UNRAVELING THE CENTRAL MECHANISMS BEHIND CHRONIC ESTROGEN-INDUCED HYPERTENSION

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Pathology

2011

ABSTRACT

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Women are exposed to estrogen in several forms including oral contraceptive pills and hormone replacement therapy. Although estrogen was originally believed to be cardioprotective, lately, its beneficial effects are being questioned. Prolonged exposure to estrogenic preparations increased the risk for cardiovascular diseases, but the mechanisms are still unknown. The paraventricular nucleus (PVN) of the hypothalamus and rostral ventrolateral medulla (RVLM) of the brainstem are two important cardiovascular centers that are well known to be involved in the central control of blood pressure (BP) regulation. My dissertation focuses on understanding the central mechanisms resulting in chronic estrogen-induced hypertension in a rat model. In my studies, chronic estradiol exposure increased arterial pressure and heart rate in female SD rats. These hypertensive effects of chronic estrogen exposure were accompanied with increased superoxide production in the RVLM. Treatment with an antioxidant reversed estradiol-induced increase in superoxide production in the RVLM and BP. Our studies also provide evidence that chronic estradiol exposure activates the endothelin-1 (ET-1) system in both the RVLM and PVN and intracerebroventricular administration of an ET-1 receptor antagonist reduced estradiol-induced increase in BP. Taken together, these findings suggest that the cardiovascular effects of estradiol may be attributed to a central component involving oxidative stress and activation of the endothelin system.

ACKNOWLEDGEMENT

I am deeply indebted to my mentors Dr. P.S. MohanKumar and Dr. Sheba MohanKumar. Looking back at where I was, I really travelled a long way both personally and professionally. The amount of training that I received in their lab will definitely help me face all the hurdles in my life. Dr. P.S. MohanKumar is a very understanding, caring and encouraging person. These are some of the qualities that I like the best in him. He is a great source of inspiration for me in terms of his fearless heart and commitment to work. Dr. Sheba MohanKumar is a dedicated person. Her questions often made me realize the simple concepts behind the complexity of science. Her advice goes a long way. She is of tremendous support. She taught me how to have fun as well as get my work done at the same time. I am grateful to be blessed with such great mentors.

I would like to thank Dr. Gregory Fink for being such a role model. His appreciation and acknowledgement always motivates me to do well in science. I cannot thank Dr. Carrie Northcott enough, for teaching me the nuances in science both conceptually and technically. I never hesitated to contact her because of her friendly nature. She always encourages me to "rock on". Dr. Ioana Sonea is an absolute delight to work with. She is very supportive and takes out all the stress from me. She motivates me to do well both in teaching and research.

I am truly grateful to all the members of MohanKumar Lab for helping me with my research all these years. Ms. Katrina Linning, always addressed me as boy since I joined the lab, I hope by now I have grown as a man. She is one person that everyone wishes to have in the lab. I have learned a lot from her, not to mention her organizational skills. She often gets me in trouble with Dr. Kumar but more often than not it all ends well. Dr. Ebony Gilbreath is such a

sweet person to work with. She sincerely cares for others. Always ready to help. I greatly appreciate her interest in learning words from my language (Tamil) and using it at the right time.

Dr. Priya Balasubramanian is more than a colleague; she is a very good friend. We share a great rapport. I was very fortunate for having her as my colleague. I wholeheartedly thank her for her support and encouragement both in and out of the lab. I would like to specially thank the past lab members Dr. Madhu Sirivelu and Dr. Lakshmikripa Jagannathan for their timely help and support. I had a great time with all my friends with whom I spent most of my leisure time.

Lastly I am extremely grateful for the unconditional love and support of my parents, whom I missed terribly during these years of my graduate studies.

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LIST OF ABBREVIATIONS

ABP	Arterial Blood Pressure
ACE	Angiotensin converting enzyme
aCSF	Artificial cerebrospinal fluid
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ANS	Autonomic nervous system
AP	Area postrema
AT1	Angiotensin II type I receptor
BBB	Blood brain barrier
BP	Blood pressure
CNS	Central nervous system
CuZnSOD	Copper-zinc superoxide dismutase
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DOCA	Deoxy corticosterone acetate
E_2	Estradiol-17β
E-90	E_2 treatment for 90 days
ELISA	Enzyme-linked immune sorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ET-1	Endothelin-1

ET-1A	Endothelin-1 receptor A
ET-1B	Endothelin-1 receptor B
GABA	Gamma-amino butyric acid
GFAP	Glial fibrillary acidic protein
HR	Heart rate
HRT	Hormone replacement therapy
ICV	Intra cerebro-ventricular
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
IML	Intermediolateral
LC	Locus coeruleus
MAP	Mean arterial pressure
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NE	Norepinephrine
NIH	National institutes of health
NO	Nitric oxide
NTS	Nucleus tractus solitaries
O_2^-	Superoxide
OC	Oral Contraceptives
OVLT	Organum vasculosum lamina terminalis
PVN	Paraventricular nucleus
qRT- PCR	Quantitiative real time polymerase chain reaction
RAS	Renin angiotensin system

Res	Resveratrol
ROS	Reactive oxygen species
RVLM	Rostral ventrolateral medulla
SBP	Systolic blood pressure
SC	Subcutaneously
SD	Sprague-Dawley
SFO	Subfornical organ
SHR	Spontaneously hypertensive rats
SHR-SP	Spontaneously hypertensive stroke prone rats
SNA	Symapathetic nerve activity
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween
TH	Tyrosine hydroxylse
WHI	Women’s health initiative

CHAPTER 1

INTRODUCTION

1. Statement of Purpose

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in women [1]. Blood pressure (BP) increases after menopause in women and therefore, hypertension is more prevalent in women compared to men of the same age [2]. Since premenopausal women have lower BP compared to age-matched men, estrogens were thought to play a protective role against hypertension. A large number of studies subscribe to this idea. Estrogens are thought to improve lipid profile [3], decrease vascular resistance [4] and modulate activity of discrete areas of the brain that are involved in cardiovascular regulation [5, 6]. However, recent reports from the Women's Health Initiative (WHI), National Institute of Health (NIH) have provided evidence that Hormone Replacement Therapy (HRT) using estrogen alone or a combination of estrogen plus progestin does not confer cardiac protection and may in fact increase the risk for coronary heart disease among postmenopausal women [7].

Besides older women who are on HRT, younger women who take oral contraceptives (OC) are also at risk for developing cardiovascular disorders. Approximately 5% of those patients who take a preparation containing more than 50 μg of estradiol-17β (E_2) have hypertension with BP readings of greater than 140/90 mmHg [8]. The risk for hypertension associated with OC appears to be related primarily to the estrogen rather than the progestin component of the preparation, because women taking progestin-only contraceptives are not at such increased risk [9]. Therefore it is important to understand the mechanisms by which chronic exposure to low levels of estrogen (in this case, E_2) may lead to hypertension.

The central control of BP occurs through highly complex and intricate mechanisms, many of which we are only beginning to understand [10, 11]. It involves a variety of mediators such as neuropeptides, superoxide (O_2^-), nitric oxide (NO), neurotransmitters etc. [11-14].

Several parts of the CNS such as the cortex, limbic system, the hypothalamus, the brainstem, and the autonomic nervous system, all play significant roles in maintaining BP [10]. Two brain regions, the rostral ventrolateral medulla (RVLM) of the brainstem and the paraventricular nucleus (PVN) of the hypothalamus. These two regions have direct efferent projections to the intermediolateral (IML) cell column of the spinal cord and can affect the sympathetic nerve activity (SNA) and in turn can play a crucial role in the central control of BP regulation [15].

The overall objective of this dissertation is to understand the central mechanisms involved in chronic estrogen-induced hypertension, specifically examining two regions of the brain namely the PVN of the hypothalamus and the RVLM of the brainstem. The first aim of my research is to establish a model to study the effects of chronic E_2 exposure on BP in young female SD rats. Subsequent studies will be focused on understanding the central mechanisms mediating the effects of chronic E_2's exposure on BP. We explored the possibility for the involvement of oxidative stress, neuroinflammation and activated endothelin system in the PVN and RVLM in chronic E_2-induced hypertension and will use mechanistic approaches to further confirm their role in this model. Collectively, these studies will provide valuable insights into the mechanisms by which chronic low-dose exposure to E_2 causes hypertension and draw attention to potential cardiovascular risks faced by women exposed to E_2 on a chronic basis.

2. Hypertension

According to the Seventh Report of the Joint National Committee (JNC-7) on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, Hypertension is defined as a continuous elevation in systemic arterial pressure with systolic blood pressure (SBP) in the range

of 140 mmHg or higher and/or diastolic blood pressure (DBP) of 90 mmHg or more [16]. Hypertension is a consistent and independent risk factor for several CVDs such as heart attack, heart failure, stroke and chronic kidney disease [16]. The increased risk of CVD with BP levels previously considered normal lead to the introduction of a new term called "prehypertension". The BP ranging from 120-139 mmHg systolic and/or 80-89 mmHg diastolic are categorized as prehypertensive. The individuals in this category are at high risk for the development of hypertension in the future. According to the National Health and Nutritional Examination Survey (NHANES) about 74.5 millions adults in United States are hypertensive [17]. The prevalence of hypertension varies with age in both men and women. Women are less hypertensive than men until 45 years of age, from 45-64 the percentage is similar between the sexes, however after 64 the trend reverses and women become more hypertensive than men. This clearly shows the need for thorough understanding of the mechanisms of hypertension in women in order to develop different therapeutic strategies.

3. Estrogen, Women and BP

Estrogen is a steroid hormone predominantly produced from the granulosa cells of the ovary. The pleiotrophic actions of estrogen are mediated through its nuclear receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). Binding of estrogen to its receptors forms a steroid receptor complex, which then translocates to the nucleus to bind with the estrogen responsive elements in the promoter region and regulates the gene expression of estrogen responsive genes. Apart from the peripheral tissues, the two estrogen receptors subtypes are also widely distributed in the central nervous system. Several beneficial effects of estrogen treatment including improvement in memory functions, prevention of bone loss and decrease in the

incidence of cardiovascular diseases have been previously documented [18, 19]. However, several clinical and epidemiological studies have questioned this hypothesis. In addition to endogenous estrogen, women are exposed to exogenous estrogen in the form of oral contraceptives, hormone replacement therapy and synthetic estrogens like bisphenol-A, which results in increased circulating estrogen levels and this has been attributed to the increased cardiovascular risk in women.

Postmenopause in women

Menopause defines the culmination of reproductive function in women. On an average, the menopausal age in women is approximately 51.5 years [20]. Menopause is characterized by increased risk for cardiovascular disorders, among which hypertension is the leading cause of increase in the morbidity and mortality in post menopausal women. Though, premenopausal women have lower BP compared to men of their same age, the trend is reversed after menopause resulting in higher prevalence of hypertension in postmenopausal women than age matched men [2]. It has been reported that around 60% of women older than 65 years are hypertensive [21]. The prevalence of hypertension is 4-fold higher in post menopausal women compared to the premenopausal even after corrected for age and body mass index [22]. The BP in the postmenopausal women does not increase immediately after menopause, it takes a longer period of time [23] the mechanisms responsible for the increased BP in postmenopausal women are still unknown. However, because the ovary stops producing estrogen after menopause, it was believed that the lack of estrogen in postmenopausal women was responsible for the increased BP. Hence it was hypothesized that replacing the loss in estrogen via estrogen or estrogen/progesterone preparations would alleviate the menopausal risk factors. Hence a

combination of estrogen and progestin or estrogen alone preparations were given as HRT for post menopausal women to alleviate the menopausal risk factors. However, two trials conducted by WHI using conjugated equine estrogen at a dose of 0.625 mg/d showed a significant increase in systolic blood pressure (SBP) in these post menopausal women [24]. Oral estrogen administration, either opposed or unopposed, was found to promote systolic hypertension in postmenopausal women [25, 26]. Although the magnitude of increase in BP was only between 1 and 2 mmHg, similar increases in SBP and pulse pressure are associated with increased risk for coronary atherosclerosis [27] and development of cardiovascular events [26] in large clinical trials. Increased sodium retention and increased angiotensinogen synthesis have been attributed as some of the possible mechanisms behind estrogenic HRT induced increase in BP [28]. Further using a combination of estrogen and progestin or estrogen alone showed no cardiovascular health benefit and in fact increased the risk for coronary heart disease and stroke [24, 25].

Perimenopause in women

Perimenopause is the transition period between the reproductive years and menopause (cessation of menses) in midlife women. It is characterized by complex hormonal changes. It typically begins when women are around 40 years of age and can last up to 3 to 4 years. It starts with periods of irregular menstrual cycles leading to symptoms such as breast tenderness, night sweats and mid-sleep wakening [29]. It was initially believed that there was a gradual decline in production of ovarian hormones during the perimenopausal period [30]; however, it was later understood that there was fluctuations in the hormonal production and in fact there was an increase in estrogen levels during perimenopause [31, 32]. Perimenopause has been associated

with increased risk for CVD's, type 2 diabetes and osteoporosis [33]. There is a possibility that the increase in estrogen levels seen during the perimenopausal period could predispose the women to hypertension and other cardiovascular disorders observed in the post menopausal period.

Premenopause in women

According to United States Bureau of the Census, approximately 62 million women across the US are in their reproductive age. In 2006-2008, approximately 62% of the women in their reproductive age used at least one method of contraception. Among the different contraception methods, OC pills were the leading contraceptive method used by 17.3% of women. OC have been used worldwide for more than 30 years. Their ability to cause hypertension became evident shortly after their introduction in clinical medicine. A slight increase in BP was observed clinically in women on OC containing > 50μg estradiol [8]. This increase was specific to estradiol containing pills, as there were no similar increases in BP observed in progestin-only pills [9]. As the dose of estradiol used in oral contraceptive was reduced to pills containing less than 30μg of estradiol the occurrence of OC-induced hypertension was also lowered. However, in a cohort study, women using low dose OC had an increased risk for the development of hypertension compared to the non-users [34]. Similarly studies conducted using low-dose OC in a 24-hour ambulatory BP monitoring showed about 6-8 mmHg increase in BP [35]. Further cessation of OC pills usage has been shown to reduce the increase in BP observed in women using OC [36]. However, the mechanism by which OC increases BP remains as a mystery.

