolism, increasing steady-state levels non-significantly compared to ovary-intact rats, yet enough to increase Ang II/I ratio.

<u>Rats 3 weeks post-MI.</u> In all three groups of MI rats, LV ACE mRNA abundance and activity were increased with proximity to the infarct scar to a maximum of 5-8 fold

within the infarct scar itself, compared to their respective sham values. This upregulation of LV ACE post-MI is consistent with previous studies in male rats: ACE mRNA abundance is increased as early as 4 days post-MI and remains increased at least until 7 days post-MI; ACE activity is increased by 7 days and remains increased at 12-13 weeks post-MI (Passier et al., 1995; Sanbe et al., 1995; Schieffer et al., 1994; Kobayashi et al., 1998). ACE density is increased several-fold at 4 and 8 weeks post-MI in all ventricular areas, including the scar, the remote LV, the septum and the RV (Tan et al., 2004; Johnston et al., 1991; Sun & Weber, 1996).

The increase in ACE also occurred in the LV of OVX+high E2 rats, despite a significant suppression of ACE mRNA abundance and activity in their respective shams. Indeed, the percent increases in LV ACE activity were significantly larger in OVX+high E2 rats compared to the other groups. However, the absolute levels of LV ACE activity were similar between MI groups. The increases in ACE mRNA abundance and activity post-MI were therefore not attenuated by the presence of high levels of circulating E2, as might be expected, given its negative regulation in sham rats. The mechanism of this reversal in LV ACE regulation remains to be investigated.

In ovary-intact MI rats, AT_1R density increased with proximity to the infarct scar to a maximum of 5-8 fold within the infarct scar itself. This is consistent with previous studies in male rats. AT_{1A}, is increased in the remote LV and the scar 1 week and 4 weeks post-MI (Nio et al., 1995; Iijima et al., 1998). AT_1R density is increased at 4 weeks, 8 weeks and 8 months post-MI, in the infarct area, remote LV and in the RV (Lefroy et al., 1996; Tan et al., 2004; Sun & Weber, 1994). This increase in AT_1R may increase the responsiveness to Ang II present in the LV of these rats.

Steady-state Ang I and II levels were unchanged in the LV remote from the infarct scar in all three groups of rats post-MI. The changes in infarct scar Ang I and II are discussed according to E2 status.

At 3 weeks post-MI, ovary-intact MI rats exhibited a 377% increase in ACE activity in the infarct scar compared to their respective shams. Infarct area Ang I, but not Ang II or Ang I/II ratio were elevated in these rats compared to sham. In contrast, OVX+veh MI rats exhibited a 275% increase in ACE activity, and exhibited increases in infarct Ang I and II compared to their shams. Similar to ovary-intact MI rats, infarct Ang II/I ratio was not increased compared to sham. OVX+high E2 MI rats exhibited a 744% increase in ACE activity, and in contrast to the other two MI groups, exhibited increases in Ang I, Ang II and Ang II/I ratio compared to their shams. In ovary-intact MI rats the changes in Ang peptide concentration are similar to those which occur in male rats at approximately this time post-MI (Gaertner et al., 2002; Leenen et al., 1999). The increase in LV ACE activity appears to not necessarily translate into an increase in steady-state Ang II levels in these rats. However, increased metabolism/turnover of Ang peptides in the LV may not allow Ang II accumulation. Because these rats also exhibited a several-fold graded increase in LV AT_1R density, they likely have increased sensitivity to the levels of Ang II that are present. In OVX+veh and OVX+high E2 MI rats, the increase in infarct ACE activity was associated with an increase in steady-state levels of infarct Ang II compared to their respective shams and compared to ovary-intact MI rats. This may indicate either increased production or decreased metabolism of Ang II in these rats compared to the ovary-intact MI rats. It is not clear why only OVX+high E2 MI rats exhibited an increase in infarct Ang II/I ratio compared to sham and compared to the other MI groups given that all three MI groups exhibited similarly increased ACE activity. The

mean of the infarct Ang II/I ratio in OVX+high E2 rats contains data from only 3 rats, and must be regarded with caution. In addition, OVX+high E2 MI rats tended to have lower Ang I and higher Ang II than the other MI groups, perhaps reflecting relatively minor differences in production and metabolism of those peptides that ultimately result in a significantly different Ang II/I ratio.