Taken together, all these findings have raised an immediate need for understanding the effects of chronic E_2 exposure on the cardiovascular system.

4. Basic neural mechanisms involved in the development of hypertension

The role of the CNS in the development and maintenance of hypertension is well known [37-40]. Several regions of the brain including the cortex, limbic system, hypothalamus and brainstem are known to regulate BP. BP regulation involves both neural and humoral components. The neural component comprises of the autonomic nervous system (ANS) including the sympathetic and parasympathetic nervous system, the ANS plays an important role in linking the CNS and the peripheral effector organs like heart, kidney and the vasculature that regulate the BP. Parasympathetic control of BP is provided through the vagal nerve arising from the dorsal motor vagal nucleus, which has direct effects on the heart [11] .

BP is a function of cardiac output and total peripheral resistance; both are in part regulated by the sympathetic outflow from the brain and the brainstem [10]. Afferent signals from the arterial baroreceptors, chemoreceptors and cardiopulmonary baroreceptors project to the brain stem nuclei, the nucleus tractus solitarius (NTS) through the glossopharyngeal nerve [10]. The rostral ventrolateral medulla (RVLM) receives input from NTS through the second order neurons which then determines the basal sympathetic tone through its preganglionic sympathetic efferent inputs to the intermediolateral (IML) cell column of the spinal cord [41]. PVN and supraoptic nucleus also receive input from NTS and consequently modulate adrenocorticotropic hormone and vasopressin release which also regulates BP through peripheral actions. Apart from RVLM, raphe pallidus nucleus, A5 catecholamine cell bodies, and the PVN of the hypothalamus are also known to have direct neuronal projections to the IML cell column

of the spinal cord [11]. From the IML column of the thoracolumbar spinal segments, the preganglionic sympathetic neurons extend to the sympathetic ganglia from where the postganglionic fibers extend to the heart, blood vessels and the kidney. Retrograde labeling studies using fluorogold demonstrated that the sympathetic ganglia and the adrenal medulla are innervated by preganglionic sympathetic neurons originating from different segments of the thoracolumbar part of the spinal cord [42]. Postganglionic fibers that project to the adrenal medulla facilitates the release of medullary catecholamines to cause rapid effects on peripheral blood vessels thus modulating BP [11, 43]. These neuronal networks aid in the short and long term control of BP. Dysfunctional baroreceptor neuronal reflexes and increased sympathetic outflow through preganglionic neurons could contribute to hypertension [10]. Elevated SNA has been associated with hypertension in humans with essential hypertension and in various animal models including spontaneously hypertensive rats (SHRs), Dahl salt-sensitive rats, and deoxycorticosterone acetate (DOCA)-salt rats [44-47].

Humoral control of BP is provided through circulating hormones such as Angiotensin II (Ang II), vasopressin, aldosterone and glucocorticoids [48, 49]. Apart from peripheral actions in the kidney, heart and blood vessels, these hormones also act centrally and helps integrate blood borne signals with the central neural networks that regulate BP. While steroid hormones like aldosterone can freely diffuse through the blood brain barrier (BBB), peptidergic hormones like Ang II cannot cross BBB and however do have direct actions in the circumventricular organs [subfornical organ (SFO), organum vasculosum lamina terminalis (OVLT) and area postrema (AP)], which inturn sends signals to PVN, RVLM or NTS through efferent projections to affect SNA. They can also act at the level of endothelial cells to release mediators such as NO and prostaglandins which diffuses through the BBB to affect SNA [50, 51].

5. Role of RVLM and PVN in the regulation of BP

RVLM and BP regulation

RVLM plays a crucial role in the control of BP, heart rate (HR) and the basal sympathetic tone [15]. Retrograde labeling studies have demonstrated afferent inputs to the RVLM from the NTS, caudoventrolateral medulla, PVN and lateral hypothalamic nuclei of the hypothalamus [52, 53]. RVLM contains C1 epinephrine cell bodies which have been shown to have a baroreceptor modulated rhythm and these neurons project to the preganglionic neurons in the IML of the spinal cord. The final sympathetic processing in the brain takes place in the RVLM based on the all the afferent inputs and the sympathetic excitatory projections are transmitted to the spinal cord [54]. RVLM neurons play a pivotal role in the baroreceptor reflex, which is a major compensatory mechanism that responds to any changes in arterial blood pressure (ABP) [55]. Some of the RVLM neurons also project to the hypothalamic centers including PVN which is also involved in the modulation of baroreceptor mediated excitatory drive [10], however the phenotype of these neurons still remains unclear. A basal level of SNA, which is required for the short and long term control of BP is largely determined by the RVLM barosensitive neuronal activity. Basically, the activity of RVLM neurons depends on various neuropeptides and neurotransmitters including GABA, glutamate, acetylcholine, serotonin, corticotropin-releasing factor, oxytocin, substance P, vasopressin etc. All these are present in the nerve terminals that synapse into C1, the presumed BP-regulating neurons [10]. Hyperactivity of RVLM is accompanied with increase in BP and heart failure [37]. Further, drug-induced inhibition or lesioning of the RVLM produced a significant drop in arterial pressure suggesting its significant role in BP regulation [56, 57].

PVN and BP regulation

The PVN acts as an important regulatory site for not only BP and the SNS but also many physiological functions such as feeding, regulation of stress axis activity, etc. [58]. It is broadly divided into a medial parvocellular and lateral magnocellular portion [58]. The former includes 2 types of cells: neurosecretory cells projecting to the median eminence and the preganglionic autonomic cells caudally projecting to the brain stem nuclei (NTS and RVLM) and to the IML cell column of the spinal cord [59]. On the other hand, the magnocellular portion of the PVN consists of vasopressin and oxytocin neurosecretory cells projecting to the posterior pituitary [60]. PVN receives a wide array of afferent inputs from the hypothalamic regions like SFO, medial septum/diagonal band of broca, median preoptic nucleus, arcuate nucleus, suprachiasmatic nucleus, pons (lateral parabrachial nucleus), and brain stem (NTS, dorsal motor nucleus of the vagus, and the ventrolateral medulla). Some of the afferent inputs to the PVN originating from circumventricular organs like SFO enable PVN to integrate blood borne endocrine signals into a single autonomic output. It is also noteworthy to mention that anatomical studies have confirmed the presence of GABA (in and around the nucleus) and Glutamate (within the nucleus) interneurons in the PVN which also modulate the excitability of PVN neurons [61, 62] .

Lesioning of the PVN has been shown to inhibit development of hypertension in various models of hypertension including 1-kidney, 1-clip model of hypertension, SHR and DOCA [63-65]. Further evidence for the role of the PVN in hypertension comes from the study where the basal firing rate of preautonomic neurons in the PVN was shown to be lower in normotensive versus SHR rats [66]. Microinjection of norepinephrine (NE) or alpha adrenergic agonist, clonidine, into the PVN has been documented to increase mean arterial pressure in conscious rats

[67, 68]. All these studies suggest that PVN is the key hypothalamic nuclei involved in BP regulation.

Several neuropeptides (Ang II, ET-1), pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), NO, reactive oxygen species (O_2^-), and neurotransmitters (GABA, Glutamate, NE, 5-HT) are involved in PVN and RVLM-mediated regulation of BP.

6. Role of Angiotensin II and Endothelin-1 in the regulation of BP

Angiotensin II in the RVLM and PVN

Ang II, a peptide hormone produced by the activation of the renin-angiotensin system (RAS) plays an important role in the development of hypertension. The first step involved in the activation of RAS is the release of renin from the Juxta glomerular cells of the kidney. Being a proteinase enzyme, renin cleaves angiotensinogen to form angiotensin I (Ang I) in the liver. In the lungs, the inactive peptide Ang I is converted to biologically active peptide Ang II by angiotensin converting enzyme (ACE). It acts by binding with the AT1 receptors in the blood vessel to cause vasoconstriction, thus increasing total peripheral resistance and inturn BP. Also, it stimulates the release of aldosterone from the adrenal gland, which adds to the hypertensive effect of Ang II by increasing sodium and water retention in the kidneys [69].

High density of Ang II receptor, type 1 or AT1 receptors present in the centers of the brain regulating BP including the circumventricular organs (SFO, OVLT and AP) and non-circumventricular organs like NTS, RVLM and PVN provided evidence for the central role for Ang II in BP regulation through modulation of sympathetic outflow [70-72]. Ang II does not cross blood brain barrier which suggests the possibility for local production of Ang II [73]. In concordance to this idea, all the arms of the renin-angiotensin system (RAS) including renin,

angiotensinogen, angiotensin converting enzyme and AT receptors have been demonstrated in the brain itself [72].

Microinjection of Ang II in the RVLM and PVN and adenovirus mediated overexpression of constitutively active AT1 receptors in the RVLM resulted in an increase in BP [70, 74, 75], which suggests that RVLM and PVN could be some of the sites of action for Ang II-mediated hypertension. Similarly, blockade of AT1 receptors in the RVLM and the PVN have been shown to reduce the BP in several models of hypertension [76-79]. Recently, studies have demonstrated that increase in reactive oxygen species (ROS) and inflammation in the RVLM and PVN as some of the important mechanism for Ang II-mediated increase in BP [38, 80, 81].

Endothelin-1 in the RVLM and PVN

ET-1 is a 21 amino acid potent vasoconstrictor peptide synthesized denovo in the endothelial cells, smooth muscle cells, neurons and macrophages. The synthesis of ET-1 occurs in 2 steps: First, pre-pro ET-1 is converted to big ET-1 by endopeptidases and then 18 amino acids are cleaved from big ET-1 to form mature ET-1 by endothelin converting enzymes [82]. In addition to its well established direct effects in the periphery, ET-1 is now being considered as a peptidergic neurotransmitter in the CNS. Similar to RAS in the brain, all the components of the endothelin system, namely the protein and mRNA of ET-1 and its receptors ET-1A, 1B and 1C, and endothelin converting enzyme have been identified in the neurons and the glial cells [83, 84]. Central administration of ET-1 (both ICV and directly into RVLM) increased MAP and SNA in SHR, SHR-SP and DOCA-salt hypertensive rats and blockade of ET-1A but not ET-1B receptors reversed ET-1 induced increases in BP [85-87].

Several mechanisms have been postulated for central ET-1-induced increases in MAP and SNA. In the periphery, ET-1 acts as a pro-inflammatory agent by activating neutrophils and mast cells, releasing free radicals from macrophages and producing NO from endothelial cells [88]. Another possibility is that elevated levels of ET-1 could activate RAS, which in turn might increase cytokine and O_2^- levels leading to hypertension [89, 90]. Treatment of ET-1A antagonists in the lateral ventricles increased superoxide dismutase (SOD) levels in the brainstem suggesting ET-1's role in inducing oxidative stress [91]. It is possible oxidative stress marked by increase in ROS might be the common denominator in all the pathways (Ang II, ET-1) leading to hypertension.

7. Role of reactive oxygen species in the development of hypertension

ROS are produced as intermediate products during sequential one electron oxygen reduction [92]. In the first step when oxygen gains the first electron it becomes a O_2^- anion radical, and addition of the second one produces hydrogen peroxide [93]. O_2^- is unstable and is converted to hydrogen peroxide by SOD. Initially, ROS was considered as completely pathological but in the recent days ROS are being considered as an important signaling molecule involving in a number of physiological functions [94]. ROS have been shown to contribute to the pathogenesis of hypertension [95, 96]. Numerous studies reported increased O_2^- production in several animal models of hypertension, both peripherally and centrally [97, 98].

Source of superoxide

Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is the major source of O_2^- in the vasculature and in the brain [99]. Vascular NADPH oxidase consists of 2 membrane-bound components ($p22^{phox}$ and NOX ($gp91^{phox}$) and 3 cytosolic components $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ and a GTPase (Rac-1 or Rac-2) [100]. Even though NAPDH oxidase was initially identified in the phagocytes, in recent days their presence has also been demonstrated in nonphagocytic cells such as endothelium and smooth muscle cells [101-103]. The distribution of NADPH oxidase is not just limited to the peripheral tissues, it is also seen in the nervous tissues especially in the microglial cells as well [104]. While normal NADPH oxidase function is involved in memory and neuronal signaling, overproduction of ROS by NADPH oxidase might result in neuroinflamation and neurodegeneration [105]. Increase in O_2^- production mediated by NADPH oxidase, both centrally and peripherally has been positively associated with development of hypertension. In DOCA-salt model of hypertension there is increased NADPH oxidase-derived O_2^- anions in the vasculature [106]. Centrally, Ang II-induced hypertension is mediated by NAPDH oxidase-derived O_2^- production in the RVLM [39]. These studies suggest the primary role of NADPH oxidase enzyme system in the production of O_2^- and in the pathogenesis of several forms of experimental hypertension.

Peripherally, increased O_2^- production in the vessel wall of spontaneously hypertensive rats (SHR) have been reported [107]. Inhibition of O_2^- in the SHR with membrane-permeable SOD mimetic tempol (4-hydroxy-2, 2, 6, 6-tetramethyl piperidinoxyl) reduced the BP and renal

vascular resistance [108]. Centrally, increase in ROS in the RVLM has been linked to increased SNA and BP in several animal models of hypertension [38-40]. The increased ROS in the RVLM of stroke-prone spontaneously hypertensive rats (SHRSP) is accompanied with a decreased activity of SOD compared to Wistar Kyoto rats (WKY) rats. Direct microinjection of tempol in the RVLM in SHRSP reduced the BP [109]. Adenovirus mediated overexpression of manganese superoxide dismutase (MnSOD) gene in the RVLM reduced BP and SNA further confirming the role of ROS in the development of neurogenic hypertension [40].

Interaction between ROS and NO

Like O_2^-, NO is also involved in the central control of BP regulation through sympathetic nervous system. Nitric oxide synthase (NOS) is an enzyme that is involved in the formation of NO. There are three forms of NOS: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). nNOs and eNOS are constitutively expressed but iNOS is expressed only under pathological conditions such as hypertension etc. *In vitro* studies showed an increased activity of iNOS in the aorta of SHR rats [110]. These changes were augmented with aging. Adenovirus-mediated overexpression of iNOS in the RVLM leads to increase in BP 6-10 days after injection of the viral vector via activation of the sympathetic nervous system [111]. In 2-kidney 1-clip hypertension model, there is increased upregulation of iNOS mRNA in the RVLM. Microinjection studies using iNOS inhibitors decreased the mean arterial pressure (MAP) and renal SNA in the hypertensive rats. These studies suggest the possibility that NO produced by iNOS could play a role in mediating BP regulation and SNA in hypertension.

8. Role of Interleukin-1β in the control of BP

Inflammation is an important contributor to the development and progression of hypertension. Circulating levels of pro-inflammatory cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein and monocyte chemo attractant protein-1 (MCP-1) are increased in human patients with essential hypertension [112, 113]. The source of IL-1β in these patients appears to be peripheral monocytes and pretreatment with Ang II appears to increase IL-1β production from these monocytes [114]. The rise in IL-1β levels is countered by the secretion of IL-1 receptor antagonist whose levels are also elevated in hypertensive patients [115].

In addition to the peripheral effects of cytokines, recent studies have shed light on the central role of inflammation in neurogenic hypertension. Direct evidence for IL-1β's involvement in BP regulation comes from the studies in which infusion of IL-1β into the lateral ventricles [116] or directly into the PVN [117] increased BP in rats. Prevention of activation of microglial cells by treating with minocycline decreases the cytokine levels in the PVN in Ang II hypertension model suggesting that the principle source of cytokines in the brain is the microglial cells. Also, overexpression or gene transfer of the anti-inflammatory cytokine, IL-10, attenuates hypertension and hypothalamic inflammation in rats [118, 119]. Although the exact mechanisms by which central IL-1β increases SNA and inturn BP are not clear, it may be mediated through Ang II [117], ET-1 or NE.

9. Chronic estrogen animal model

Female SD rats were used to investigate the effects of chronic E_2 exposure on hypertension. Perimenopausal state is characterized by increases in estrogen levels throughout

premenstrual and follicular phases of the menstrual cycles. This period in a woman's life is also characterized by other symptoms such as increased endometrial hyperplasia, dysfunctional uterine bleeding, anovulatory cycles, etc. These features are consistent with elevated estrogen levels and are comparable to the constant estrus stage in rodents. Also, postmenopausal women with stromal hyperplasia or with nonfunctional ovarian tumors secrete significantly high levels of estrogen. Our lab has recently published a paper using this model [120]. It has been demonstrated that animals that are exposed to 60 and 90 days of E_2, an endogenous estrogen, remain in the constant estrous stage, very similar to old rats. Although these animals are not chronologically aged, they are hormonally aged and show signs and symptoms of reproductive senescence very similar to perimenopausal women. Thus this is an excellent animal model to investigate the effects of chronic E_2 exposure on hypertension.

10. Thesis objective

Menopausal transition is a highly complicated phase in a woman's life. Originally it was believed that reproductive hormones gradually declined during this period. However recent studies indicate that perimenopause is characterized by irregular and elevated levels of estrogen [121]. Moreover, clinical trials from the WHI have shown that prolonged exposure to estrogenic preparations increases the risk for coronary heart disease, stroke, and blood clots [25]. These findings have raised an immediate need for understanding the effects of chronic estrogen exposure on the cardiovascular system. The studies in the following chapters were designed to understand the effects of chronic estradiol exposure on cardiovascular system in female Sprague-Dawley (SD) rats. The overall objective is to investigate the potential central mechanisms mediating chronic estradiol-induced increases in mean arterial pressure. Each of the possible

mechanisms will be tested under separate hypothesis as shown in Fig. 1-1. 1) Chronic E_2 exposure increases mean arterial pressure in female SD rats. 2) Chronic E_2-induced increase in MAP is mediated by oxidative and inflammatory changes in the RVLM. 3) Treatment with an antioxidant, resveratrol will reverse the increase in BP, oxidative and inflammatory changes in the RVLM. 4) Chronic E_2-induced cardiovascular changes are associated with increased ET-1 activity in the RVLM and PVN and 5) Treatment with ET-1 receptor antagonist will reverse the chronic E_2-induced BP changes.

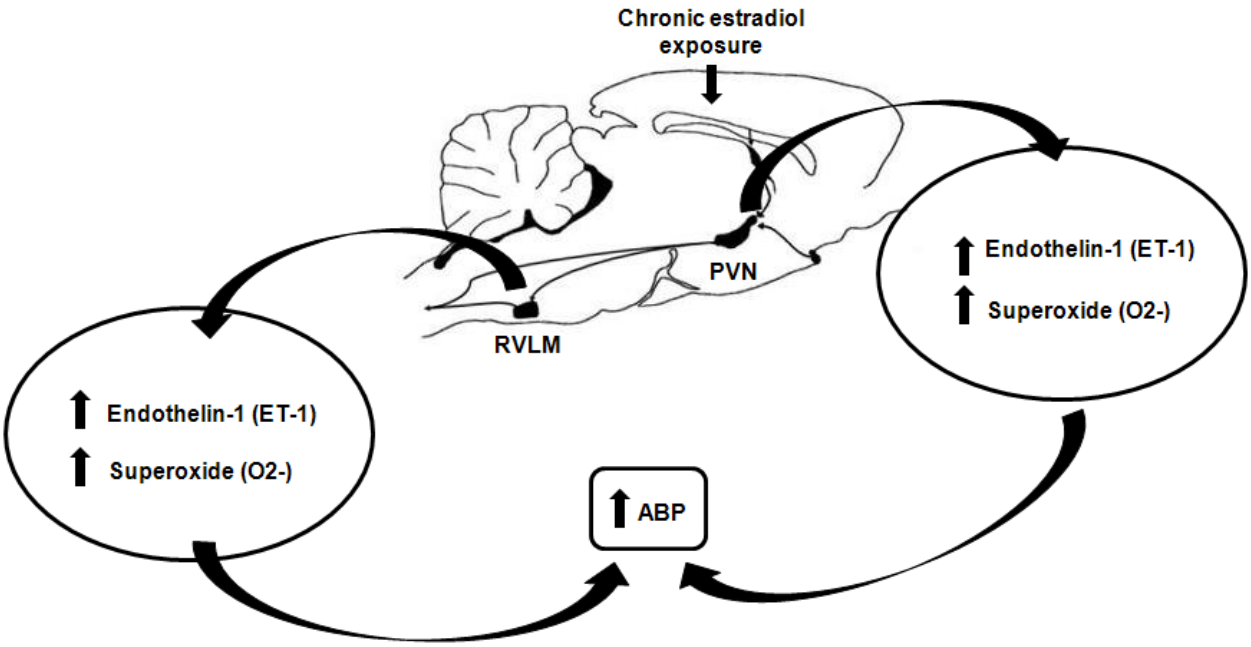


Fig. 1-1 Central hypothesis – Chronic exposure to low levels of estrogen increases arterial blood pressure (ABP) by increasing oxidative changes and endothelin-1 system activation with in rostral ventrolateral medulla (RVLM) of the brainstem and paraventricular nucleus (PVN) of the hypothalamus.

CHAPTER 2

MATERIALS AND METHODS

1. Animals

Sprague-Dawley rats

Adult (3-4 month old) female SD rats were obtained from Harlan Inc. (Indianapolis, IN). Animals were housed in temperature (23 ± 2^0C) and light controlled (lights on from 0600 to 1800h) rooms with *ad libitum* food and water. The animals were fed with regular chow diet (Teklad 8640 diet; 3.11 kcal/g, 5% fat; Harlan, Indianapolis, IN). Estrous cycles were monitored for 2 weeks by examining vaginal cytology, as described previously [120]. Animals that were cycling regularly were used in experiment. All the experiments were conducted in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and the protocols were approved by Institutional Animal Care and Use Committee at MSU.

2. Treatments

Estradiol-17β treatment

SD rats were fed with regular chow diet containing 23% protein, 72% carbohydrate, and 5% calories as fat with an energy density of 3.11 kcal/g (Teklad 8640, Harlan, Indianapolis, IN). Animals were implanted with 90-day custom made slow-release E_2 pellet (20ng/day; Innovative Research America, Sarasota, FL) subcutaneously (SC). Body weight, feed intake and water intake were monitored every week until the end of the treatment. After 90 days of exposure to E_2, all the animals were sacrificed at noon on the day of estrous. About 90% of the E_2 treated animals were in persistent estrous after 90 days treatment. The control animals were sacrificed on the day of estrous after 90 days of sham implantation for comparison to the treatment group. Immediately after sacrifice, the brains with the brainstem were removed, frozen on dry ice and

stored at -70°C until sectioned. Trunk blood was collected; serum and plasma (heparin coated tubes) were separated and stored at -70°C until further analysis.

Resveratrol Treatment

SD were divided into four groups, sham implanted (control); implanted with E_2 slow-release pellets (E-90; 20 ng/day for 90 days); control fed with chow containing 0.84g resveratrol/kg of chow (Res) ; and E_2 implanted rats fed with chow containing resveratrol (Res+E-90). Resveratrol treatment was started 7 weeks after pellet implantation and continued until the animals were sacrificed. Food intake and water intake were monitored every week until the end of treatment.

BQ-123 Treatment

SD rats were divided into four groups, 1) control+aCSF 2) control+BQ-123 (Sigma-Aldrich, St. Louis, Missouri), 3) E-90+aCSF and 4) E-90+BQ-123. Osmotic minipumps (Alzet model 2002 Osmotic Minipump; DURECT Corporation, Cupertino, CA) were filled with either BQ-123 (400μg/hr dissolved in aCSF) or aCSF vehicle and delivered at 0.5μl/hr. The osmotic minipumps were primed at 37°C in sterile saline overnight before implantation. The pumps were placed subcutaneously between the scapulae and connected to the ICV cannula with polycarbonate tubing. Rats received continuous ICV infusion of BQ-123 or vehicle via the osmotic minipumps for 14 days until the end of E_2 treatment.

3. Surgeries

Radiotelemetry Transmitter Implantation

A TA11-PA-C40 radiotelemetry transmitter (Data Sciences International, St. Paul, MN) was used for the measurement of MAP, HR, SBP and DBP. The tip of the transmitter catheter was placed in the abdominal aorta through the femoral artery under general anesthesia. The body of the transmitter was placed in a subcutaneous pocket in the abdomen. Ketoprofen (30mg/kg) was administered once for anti-microbial prophylaxis. Data was stored and analyzed using the Dataquest A.R.T. software (Data Sciences International, St. Paul, Minnesota).

ICV cannula implantation

Adult female SD rats were cannulated under isoflurane anaesthesia with a L-shaped stainless steel cannula (Alzet Brain Infusion Kit, Cupertino, CA) aimed at the lateral ventricles (0.3 mm posterior and 1.6 mm lateral to bregma) and connected to the osmotic minipump. Two spacers each at 0.5mm were added to the cannula before their implantation to reduce the length of the cannula to 4mm below the surface of the skull.

4. Brain / brainstem microdissection

Palkovits's microdissection procedure was used to isolate the paraventricular nucleus (PVN) as described previously [122]. Briefly, 300-µm serial sections of brain and brainstem were obtained using a cryostat (Slee Mainz, London, UK). The sections were transferred to microscope slides and placed on a cold stage maintained at –10°C. The PVN was microdissected from sections using a 500µm diameter punch and stored in -70°C until analysed for O_2^- measurement. Separate set of PVN was used for measuring the gene expression and

protein levels by quantitative Real-Time PCR (qRT-PCR) and western blot respectively. Another set of microdissected PVN were used to analyze IL-1β and NO using ELISA and Griess assay respectively. RVLM of the brainstem was microdissected, using Palkovit's micropunch technique. The punches were stored immediately at -70°C until processed for O_2^- measurement. Another set of microdissected brainstem tissues were subjected to detection of NO and IL-1β using Griess assay and ELISA respectively. Separate set of RVLM was used for the detection of mRNA expression by qRT-PCR. Adequate care was taken before mounting these sections to microscopic slides to avoid possible contamination with RNase's. RNaseZap (Sigma-Aldrich Co., St. Louis, MO) was used to wipe the microscopic slides and other instruments before mounting sections.

5. Superoxide Measurement

O_2^- levels from the RVLM region were measured using a lucigenin O_2^- chemiluminescence assay adapted from Rey et al. [123]. RVLM punches were placed in HEPES buffer [119 mmol NaCl, 20 mmol HEPES, 4.6 mmol KCl, 1.0 mmol $MgSO_4$, 0.15 mmol Na_2HPO_4, 0.4 mmol KH_2PO_4, 5 mmol $NaHCO_3$, 1.2 mmol $CaCl_2$, 5.5 mmol glucose (pH 7.4)]. Diethyldithiocarbamate (DDC; a SOD inhibitor; 10 mmol) was added and incubated at 37°C for 30 min. Lucigenin (5µmol/L) was added and the samples were incubated for 10min at 37°C. Chemiluminescence measurements were obtained using a model TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA) for 10 readings (30 seconds/reading). Tiron (10 mmol/L; a O_2^- scavenger; Sigma, St. Louis, MO) was added and incubated for 15 min at 37°C and 10 additional

readings were taken. The relative amount of O_2^- was determined by taking the average of 2^{nd}-9^{th} readings prior to the addition of tiron, and subtracting the average of the 7^{th}-10^{th} readings after the addition of tiron. O_2^- generated was reported as change in chemiluminescence/min/mg tissue weight.

6. ELISA

A commercial ELISA kit (TiterZyme Kits, Assay Designs, Ann Arbor, MI) was used to measure IL-1β levels in the PVN and RVLM punches. The samples were assayed in duplicates. The assay was performed based on the manufacturer's guidelines. Protein concentrations in the punches were measured and the IL-1β values were expressed as pg/μg of protein.

7. Griess Assay

Total nitrate levels in both the PVN and RVLM were measured using a commercially available kit (total nitric oxide assay kit; Assay Designs, Ann Arbor, MI). Nitric oxide cannot be measured directly, so its breakdown product, nitrate is enzymatically converted to nitrite using nitrate reductase and measured colorimetrically.

8. Protein Estimation

Protein concentrations in the brain tissue homogenates (10 μl) were determined using the micro-Bicinchoninic acid (micro BCA) colorimetric assay (Pierce, Rockford, IL). The absorbance values of the sample were obtained at 562 nm using an ELX 800 microplate reader

(Biotek Instruments, Winooski, VT). The concentrations of IL-1β and NO were expressed as pg/μg protein.