4.4. Regulation of brain ACE and AT_1R by E2 status and MI

Five weeks after OVX, ACE density was increased in the SFO, PVN and MnPO and AT_1R density was increased in all four nuclei studied: the OVLT, SFO, PVN and MnPO. Each of these increases were prevented by treatment with regular E2, and were reversed to decreases or tended to be reversed to decreases by high E2. The present study is the first to measure E2 regulation of ACE in the brain. This is also the first study to measure changes in AT_1R regulation by OVX alone. The regulation of brain AT_1R by E2 in OVX rats has been previously reported. For example, in OVX rats, regular E2 treatment decreased the number of SFO neurons that are positive for AT_1R immunoreactivity (Rosas-Arellano et al., 1999). Treatment of OVX rats with high E2 reduced AT_1R density by 20% in homogenates of the preoptic area (Jonklaas & Buggy, 1985) and decreased AT_1R mRNA abundance by 40% in hypothalamus-septum-thalamus tissue blocks and AT_1R density in the SFO by 30% (Kisley et al., 1999). These actions may reduce the activity of angiotensinergic neurons as moderate sc E2 treatment decreased the responsiveness of SFO neurons to Ang II injected into the carotid artery or microinjected into the SFO itself (Tanaka et al., 2001a; Tanaka et al., 2001b). AV3V brain slices from OVX rats treated with E2 exhibited decreased firing rate in response to perfusion with Ang II compared to those taken from vehicle-treated rats (Akaishi & Homma, 1996).

In ovary-intact MI rats, ACE density was increased by a modest 8% in the PVN and MnPO compared to their respective shams. AT_1R was increased by ~10% in the SFO and PVN. OVX+veh MI rats exhibited elevated OVX-induced baseline densities and did not exhibit increased ACE densities compared to sham. They did exhibit ~15% increases in AT_1R density in the PVN and MnPO post-MI over and above the 20-25% OVX-induced increases. OVX+high E2 MI rats exhibited significant increases in ACE (all nuclei) and AT_1R density (OVLT, SFO, PVN) compared to sham. OVX+veh MI rats, in all cases, had higher absolute densities compared to the other groups of MI rats, but only in two cases did these nuclei exhibit significant increases compared to sham: AT_1R in the PVN and MnPO. Ovary-intact and OVX+high E2 MI rats had very similar absolute densities post-MI.

In male rats activation of the brain RAS plays an essential role in sympathetic hyperactivity and the progression of LV dysfunction post-MI (Leenen et al., 1999; Zhang et al., 1999; Wang et al., 2004). Both ACE and AT_1R densities are increased in the SFO, OVLT, PVN and MnPO post-MI (Tan et al., 2004). In male rats, the increase in sympathetic drive post-MI may be mediated by several mechanisms. The first involves an increase in a plasma aldosterone (Lal et al., 2004) and/or Ang II (Leenen et al., 1999) post-MI that has been proposed to increase ACE and AT_1R density in the SFO and OVLT (Porter et al., 1999; Mollenhoff et al., 2001; Wilson et al., 1988). This activation may in turn lead to increased activation of these nuclei and consequently increased expression of RAS components in the PVN and MnPO inside the BBB (Tan et al., 2004). Whether plasma aldosterone levels are altered in these groups of female rats post-MI was not evaluated. OVX+high E2 rats exhibited increased Ang II levels post-MI, but the absolute levels were no different than ovary-intact sham rats. It is therefore uncertain to what

extent the activation of the circulatory renin-angiotensin-aldosterone system contributes to the increased expression of these brain RAS components post-MI.

The second mechanism of brain RAS activation post-MI occurs via activation of the cardiac afferent reflex. This reflex loop originates within the LV, is sympatho-excitatory, and is activated by changes in cardiac pressures and dimensions that occur post-MI, including ischemia (Inoue et al., 1988). In male rats, the gain of this reflex is increased post-MI. That is, the same amount of stimulation of cardiac afferents leads to a greater increase in efferent sympathetic activity in MI rats versus sham rats. This increase in gain is mediated by activation of the AT_1R in the PVN (Zhu et al., 2004) but may also be a result of activation of mineralocorticoid receptors (for aldosterone) in the brain (Lal et al., 2004). In the present study the PVN is the only nucleus with consistent increases in ACE and/or AT_1R in all three groups of rats post-MI. The increase in PVN AT_1R occurs even in OVX+veh MI rats despite their higher baseline densities.