9. Radioimmunoassay

Estradiol levels in serum separated from trunk blood were measured by double antibody radioimmunoassay by Dr. A.F. Parlow, National Hormone and Pituitary Program, NIDDK. Samples were assayed in duplicate. The values were expressed as pg/ml.

10. Western Blot

The PVN punches were solubilized in lysis buffer [0.5 mmol/l Tris·HCl (pH 6.8), 10% SDS, and 10% glycerol] with protease inhibitors (0.5 mmol/l PMSF, 10 μg/μl aprotinin, and 10 μg/μl leupeptin). An ultrasonic processor was used to homogenize punches (1–2 s pulses, with intermediate vortexing), which were centrifuged for 10 min at 5,000 rpm at 4°C. Supernatant was collected, and protein concentration was determined using a bicinchoninic acid protein assay (Sigma). Proteins (4:1 dilution in denaturing sample buffer, boiled for 5 min) were separated on SDS-polyacrylamide gels and transferred to Immobilon-P membranes. Membranes were blocked for 3 h [Tris-buffered saline (TBS)-Tween (TBS-T), 4% chick egg ovalbumin, and 2.5% sodium azide]. Blots were probed overnight at 4°C with primary antibody [ET-A (1:200 dilution; Alomone Labs, Israel) and tubulin (1:1,000 dilution; Millipore; Temecula, CA)], rinsed in TBS-T with a final rinse in TBS, and incubated with the appropriate secondary antibody for 1 h at 4°C. The ET-A antibody had been tested previously with appropriate positive controls in

the laboratory. Blots were then incubated with ECL reagents for visualization of the bands. The intensity of the bands was measured using NIH's Image J software.

11. Quantitative RT-PCR

RNA extraction and cDNA synthesis

The RNA was extracted from the brain and brainstem punches using MELT Total Nucleic Acid Isolation System (Ambion Inc, Austin, TX) according to manufacturer's instruction. The tissue was digested using the Multi-Enzymatic Liquefaction of Tissue (MELT) mix given in the kit. The RNA was eluted in a volume of 500 µl, after on-bead Turbo DNase digestion (Ambion). The quality of the RNA was assessed using Nanodrop spectrophotometer and the good quality RNA samples with a 260/280 ratio in the range of 1.7-2.2 were only used for cDNA synthesis. First strand cDNA was synthesized by reverse transcribing 400 ng of total RNA using RT^2 First Strand Kit (SABiosciences, Frederick, MD).

qRT-PCR

The cDNA samples from RVLM and PVN were used to perform quantitative real-time PCR (qRT-PCR). RT^2 Real-Time PCR SYBR Green/ROX Master Mix (SABiosciences, Frederick, MD), cDNA samples, and the appropriate amount of RNA nuclease-free water were combined. The volume/well equated to 12.5 µL of PCR master mix, 2 µL of cDNA, 1µL each of forward and reverse primer and 8.5 µL of water. The total reaction volume equaled to 25 µL. The forward and reverse primers for ET-1, ET-1A, ET-1B, AT-1, iNOS, IL-1β, NADPH oxidase subunits and CuZnSOD were purchased from Integrated DNA Technologies (Coralville, IA). The reactions were performed in the Applied Biosystems 7500 Real-Time PCR System (Applied

Biosystems) with the following run method: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec and 72°C for 35 sec. At the end of amplification, a melting curve analysis was done by heating the PCR products to 65°-95°C and held for 15 sec at increments of 0.2°C, and the fluorescence was detected to confirm the presence of single amplification product. After obtaining the C_T values, the values are compared between the control and treatment group according to $2^{-\Delta\Delta CT}$ method.

12. Statistical Analysis:

All statistical procedures were performed using STATVIEW software unless specified otherwise. Changes in MAP, HR, SBP and DBP profiles were analyzed by repeated measures ANOVA followed by post hoc Fisher's LSD. The average values were compared using one way ANOVA followed by post hoc Fisher's LSD. Changes in NO levels, IL-1β, ET-1, Ang II levels were analyzed by unpaired student's t-test. The differences in the fold change in the expression of genes in the PCR array and q-PCR were analyzed by unpaired student's t-test.

CHAPTER 3

CHRONIC ESTRADIOL-17β EXPOSURE CAUSES HYPERTENSION IN ADULT FEMALE SPRAGUE-DAWLEY RATS

1. Introduction

Cardiovascular disease remains one of the leading causes of morbidity and mortality in women. Postmenopausal increases in BP in women make hypertension more prevalent in women compared to men of the same age [2, 124]. Since premenopausal women have lower BP compared to age-matched men, estrogens were thought to play a protective role against hypertension. Estrogens are reported to improve lipid profile [3], decrease vascular resistance [4] and modulate the activity of brain nuclei involved in cardiovascular regulation [5, 125]. However, recent reports from the Women's Health Initiative study, National Institute of Health [126] have provided evidence that Hormone Replacement Therapy (HRT) using estrogen alone or a combination of estrogen and progestin does not confer cardiovascular protection, and may actually increase the risk for coronary heart disease among postmenopausal women [7]. These clinical studies have brought to light the importance of clinical and basic research in understanding the role of estrogen in BP regulation [127]. Oral estrogen administration, either given alone or in combination with progestins, was found to promote systolic blood pressure in postmenopausal women [25, 26]. Although the magnitude of the increase in BP was only between 1 and 2 mmHg, similar increases in systolic BP and pulse pressure are known to be associated with a higher rate of progression of coronary atherosclerosis [27] and development of cardiovascular events [26] in large clinical trials.

In addition to women who are on HRT, younger women who take oral contraceptives are also at increased risk for potentially developing cardiovascular disorders. Oral contraceptives have been used worldwide for over 30 years. Most women taking oral contraceptives have been found to have small elevations in BP of ~2mm of Hg [8]. In addition, approximately 5% of women over 35 years of age who take a preparation containing more than 50 μg of E_2 have

significant elevations in BP greater than 140/90 mmHg [8]. Estrogen rather than progesterone, appears to be the primary cause for increases in BP observed with oral contraceptives because women taking progestin-only contraceptives do not have significant elevations in BP [9]. Taken together, these studies suggest a negative correlation between chronic estradiol exposure and cardiovascular health in women. In order to elucidate the mechanisms behind these clinical findings, it is important to develop an animal model which simulates chronic estradiol exposure in women. We hypothesize that chronic estradiol exposure increases mean arterial pressure in adult female SD rats (Fig. 3-1) and to test this hypothesis, we exposed young, adult cycling female SD rats to low levels of E_2 for a period of 90 days and assessed their BP parameters.

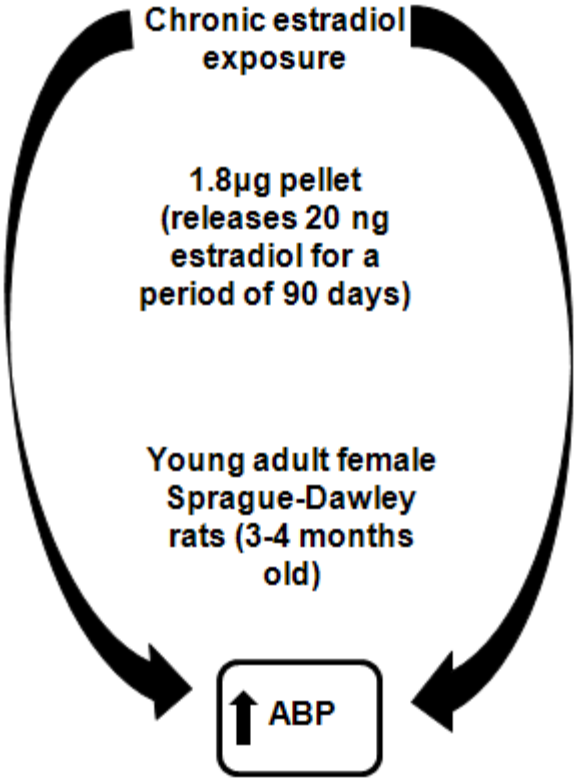


Fig. 3-1 Hypothesis 1 Chronic exposure to low levels of estrogen increases arterial blood pressure (ABP) in young adult female Sprague-Dawley rats.

2. Experimental Design

In this experiment, the effects of chronic estradiol exposure on BP parameters were evaluated. The animals were divided into 2 groups (n=4/group); sham-implanted (control); or implanted with E_2 (20 ng/day, 90-day slow-release pellet; Innovative Research America, Sarasota, FL). After 60 days, sham and E_2-treated rats were implanted with radiotelemetry transmitters to monitor BP. After 90 days of exposure to E_2, all the animals were sacrificed at noon on the day of estrous. Most of the E_2 treated animals were in persistent estrous after 90 days treatment. The control animals were sacrificed on the day of estrous after 90 days of sham implantation for comparison to the treatment group.

3. Results

Chronic exposure to estradiol-17β causes hypertension

The daily average profiles and the average mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) during the 10-11th week of treatment in control and E_2-treated rats are shown in Fig. 3-2 to 3-5. The MAP in control animals remained steady over the entire period of observation. In contrast, E_2 treatment increased MAP significantly ($p<0.05$; Fig. 3-2A). The average MAP (mean±SE, mmHg) measured during the 10-11th week of observation in control rats was 105.1±0.7. In contrast, E_2 exposure increased MAP significantly to 119.6±0.8 ($p<0.05$; Fig. 3-2B). The HR profile of E_2-treated rats had a tendency to increase when compared to control rats during the entire period of observation (Fig. 3-3A). The average HR (mean±SE, beats/min) during the 10-11th week of treatment in E_2-treated rats (371.7±1.5) was significantly elevated compared to control rats (354.5±1.4; $p<0.05$; Fig 3-3B). Similarly, the SBP and DBP profiles in E_2-treated were significantly elevated in E_2-treated rats compared to control rats ($p<0.05$; Figs. 3-4A and 3-5A). E_2 exposure also increased the average SBP and DBP (mean±SE, mmHg; 140.0±0.7 and 101.1±0.8 respectively) significantly compared to control rats (125.4±0.7 and 88.0±0.7 respectively; $p<0.05$; Figs. 3-4B and 3-5B).

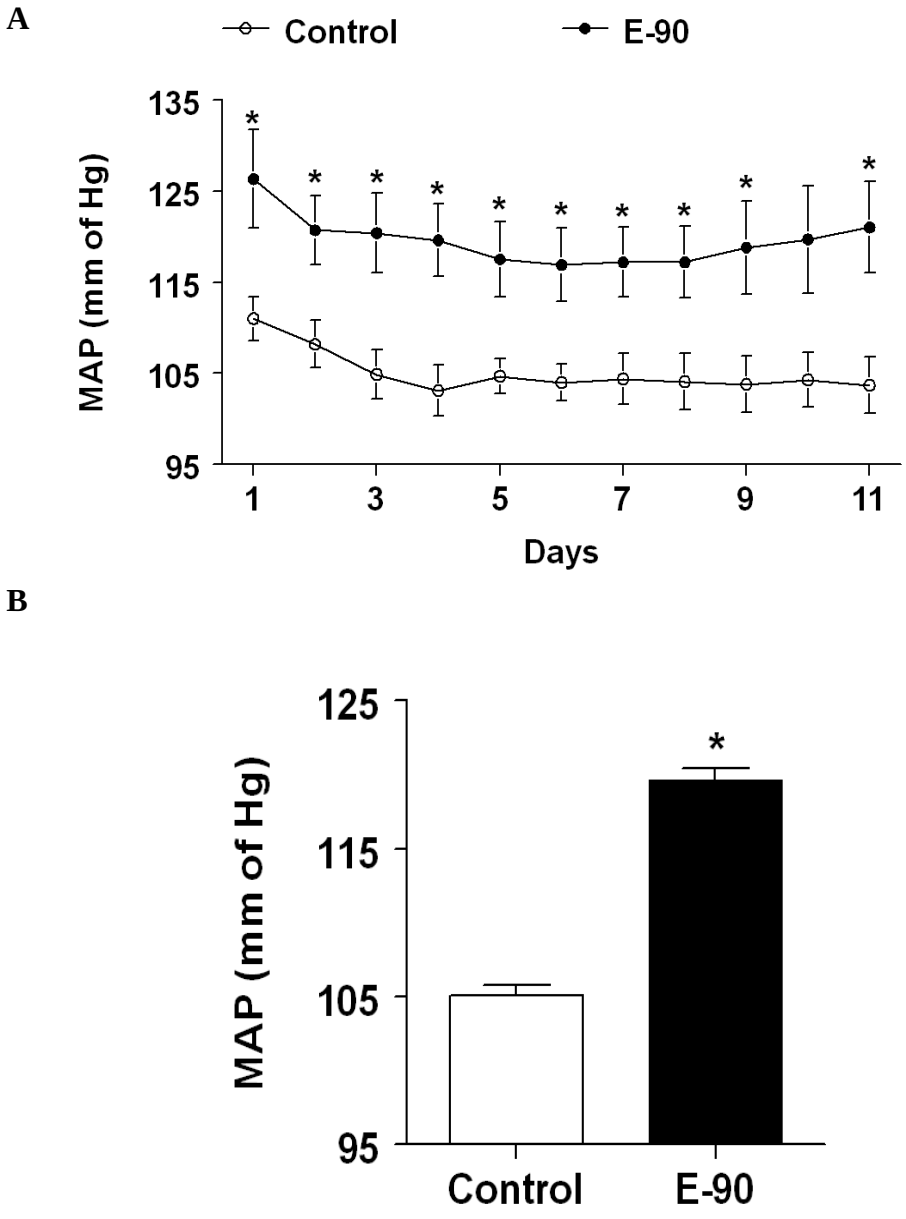


Fig. 3-2 Effect of chronic E_2 exposure on Mean Arterial Pressure (MAP).