The regulation of brain ACE and AT_1R post-MI may be related to LV function. In the SFO and PVN, AT_1R density increases in OVX+veh and OVX+high E2 rats as infarct size increases. This is consistent with a functional relationship between increased brain RAS activity and infarct size that has been shown in male rats (Tan et al., 2004). In ovary-intact rats, however, this relationship is negative, perhaps implicating a factor other than E2 affecting AT_1R density in the SFO and PVN post-MI. It is not immediately clear based on the current data what causes these different relationships. However, this unknown factor, removed by OVX, seems to modify the activation of ACE and AT_1R in the SFO and PVN post-MI, especially in rats with large infarcts.

4.5. Regulation of LV function and heart rate by E2

Five weeks after OVX and E2 or vehicle treatment, LVEDP was similar across sham groups. LVPSP and +dP/dt tended to be higher in OVX+veh and OVX+high E2 sham rats compared to ovary-intact sham rats. However, the ovary-intact rats were studied in a different experiment than the other two groups, and thus it cannot be excluded that the changes in these parameters are due to inter-experimental variability. Previous studies measured in vivo LV function post-OVX. Nine to 10 weeks after OVX, LVEDP and LVPSP were not changed compared to ovary-intact rats or OVX rats treated with E2 at physiological levels (Hügel et al., 1999). In contrast, 3 weeks after OVX, LVEDP was increased by 6 mmHg, LVPSP was increased by 27 mmHg and -dP/dt was increased by 20%. Six weeks after OVX, LVEDP had normalized and LVPSP and -dP/dt remained increased. The OVX-induced increases were normalized by replacement of E2 at physiological levels (Mercier et al., 2002). Altogether, these studies suggest that changes in LV function during the early stages of E2 deficiency may normalize after several weeks.

OVX+high E2 rats, both sham and MI, exhibited modest, yet statistically significant 6-12% decreases in heart rate. These data are consistent with previous reports using high-dose estrogen (Fregly et al., 1977; Salgado et al., 1995). This bradycardia may be a result of the decreased activity of the circulatory, cardiac and/or brain RAS in these rats, i.e. reduced direct chronotropic action of Ang II in the heart (Kobayashi et al., 1978), decreased sympathetic tone (He et al., 1998; El-Mas & Abdel-Rahman, 1998; Mohamed et al., 1999) or increased parasympathetic tone (Saleh & Connell, 2000).

4.6. Potential mechanisms of regulation of ACE and AT_1R by E2

While the present study did not investigate the mechanism of regulation of ACE and AT_1R by E2, several groups have performed such studies in specific tissues.

ACE. Incubation of ACE purified from rat lung with E2 does not result in a change in ACE activity (Gallagher et al., 1999). This suggests that regulation of ACE activity by E2 does not occur via a direct molecular interaction. Many actions of E2 require estrogen receptors and all tissues examined in this study express one or both of the ERs (Couse et al., 1997; Mohamed & Abdel-Rahman, 2000; Andersson et al., 2001; Kuiper et al., 1997; Grohe et al., 1998; Grohe et al., 1997; Wu et al., 2003; Laflamme et al., 1998; Shughrue et al., 1998). The classical action of E2 is to act via the ERs to up- or down-regulate gene transcription. The present experiments suggest that, at least in the kidney and heart, E2 deficiency increases and E2 dose-dependently decreases the abundance of ACE mRNA. Gallagher and colleagues (1999) also found that kidney ACE mRNA transcript abundance was decreased by E2 treatment of OVX rats. This suggests that the rate of transcription and/or the stability of ACE transcripts are decreased by E2. While the promoter region of ACE does not contain a consensus estrogen response element, it does contain AP-1 and SP-1 response elements (Shai et al., 1990), known to act cooperatively with the E2/ER complex to regulate other genes (e.g. Tanaka et al., 2000; Schultz et al., 2005).

The regulation of AT_1R by E2 has been studied in greater depth. E2 downregulates AT_1R mRNA abundance, likely in a tissue-specific manner. Similar to ACE, the promoter region of the AT_1R gene also contains AP-1 and SP-1 elements (Ichiki et al., 1998). In hypothalamic-septum-thalamic homogenates, E2 decreases AT_1R mRNA abundance (Kisley et al., 1999). In cultured aortic VSMCs, high E2 treatment decreases AT_1R mRNA abundance. However, the mechanism was not via a decrease in the rate of AT_1R mRNA transcription, as measured with a nuclear run-on assay. Rather, transcript half-life was shortened from 5 to 2 hours (Nickenig et al., 2000). In the adrenal cortex, E2

down-regulates the AT_1R via a different mechanism. Treatment of adrenal cortical cells with E2 for 8 days did not change AT_1R mRNA abundance, but decreased its density on cell membranes. E2 increased the association of cytosolic RNA binding proteins to the 5' leader sequence of the AT_1R transcript. During the translation process, these proteins interfered with ribosomal scanning and decreased AT_1R translation efficiency (Wu et al., 2003). The mechanism of AT_1R regulation by E2 appears to be tissue-specific, insofar as the regulation in VSMCs and adrenal cortex are very different. This is consistent with the known literature about E2/ER transcriptional regulation. Although both tissues contain ERα and ERβ, they likely express very different combinations of transcriptional modifiers that may account for the differential regulation by E2/ER. The precise mechanisms of regulation of AT_1R by E2 status in the heart, brain, adrenal medulla and kidney remain to be clarified.

4.7. Pathophysiological implications of regulation of RAS by E2

LV dysfunction post-MI. Rats from all three E2 status groups exhibited similar infarct sizes, similar increases in LVEDP and LV dimensions, and similar decreases in LVPSP, ejection fraction and fractional shortening at 3 weeks post-MI. LV dysfunction was therefore similar across MI groups. Studies in rats examining the effects of E2 status on changes in *in vivo* LV function post-MI are few. Smith and colleagues (2000) demonstrated that at 10-11 weeks post-MI, ovary-intact rats and OVX+veh rats had similar infarct sizes: (35 vs. 27%, respectively), and OVX+regular E2 rats had larger infarct sizes than OVX+veh rats (42%). *In vivo* LV function was not measured in this study and so the consequences of the change in infarct size are not known. In a study by Nekooiean & Pang (1998), at 7 weeks post-MI, OVX rats treated with E2 at physiological levels had similar infarct sizes compared to OVX+veh rats (31 vs. 34%, respectively).

OVX+E2 rats exhibited significantly smaller increases in LVEDP post-MI than OVX+veh rats (Figure 1.1) perhaps indicative of a minor improvement in LV function independent of infarct size. However, LVPSP and +dP/dt were similarly decreased in both groups of rats. The present study demonstrates that changes in the circulating levels of E2 do not confer any benefit or detriment to infarct size or to global LV function in female rats 3 weeks post-MI.

Given the overall lack of regulation of LV and brain RAS by E2 at 3 weeks post-MI, the absence of effects on the degree of LV dysfunction are not surprising. In sham rats, OVX upregulated and high E2 downregulated LV ACE and AT_1R. These changes did not translate into a greater increase in LV ACE in E2-deficient rats post-MI, nor did they translate into attenuation of the increase in LV ACE in OVX rats treated with high E2; the ultimate levels of LV ACE were similar in all three MI groups. In male rats, AT_1R and ACE are increased several-fold in the LV of rats exhibiting large infarcts and moderate LV dysfunction. However, LV ACE and AT_1R are also upregulated to similar levels in rats with small infarcts and less severe LV dysfunction (Tan et al., 2004). Thus the degree of activation of LV ACE and AT_1R may not directly correlate with LV dysfunction; even if E2 status regulated LV ACE in MI rats in this study, this regulation may not have altered the progression of LV dysfunction.