A: Line graph depicting MAP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats. B: Bar graph showing the average MAP values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

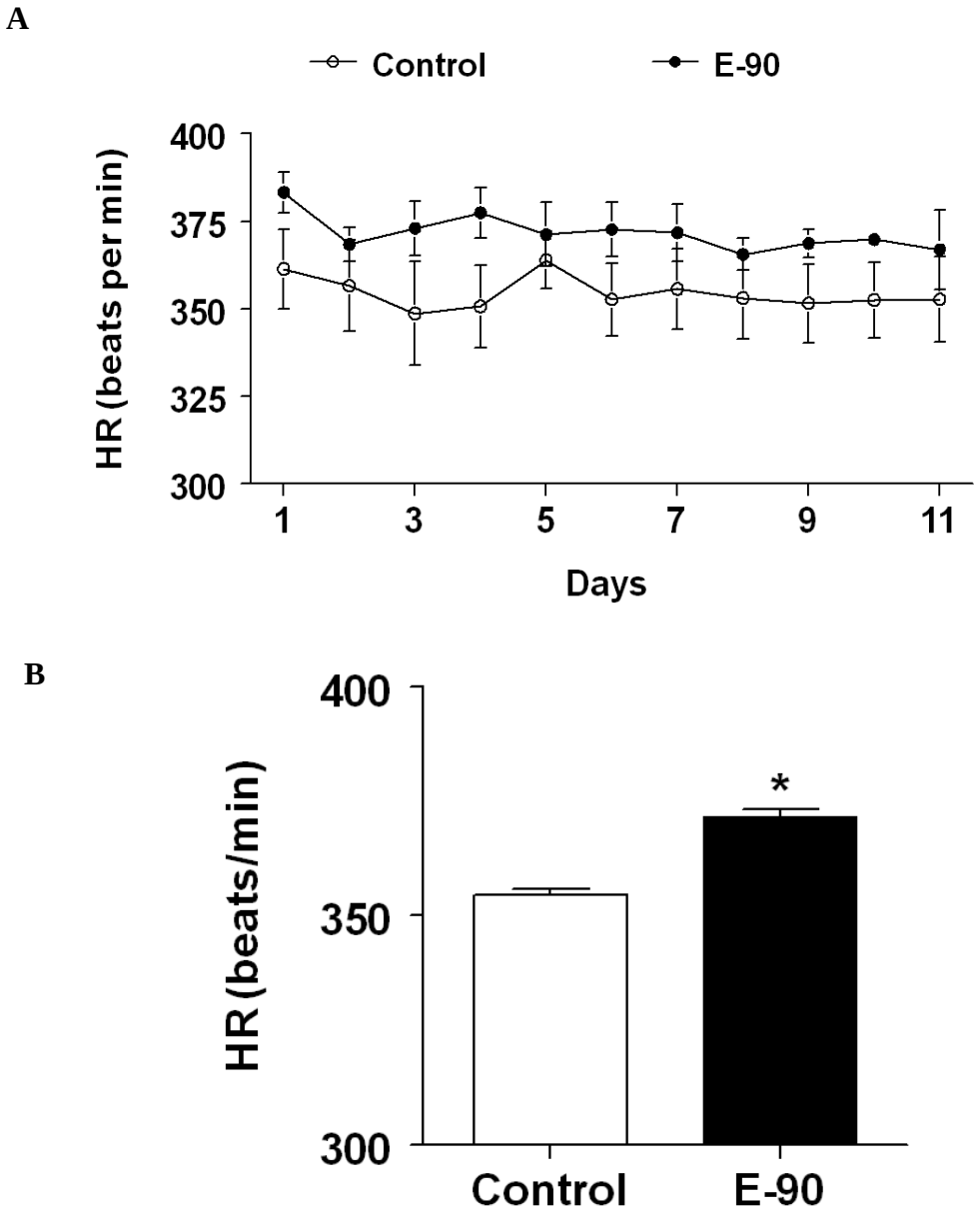


Fig. 3-3 Effect of chronic E_2 exposure on Heart Rate (HR).

A: Line graph depicting HR (beats/min): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats. B: Bar graph showing the average HR values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

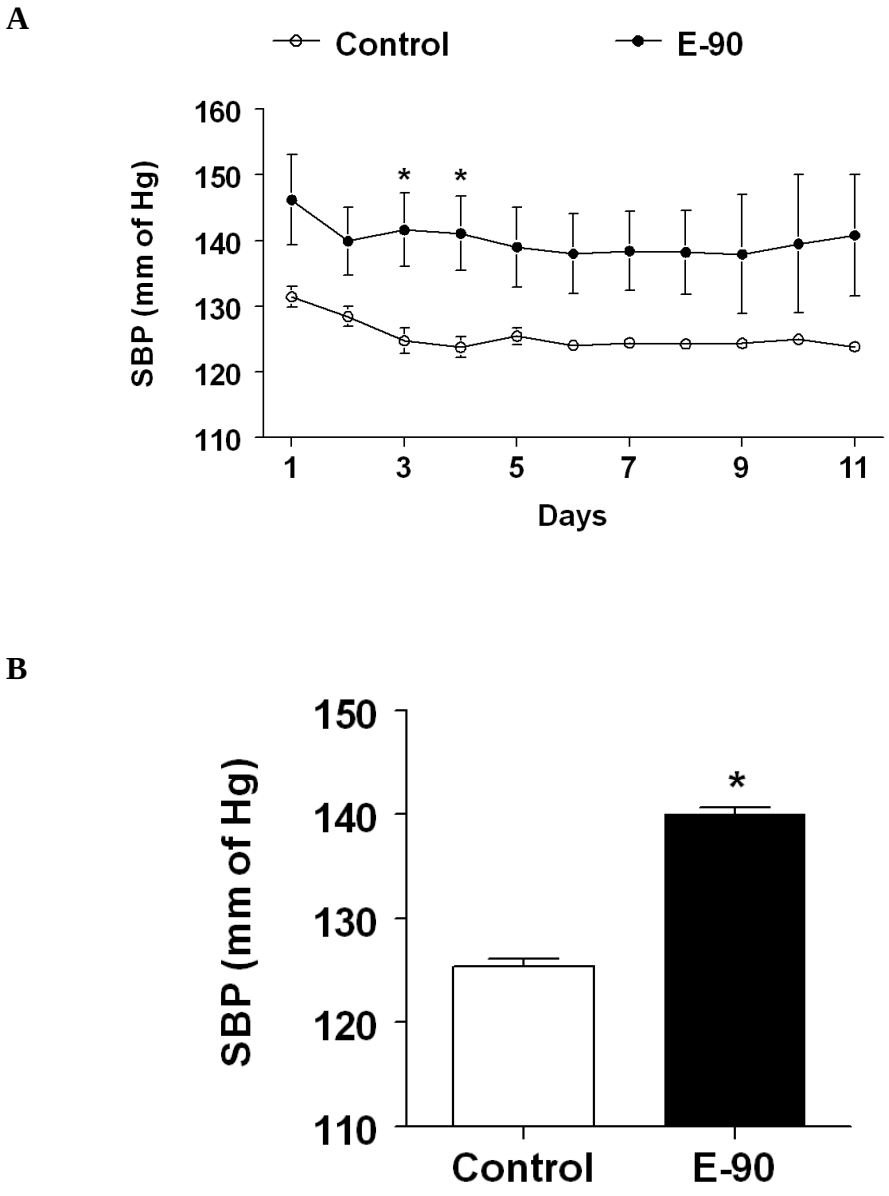


Fig. 3-4 Effect of chronic E_2 exposure on Systolic Blood Pressure (SBP).

A: Line graph depicting SBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats. B: Bar graph showing the average HR values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

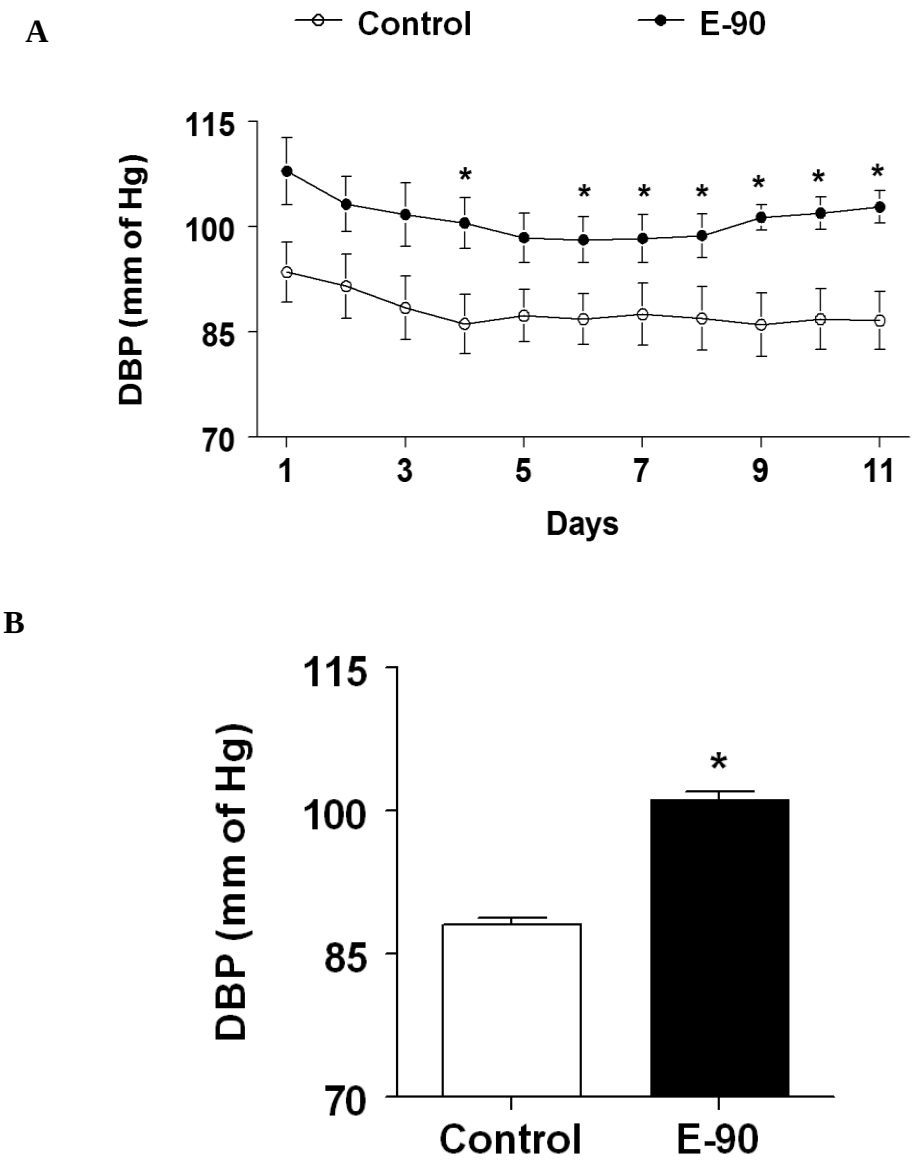


Fig. 3-5 Effect of chronic E_2 exposure on Diastolic Blood Pressure (DBP).

A: Line graph depicting DBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats. B: Bar graph showing the average HR values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

4. Discussion

The results from the present study demonstrate that chronic exposure to low levels of E_2 causes increase in BP. Estrogen was originally believed to prevent hypertension based on the observation that postmenopausal women have a higher incidence of hypertension compared to age-matched men [128-130] and, therefore, HRT containing estrogenic preparations were believed to reduce BP in these women [131]. Several possible mechanisms by which estrogens could decrease BP have been suggested. There is evidence that estrogen increases acetylcholine-induced, NO-mediated relaxation of aorta in male SHR rats [132] through upregulation of eNOS [133]. Also, estrogen is believed to act on brainstem autonomic centers to decrease SNA [134, 135], causing reduced vasomotor tone and lowered BP.

Contrary to the belief that estrogens can lower BP, there is evidence indicating that repeated exposure to low levels of estrogens, as in the case of oral contraceptives, can cause an increase in BP. Numerous human clinical studies have associated chronic use of contraceptive pills with increase in BP; in one study about 30% of the women aged between 15-44 were having malignant increase in BP. In another study there was a mild increase in BP in most women over the age of 35 years with about 5% of the women having high BP. [8, 34, 136]. Similar results have been seen in studies using female SD rats, where exposure to a combination of 1µg of ethinyl estradiol and 10µg of norgestrel or 1 µg of ethinylestradiol alone for 10 weeks resulted in concomitant increases in systolic and mean arterial BP [137-139] In another study, female SD rats were injected with 0.2µg of ethinyl estradiol six days per week exhibited significant increases in systolic BP of 17mm of Hg in 6 weeks and 32mm of Hg in 12 weeks [138].

The increase in MAP, HR, SBP and DBP with chronic E_2 exposure that was observed in the present study is supported by the observations mentioned above [137-139]. The main

difference between these and the present study is the dose of E_2 used and duration of exposure. While the doses used in other studies ranged from 0.2 to 10 µg of various estrogenic preparations, we were able to observe increases in cardiovascular parameters with 10-fold less concentration of E_2 (0.02 µg or 20 ng) for a period of 13 weeks.

In contrast to our present study, Brosnihan et.al, have shown that 3 weeks of estrogen treatment decreased MAP and significantly decreased Ang II-induced pressor response in female ovariectomized rats which was accompanied by significant reduction in plasma Ang II levels [126]. However, the dose of E_2 used in their study was 1.5 mg/day which yielded plasma concentrations of 190±20 pg/ml. With a similar dosage of E_2, Gimenez et.al, demonstrated enhanced hypotensive responses to administration of an ACE inhibitor in E_2-treated 18 months old female SHR rats [140]. When using 1.5 mg/day, the serum E_2 concentrations were approximately 2.5 fold higher than those that have been found in the present study, where serum E_2 concentrations were 68.2±4.07 pg/ml in animals that were treated with E_2 alone. Previous studies also used a much shorter E_2 exposure time frame than those used in the present study, thus complicating the comparisons. In the present study, we monitored changes in BP only 9 weeks after E_2 implantation. Further studies are needed to determine exactly when the changes in BP become apparent in these animals.

In conclusion, our results support the hypothesis that chronic exposure to low levels of E_2 causes hypertension in young SD female rats.