In the brain, the absolute densities of ACE and AT_1R were significantly, yet modestly, higher in OVX+veh MI rats compared to the other MI groups. The only nuclei that exhibited higher AT_1R densities than the other groups and increased densities compared to sham were the PVN and the MnPO. In male rats, like in the LV, brain ACE and AT_1R densities are increased post-MI in rats exhibiting large infarct sizes and moderate LV dysfunction. In contrast to the LV, the activation of brain ACE and AT_1R in

rats exhibiting small infarcts and less severe LV dysfunction occurred for the most part to a lesser degree compared to rats with large infarcts and moderate LV dysfunction (Tan et al., 2004). Thus in certain brain areas, the activation of these RAS components may be correlated with degree of LV dysfunction. In the present experiments, as noted above, OVX+veh MI rats exhibited increased AT_1R density in the PVN and MnPO compared to their respective shams and increased density in many areas compared to the other MI groups. However, they did not exhibit worsened LV dysfunction at this early time-point (i.e. 3 weeks) post-MI. Brain ACE and AT_1R densities in OVX+veh MI rats were increased rather modestly, by ~20%, compared to the other MI groups. These increases appear to be insufficient to result in significantly worsened LV dysfunction. However, previous studies in male rats demonstrated that blockade of the brain RAS post-MI attenuated the degree of LV dysfunction measured at 8 weeks post-MI. It is conceivable that brain ACE and AT_1R in OVX+veh rats has not yet, at 3 weeks post-MI, reached levels sufficiently higher than the other MI rats to result in worsened LV dysfunction.

Several future experiments could address the above issues. First, a longer duration of follow-up post-MI would determine the effects of E2 status on the tissue RASs during long-term LV remodeling. Second, intact female rats could be treated with icv losartan following MI to ensure that the brain RAS is indeed a mediator of the sympathetic hyperactivity-induced LV dysfunction post-MI in female rats as it is in male rats. Alternatively, female rats expressing brain-specific antisense against Agt (Schinke et al., 1999) could be used. Experiments similar to those presented here could be performed: female rats with differing levels of circulating E2 could be followed for short and long-term (3 and 6-12 weeks post-MI) and sympathetic activity could be measured, to determine whether the differences in brain RAS components lead to parallel differences in

sympathetic drive and LV dysfunction. To specifically implicate the brain RAS, ideally, icv administered E2 could also be used. Unfortunately, E2 is highly lipid soluble and chronic infusions would readily leak into the periphery. Instead, sc E2 could be used in concert with icv-administered estrogen receptor antagonists to selectively block brain ERα (e.g. methyl-piperidino-pyrazole (Sun et al., 2002) or β to determine 1) whether E2 is acting at its receptor in the brain to mediate the effects and 2) which receptor subtype mediates the effects. These compounds could also be microinjected into discrete brain nuclei (e.g. PVN, SFO) to determine which nucleus mediates the effects. Alternatively, ER knockout mice (ERα or β or double knockouts)(Krege et al., 1998; Dupont et al., 2000) would help elucidate the importance of E2 in the development of LV dysfunction post-MI.

Hypertension post-OVX. Removal of both ovaries in sexually-mature Wistar female rats increased BP by 10-23 mmHg in several studies (Mercier et al., 2002; Hernandez et al., 2000; Milsted et al., 1998; Brosnihan et al., 1997a) but not others (He et al., 1998; Nickenig et al., 1998a; El-Mas & Abdel-Rahman, 1998). These increases was prevented by systemic treatment with E2 or the AT_1R antagonist irbesartan (Pham-Dang et al., 2003; Mercier et al., 2002). Female Dahl S rats fed a diet containing low to normal NaCl (0.1% or 0.5%) exhibited progressive increases in BP with age; this age-induced increase in BP was exacerbated in OVX rats: systolic BP was increased by an additional 25 mmHg (Harrison-Bernard et al. 2003) and mean arterial pressure was increased by an additional 15 mmHg (Hinojosa-Laborde et al., 2004). The OVX-induced increases are prevented by E2 treatment at physiological levels and by systemic treatment with the AT_1R antagonist candesartan (Harrison-Bernard et al., 2003) and were reversed to decreases by E2 treatment at high levels (Hinojosa-Laborde et al., 2004). The efficacy of

both E2 and AT_1R antagonists at preventing the increases in blood pressure suggests that the mechanism of OVX-induced increases in BP involve a increase in activity of the RAS. The OVX-induced increase in ACE and AT_1R in several tissues as demonstrated in this study is consistent with this notion. However, it is not certain whether local tissue RAS activation is responsible for this OVX-induced effect, and if so, which tissue RASs, alone or in combination, are responsible. Several future studies could be performed to address this question. The administration of losartan icv around the time of ovariectomy would determine whether the brain RAS, at least in part, is involved in the mediation of the hypertension. Alternatively, female rats expressing brain-specific antisense against Agt could be used. The contributions of the other local tissue RASs could be elucidated by using mice with tissue-specific RAS component knockouts (e.g. Agt KO in heart and kidney as characterized by Kang et al., (2002)).