CHAPTER 4

CHRONIC ESTRADIOL-17β EXPOSURE INCREASES SUPEROXIDE PRODUCTION IN THE ROSTRAL VENTROLATERAL MEDULLA (RVLM) AND CAUSES HYPERTENSION: REVERSAL BY RESVERATROL

1. Introduction

The central nervous system (CNS) plays an important role in the development and maintenance of hypertension [37-40]. The cortex, limbic system, hypothalamus, brainstem and the autonomic nervous system are all known to be involved in the maintenance of BP. The rostral ventral lateral medulla (RVLM) of the brainstem and the paraventricular nucleus (PVN) of the hypothalamus are two critical areas that are involved in the regulation of SNA and BP [15, 141]. The RVLM and PVN have direct neuronal projections to the intermediolateral cell column (IML) of the thoraco-lumbar spinal cord. They integrate the sympathetic outflow and excitatory input to preganglionic sympathetic neurons in the spinal cord [142, 143]. The PVN also affects the SNA indirectly by its lateral projections to the RVLM. The RVLM is involved in maintaining basal vasomotor tone and an increase in RVLM activity is associated with hypertension and heart failure [37].

In our previous study (chapter 3), we demonstrated elevated mean arterial pressure (MAP) in female SD rats after chronic (90 days) estrogen exposure. However, the central mechanisms behind chronic estrogen-induced increases in MAP are still unclear. Recently, oxidative stress associated with increased ROS, O_2^-, in both the RVLM and PVN has been suggested to increase SNA and BP in several animal models of hypertension [38-40, 144]. The association between oxidative stress and hypertension has also been documented in human hypertension [145]. Pro-inflammatory cytokine and Interleulin-1β [90] can drive the production of ROS in the PVN, which in turn could lead to development of hypertension. Further, inhibition of brain cytokine synthesis using icv pentoxifylline has been shown to reduce O_2^- generation [90]. Central administration of tempol, a SOD mimetic has also been shown to reduce the levels of pro-inflammatory cytokines and BP in Ang II hypertensive rats [81]. These studies suggest

that cytokines and O_2^- could sustain the existing pro-inflammatory state in the brain and promote the development of hypertension.

Apart from Interleukin-1β, an increase in NO levels produced by iNOS has also been demonstrated to play a role in O_2^- generation in the RVLM [146]. Overexpression of iNOS in the RVLM increased the O_2^- levels in the RVLM accompanied by increase in mean arterial pressure [111]. Proinflmmatory cytokines, IL-1β and tumor necrosis factor α (TNF-α) has been shown to stimulate iNOS expression and increase nitrite and nitrate levels in the PVN [147]. Together, these studies suggest that interplay between oxidative stress and inflammation could play a role in chronic estradiol-induced hypertension. In the present study first we hypothesize that there is increase in oxidative stress in both RVLM and PVN in chronic estradiol-induced hypertension. Secondly, we further hypothesis that chronic estradiol exposure is associated with inflammatory changes marked by increase in Interleukin-1β and NO levels in the PVN and RVLM (Fig. 4-1). Finally, we also hypothesize that these changes could be reversed using resveratrol, a polyphenolic compound which has both antioxidant and anti-inflammatory properties.

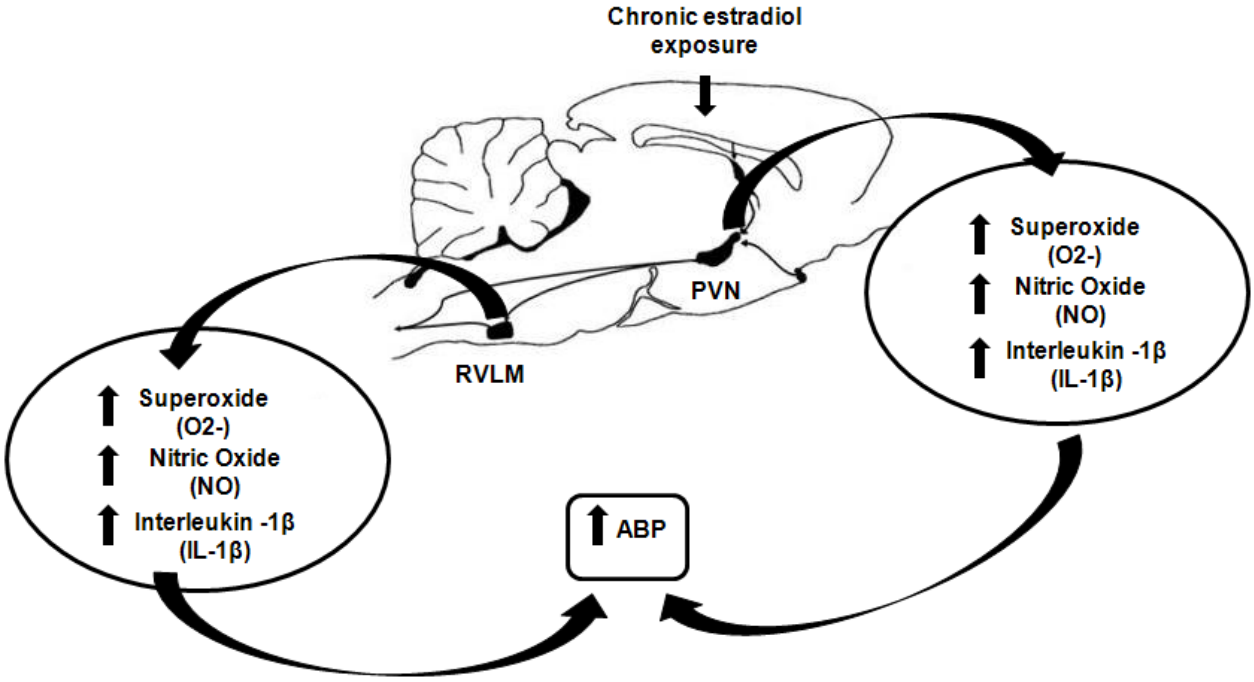


Fig. 4-1 Hypothesis 2 – Chronic exposure to low levels of estrogen increases arterial blood pressure (ABP) by increasing superoxide with in rostral ventrolateral medulla (RVLM) and paraventricular nucleus (PVN). These changes will be accompanied with increased nitric oxide and interleuckin-1β in both the regions.

2. Experimental Design

In experiment 1, the role of O_2^-, NO and IL-1β in chronic estradiol-induced increase in mean arterial pressure was assessed. The animals were divided into 2 groups (n=4/group); sham-implanted (control); or implanted with E_2 (20 ng/day, 90-day slow-release pellet; Innovative Research America, Sarasota, FL). Animals were 3-4 month old at the beginning of the experiment. After 90 days of exposure to E_2, all the animals were sacrificed at noon on the day of estrous. Most of the E_2-treated animals were in persistent estrous after 90 days treatment. The control animals were sacrificed on the day of estrous after 90 days of sham implantation for comparison to the treatment group. The brains were removed, frozen on dry ice and stored at -70°C. The trunk blood was collected and serum was separated and stored at -70°C until processed.

The brains collected from the above experiment were sectioned (300µm thick) and RVLM and PVN were microdissected using Palkovit's microdissection technique. O_2^- levels were measured from both the regions using chemiluminescence method. NO and IL-1β assays were performed in both the RVLM and PVN. Further iNOS and IL-1β mRNA expressions were measured in both the areas using quantitative RT-PCR. In order to get sufficient tissues for all the above mentioned analysis, an additional batch of animals were obtained with the same experimental design.

In experiment 2, the effect of an anti-oxidant on chronic estradiol-induced hypertension and its changes in the PVN and RVLM has been assessed. The animals were divided into 4 groups (n=6/group):

Group 1: sham-implanted (control)

Group 2: implanted with E_2 slow-release pellets (E-90; 20 ng/day for 90 days; [120])

Group 3: control fed with chow containing 0.84g resveratrol/kg of chow (Res) [148] and

Group 4: E_2 implanted rats fed with chow containing resveratrol (Res+E-90)

Resveratrol treatment was started 7 weeks after pellet implantation. Animals were implanted with telemeters on the 9^{th} week to measure cardiovascular parameters. Food intake, water intake and body weight were monitored weekly throughout the experimental period. The animals were sacrificed at the end of 90 days while in the state of estrous. Heart weight and heart to body weight ratio were recorded at the time of sacrifice.

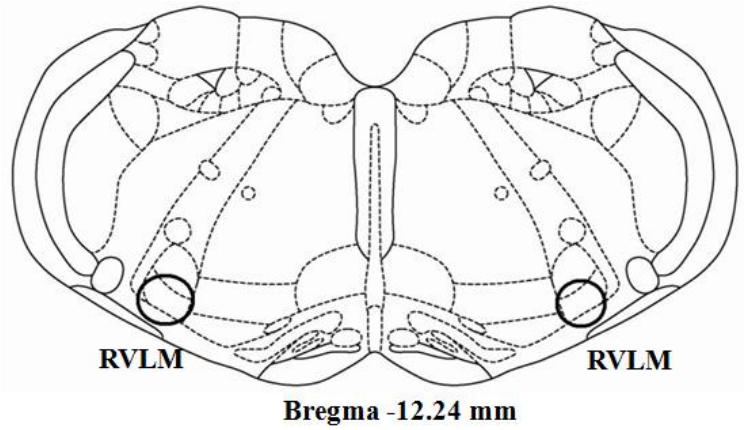
Estradiol levels were measured in the serum samples by radioimmunoassay. O_2^- levels were measured as described in the previous experiment.

3. Results

Chronic exposure to E_2 increases superoxide levels in RVLM

Chronic E_2 exposure significantly elevated O_2^- levels (mean±SE, nmol/min*mg) in the RVLM of E_2-treated rats (0.83±0.1) when compared to control rats (0.53±0.04; $p<0.01$) (Fig. 4-2).

A



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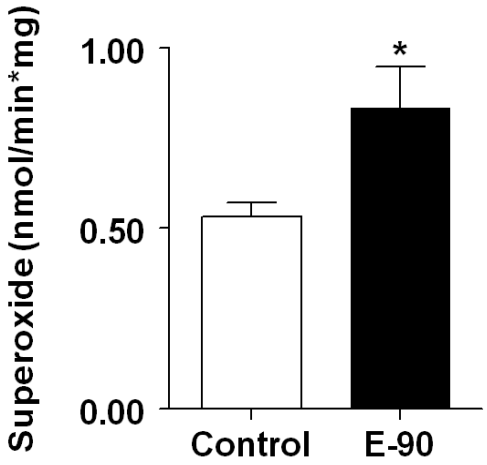


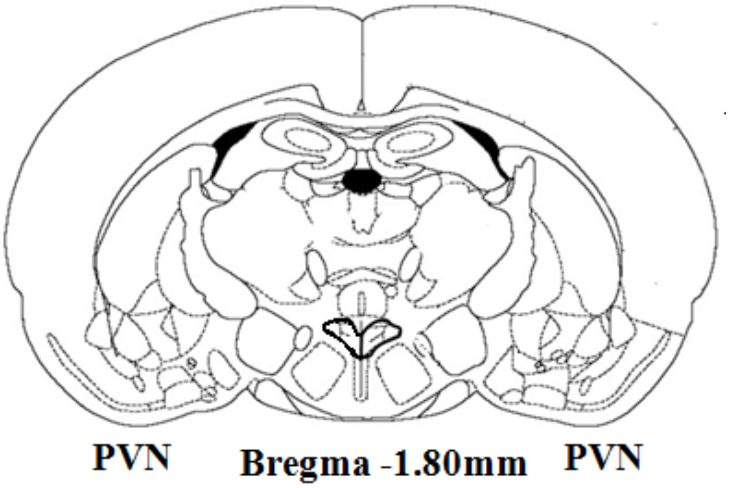
Fig. 4-2 Effect of chronic E_2 exposure on superoxide production in the RVLM.

A: Schematic coronal brain stem section showing the location of micropunches of RVLM (within circles) taken from Paxinos and Watson rat brain atlas, 6^{th} edition. The section coordinate (-12.24mm bregma) represents the distance caudal to the bregma. B: Bar graph denoting O_2^- levels measured in RVLM brainstem punches of E_2-treated (20 ng/day, 90-day slow-release pellets) and sham-implanted female SD rats (n=4 per group) using a luminometer. * indicates significant difference ($p<0.05$) from control rats.

Chronic E_2 exposure had no effect on superoxide production in the PVN

Chronic E_2 exposure produced no significant changes in the O_2^- levels (mean±SE, nmol/min*mg) in the PVN of E_2-treated rats (2.4±0.2) when compared to control rats (1.8±0.2) (Fig. 4-3).

A



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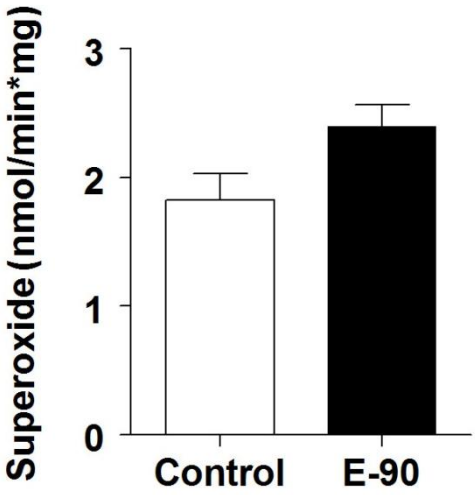


Fig. 4-3 Effect of chronic E_2 exposure on superoxide in the PVN.

A: Schematic coronal brain section showing the location of PVN (within highlighted area) taken from Paxinos and Watson rat brain atlas, 6th edition. The section coordinate (-1.8mm bregma) represents the distance caudal to the bregma. The micropunches were taken from this section of PVN. B: Bar graph denoting O_2^- levels measured in PVN punches of E_2-treated (20 ng/day, 90-day slow-release pellets) and sham-implanted female SD rats (n=4 per group) using a luminometer.

Chronic E_2 exposure upregulated the gene expression of NADPH oxidase subunits in the RVLM

The levels of NAPDH oxidase subunits mRNA expressed as a ratio of NAPDH oxidase to β-actin mRNA in control and E_2-treated animals are shown in (Fig. 4-4). Between the different NADPH oxidase subunits Nox1 and Nox2 were upregulated in chronic E_2 treated rats on chronic E_2 exposure. The mRNA expression of Nox1 was upregulated by 2 fold in the RVLM of chronic E_2-treated rats (2.5±0.7, $p<0.05$), compared to normalized control ($p<0.05$). Nox2 gene expression was also upregulated by 2 fold in chronic E_2-treated rats (2.8±0.7, $p<0.05$) compared to control rats (normalized to 1). Nox 4 was significantly downregulated on chronic E_2 exposure (0.5±0.1, $p<0.001$) compared to control rats. No changes were seen in the gene expression expression of p47phox or p22phox subunits of NADPH oxidase.

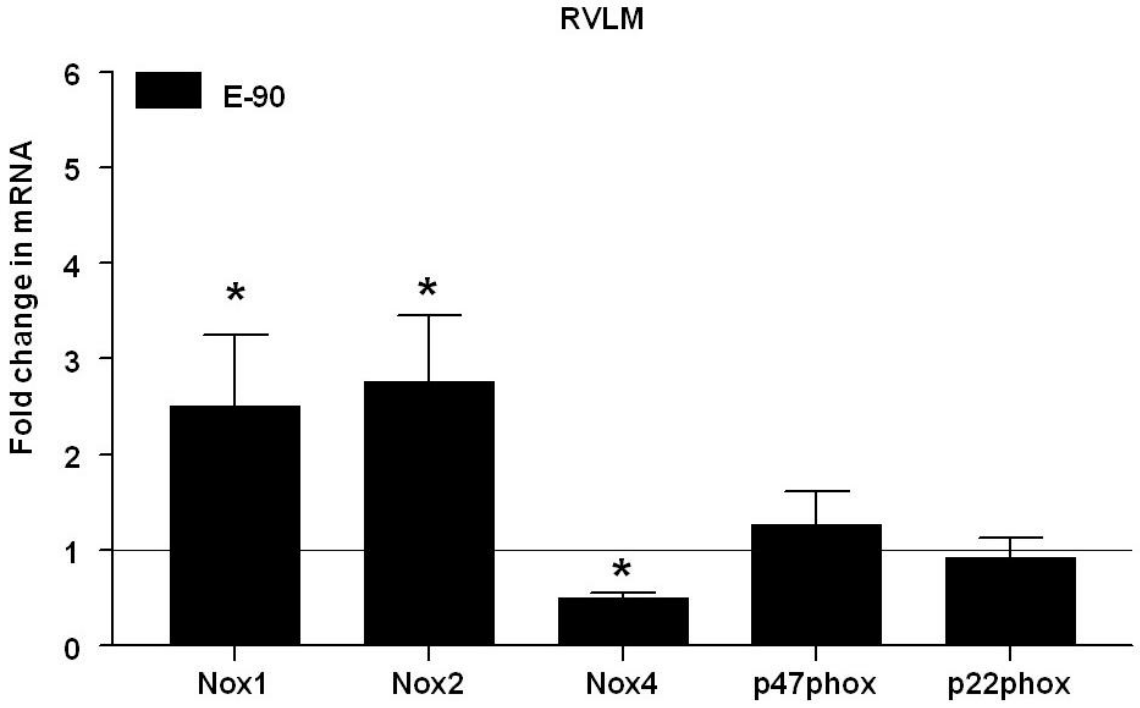


Fig. 4-4 Effect of chronic E_2 exposure on the gene expression of NADPH oxidase subunits in the RVLM

The mRNA expression levels of NADPH oxidase subunits in the E_2 treated rats. The fold change was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$. The C_t values of all the groups were normalized to control rats (n=6-7 per group). * denotes significant difference ($p<0.05$) from control group.

Chronic E_2 exposure downregulated the gene expression of superoxide dismutase in the RVLM

Chronic E_2 exposure significantly downregulated the mRNA expression of CuZnSOD in the RVLM of E_2-treated rats (0.21±0.02) when compared to control rats (1.01±0.11, $p<0.001$) (Fig. 4-5).

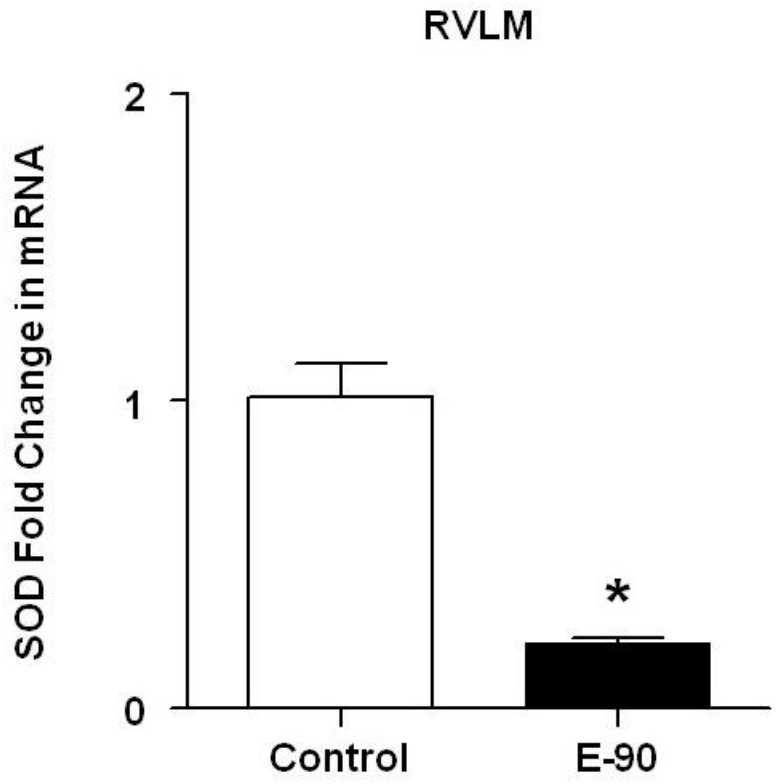


Fig. 4-5 Effect of chronic E_2 exposure on the gene expression of superoxide dismutase in the RVLM

The mRNA expression of CuZnSOD in the control and E_2 treated rats. Fold changes were normalized to β-actin control (n=3-4 per group). * denotes significant difference ($p<0.05$) from control group.

Chronic E_2 exposure had no effect on nitric oxide (NO) levels in the RVLM

The NO levels were measured in the form of nitrate concentrations (means±SE; μM/μg protein) in the control and E_2-treated rats. There was no difference in the NO level between the control (0.2±0.03) and E_2-treated rats (0.24±0.02) (Fig.4-6).

Chronic E_2 exposure had no effect on iNOS mRNA expression in the RVLM

The gene expression of iNOS was not significantly different between the E_2-treated rats (1.54±0.59) and control rats (1.84±0.77) in the RVLM (Fig. 4-7).

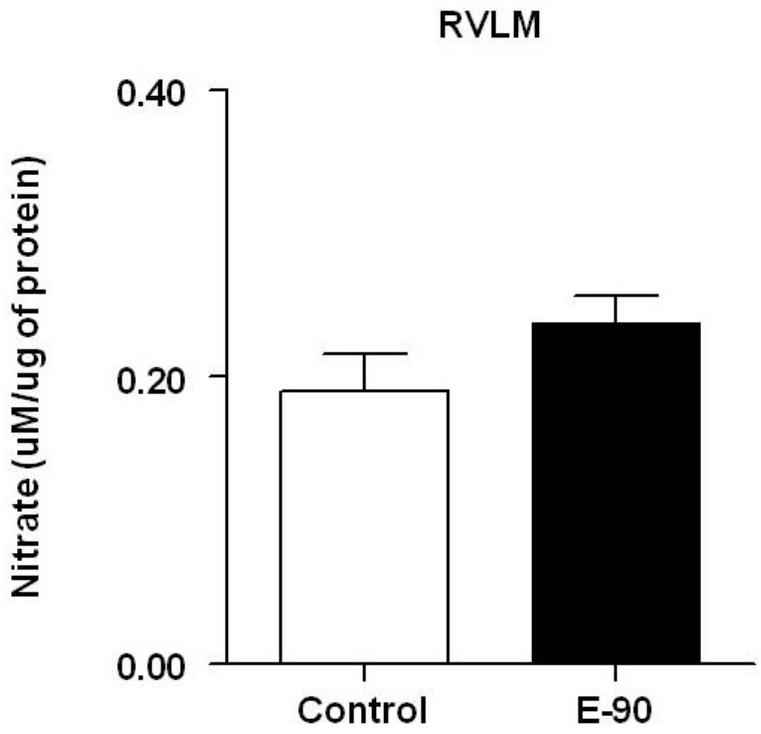


Fig. 4-6 Effect of chronic E_2 exposure on the nitric oxide levels in the RVLM

The total nitrate concentrations (means ± SE; μM/μg protein) in the RVLM of control and E_2-treated rats (n=5 per group).

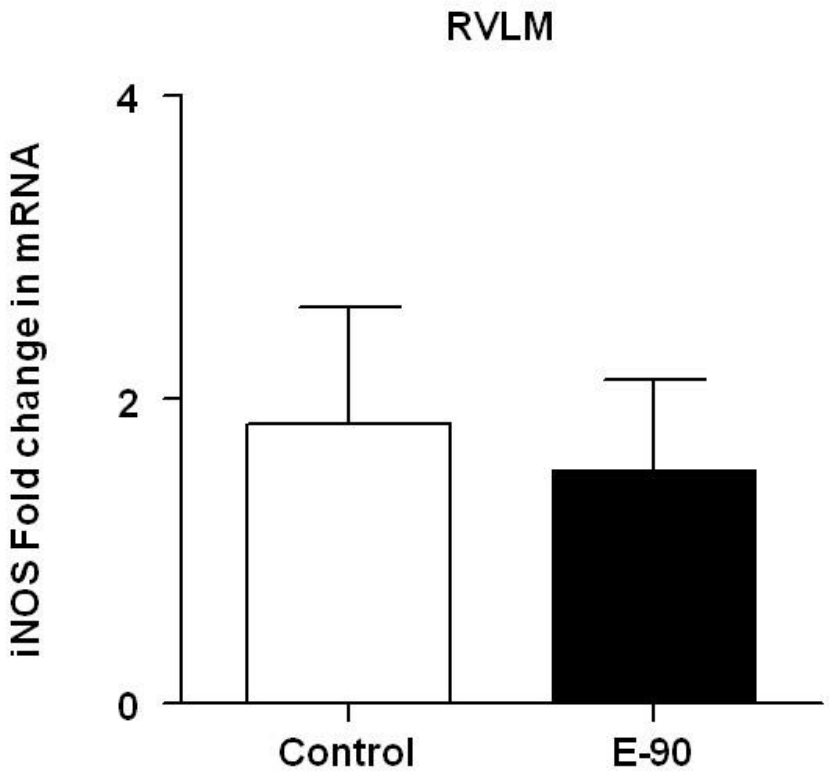


Fig. 4-7 Effect of chronic E_2 exposure on the gene expression of iNOS in the RVLM

The mRNA expression level of iNOS from control and E_2 treated rats. Fold changes were normalized to β-actin control (n=5 per group).

Chronic E_2 exposure had no effect on nitric oxide levels in the PVN

The NO levels (means±SE; μM/μg protein) in the PVN of control and E_2-treated rats are shown in Fig. 4-8. There was no difference in the PVN NO levels between control (0.08±0.01) and E_2-treated rats (0.07±0.01).

Chronic E_2 exposure had no effect on iNOS mRNA expression in the PVN

Similarly, the gene expression of iNOS was not significantly different between the E_2-treated (0.82±0.31) and control rats (1±0.31) (Fig. 4-9).

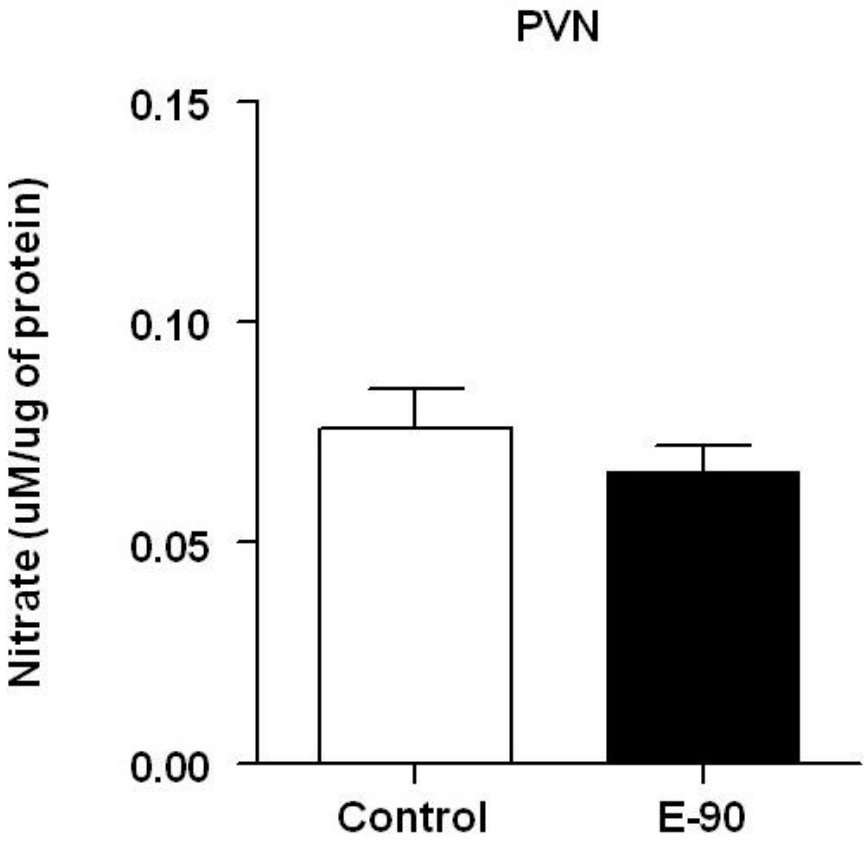


Fig. 4-8 Effect of chronic E_2 exposure on the nitric oxide levels in the PVN

The nitrate concentration (means ± SE; μM/μg protein) in the PVN of control and E_2-treated rats (n=5 per group).