Atherosclerosis. AT_1R density is increased in the media and neointima of the atherosclerotic arteries of hypercholesterolemic rabbits (Yang et al., 1998) and is increased in the arteries of hypercholesterolemic monkeys (Song et al., 1998). AT_1R blockade reduces vascular lipid deposition and endothelial dysfunction in monkeys with atherosclerosis (Takai et al., 2003). Apolipoprotein E knockout mice develop atherosclerosis (Jawien et al., 2004). Double knockout ACE/ApoE mice and double knockout AT_1R/ApoE mice exhibit reduced atherosclerotic lesion size compared to ApoE single-knockout mice (Hayek et al., 2003; Wassmann et al., 2004). These studies indicate a role for the RAS in the development of atherosclerosis. The development of lesions in ApoE -/- mice is attenuated by E2 treatment at physiological levels (Elhage et al., 1997). This effect of E2 is via ERα (Hodgin et al., 2001). It is conceivable that the inhibitory

action of E2 on the development of atherosclerotic lesions in these mice is mediated via a decrease in expression of vascular ACE and/or AT_1R.

Renal disease. ACE inhibitors are effective in slowing the loss of renal function in diabetic nephropathy in both humans (Lewis et al., 1993) and in rats (Remuzzi et al., 1998; Griffin et al., 2003). In female Dahl S rats fed a low salt diet, age-induced glomerulosclerosis and cortical tubulointerstitial fibrosis is observed by 12 months of age. Renal decline is exacerbated in OVX rats compared to ovary-intact rats, and is significantly slowed by E2 treatment at physiological levels (Maric et al., 2004). The present study and a previous study (Harrison-Bernard et al., 2003) report parallel changes in AT_1R protein levels according to E2 status (i.e. a increase due to OVX and prevention by E2). The degree of nephropathy induced by streptozin in female rats is exacerbated by OVX. The worsening is prevented by E2 replacement at physiological levels (Mankhey et al., 2005). Together, these studies suggest a potential renoprotective role for E2 via downregulation of the kidney RAS.

4.8. Conclusions

This study demonstrates that ovariectomy alone increases ACE and AT_1R in several tissues relevant to cardiovascular regulation (including the heart, brain, kidney, abdominal aorta, adrenal and lung), and that these OVX-induced increases are mediated by E2 deficiency. The negative regulation of cardiac ACE and plasma Ang II in the normal rat is fully overridden at 3 weeks post-MI, so that the absolute values of these components are similar across MI groups. In E2-deficient MI rats brain ACE and AT_1R densities were modestly higher than densities in the E2-replete MI groups. These studies demonstrate that the regulation of circulatory, cardiac and brain RAS components by E2 status in the otherwise healthy female rat 1) largely does not affect the activity of these

RASs post-MI and 2) does not confer any benefit or detriment to the progression of LV dysfunction post-MI.

4.9. Perspectives

Similar to rats, women exhibit lower circulating E2 levels following menopause or surgical ovariectomy. No study has yet assessed whether components of the tissue RASs are regulated by E2 in women. This study identifies potential benefits of premenopausal endogenous estrogens and estrogen replacement therapy following menopause: a reduction in tissue ACE and AT_1R. The present experiments demonstrate that the negative regulation of the local tissue ACE and AT_1R in the heart and brain by E2, contrary to expectation, does <u>not</u> lessen the activation of ACE and AT_1R that occurs post-MI, and therefore, as would be expected, is not associated with attenuation of the development of LV dysfunction by 3 weeks post-MI. However, the very specific regulation of these and other local tissue RASs by E2 identifies potential areas of research into the effects of endogenous and exogenous estrogens in the presence of other pathology, like hypertension, atherosclerosis, and diabetic nephropathy, where the activity of local renin-angiotensin systems is known to play an etiological and/or contributing role.

Chapter 5:

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