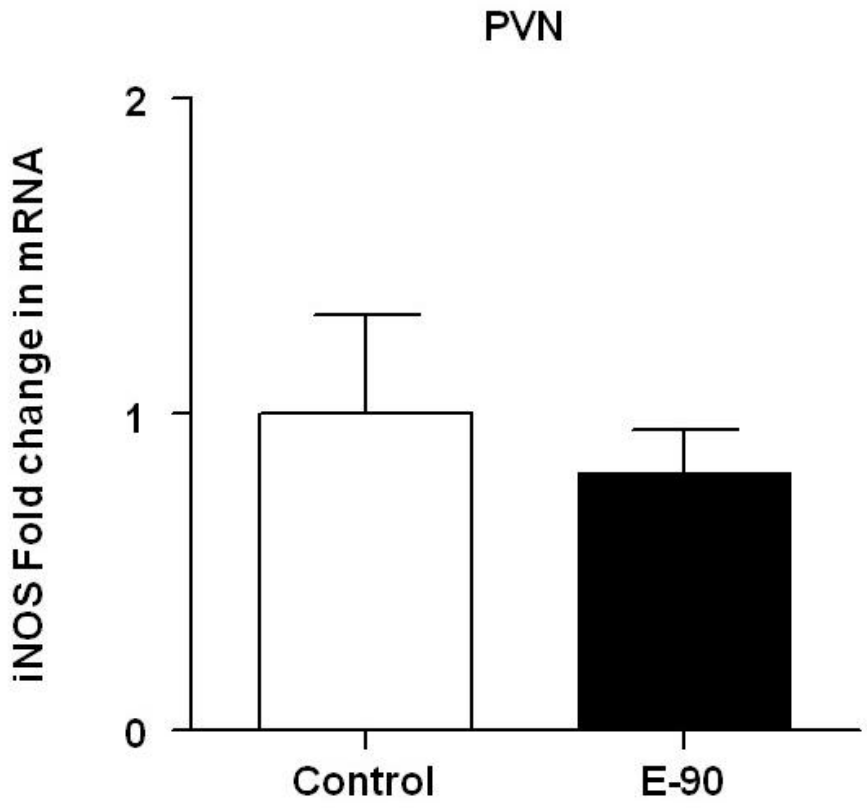


Fig. 4-9 Effect of chronic E_2 exposure on the gene expression of iNOS in the PVN

The mRNA expression levels of iNOS between the control and E_2-treated rats (n=5 per group). Fold changes were normalized to β-actin control

Chronic E_2 exposure had no effect on IL-1β levels in the RVLM

The interleukin-1β levels for the control and E_2-treated rats are shown in Fig. 4-10. There was no difference in IL-1β levels (mean±SE; pg/μg protein) in the RVLM of E_2-treated rats (11.34±0.85) and control rats (12.63±0.9).

Chronic E_2 exposure had no effect on IL-1β mRNA levels in the RVLM

The mRNA levels of IL-1β in the RVLM were also not different between E_2-treated (0.39±0.2) and control rats (2.29±1.18) (Fig. 4-11).

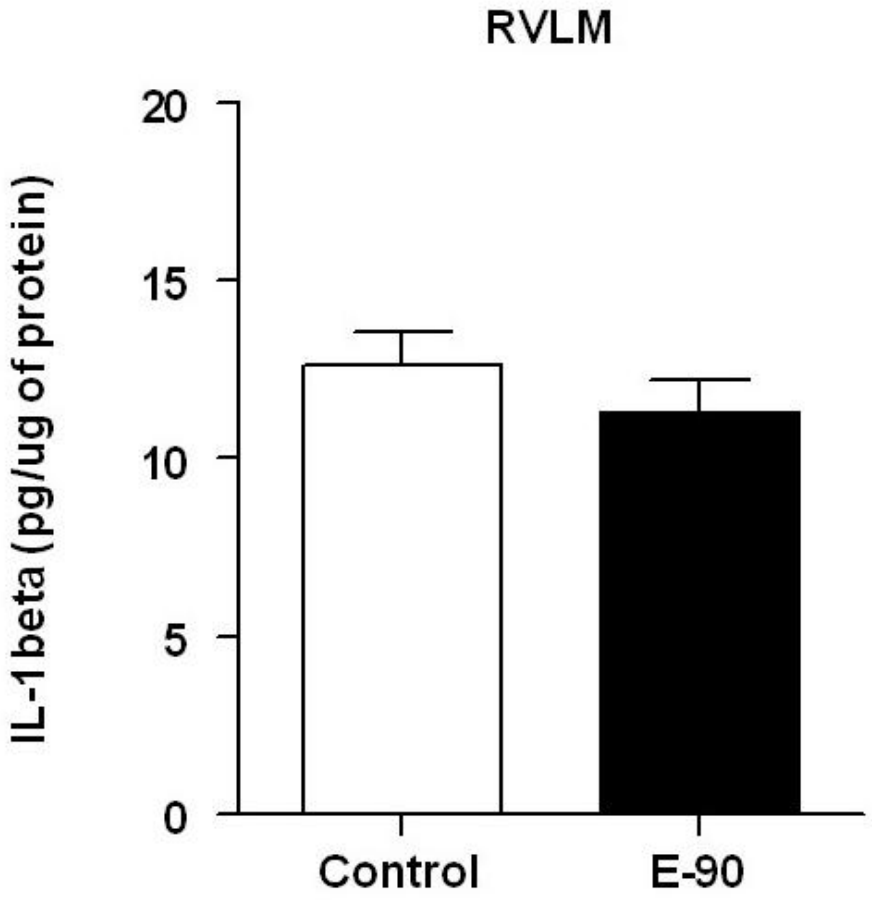


Fig 4-10 Effect of chronic E_2 exposure on the interleukin-1β levels in the RVLM

IL-1β levels from control and E_2-treated rats (n=5/group). Values are means; error bars represent SE.

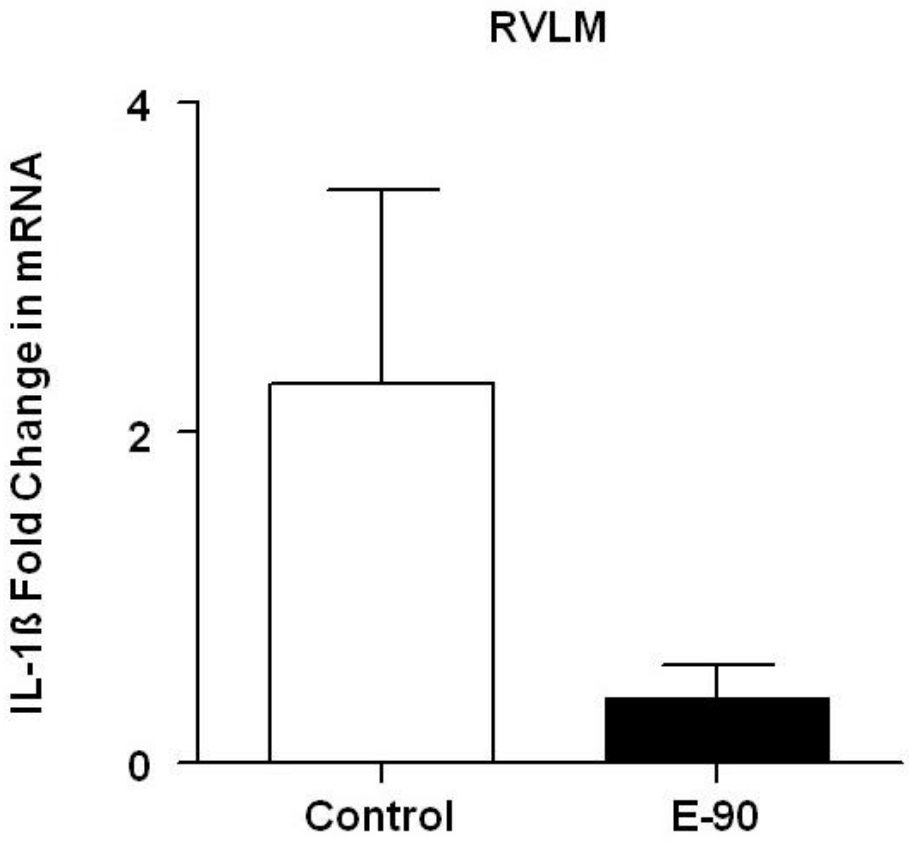


Fig 4-11 Effect of chronic E_2 exposure on the gene expression of IL-1β in the RVLM

The mRNA expression levels of IL-1β between the control and E_2-treated rats (n=5/group). Fold changes were normalized to β-actin control.

Chronic E_2 exposure had no effect on the levels of IL-1β in the PVN

The IL-1β levels (mean±SE; pg/μg protein) in the PVN of E_2-treated rats (14.1±0.72) were not significantly different from the control rats (14.87±0.5) (Fig. 4-12).

Chronic E_2 exposure had no effect on IL-1β mRNA levels in the PVN

There was no difference in the mRNA levels of IL-1β between E_2-treated (0.9±0.28) and control rats (1.2±0.24) in the PVN (Fig.4-13).

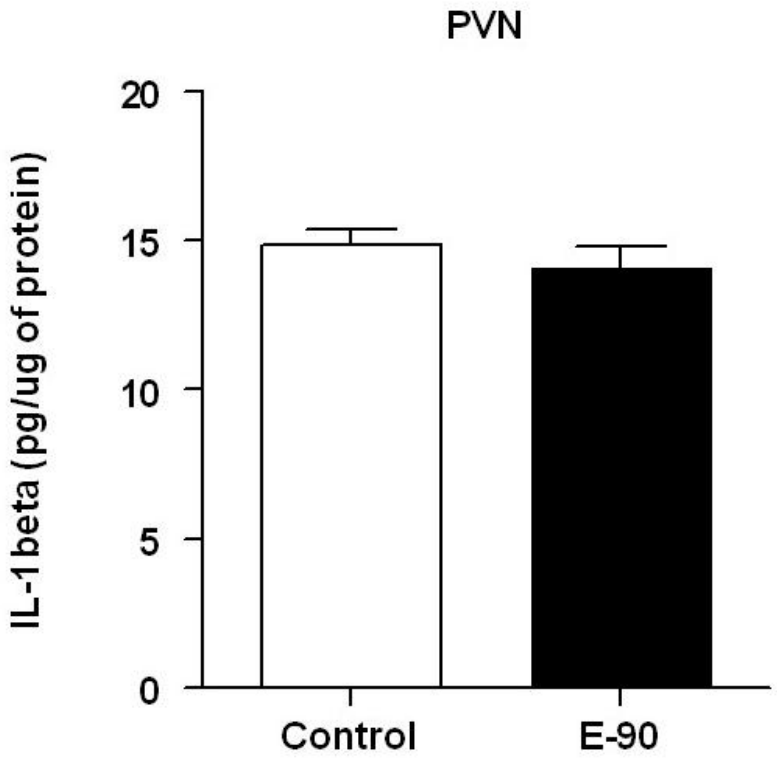


Fig. 4-12 Effect of chronic E_2 exposure on the IL-1β levels in the PVN

IL-1β levels from control and E_2-treated rats (n=5/group). Values are means; error bars represent SE.

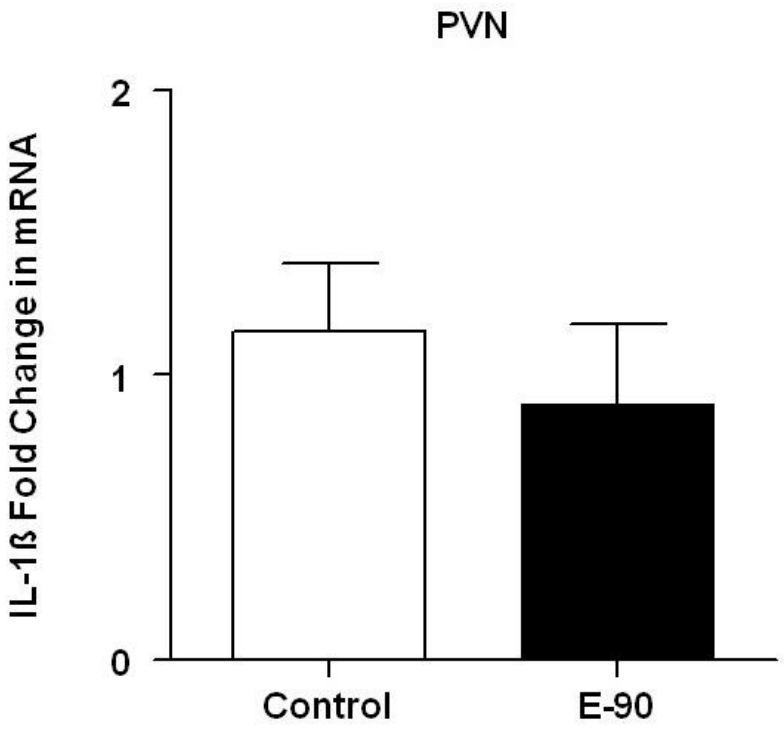


Fig. 4-13 Effect of chronic E_2 exposure on the gene expression of IL-1β in the PVN

The mRNA expression of IL-1β between the control and E_2-treated rats (n=5/group). Fold changes were normalized to β-actin control.

Chronic E_2 exposure increases food intake, water intake and body weight

The average weekly food intake (mean±SE, g/week), water intake (mean±SE, ml/week) and body weight (mean±SE, g) of control, E_2, resveratrol, resveratrol+E_2 animals are shown in Fig. 4-14 to 4-17. There was no difference in food intake between control and E_2-treated groups at the beginning of the experiment. However, food intake in the E_2 treated group appeared to increase on the 2^{nd} week (141.7±4.7), the 5^{th} week (132.1±5.3) and the 7^{th} week (127.6±3.8) compared to control rats (123.8±2.8, 117.7±2.3 and 117.6±2.5 respectively; $p<0.05$; Fig. 4-14A). The average food intake was significantly higher in E_2 -treated rats (134.6±1.7) compared to control rats (122±2.3) and those treated with resveratrol alone (123.1±1.3; $p<0.05$; Fig. 4-14B). Feeding chow containing resveratrol did not affect food intake in the control and E_2-treated groups.

The average weekly water intake in control and experimental animals are given in Fig. 4-15A. There was a tendency for increased water intake in E_2-treated rats compared to control rats in the first week and this trend continued throughout the period of observation, though the change was not statistically significant. However, the average water intake (Fig. 4-15B) was significantly higher in the E_2-treated group (316.5±5.6) compared to control rats (288.2±5.5), sham-implanted resveratrol treated group (279.6±2.9) and the resveratrol and E_2 treated group (257.5±5.8; $p<0.01$). The E_2-treated group fed chow containing resveratrol consumed less water (257.5±5.8) compared to the control group (288.2±5.5; $p<0.05$).

Changes in weekly body weight among the different groups are shown in Fig. 4-16A. There was no difference in body weight between the treatment groups at the beginning of the experiment. Although there was a tendency for body weight to increase with time, the average weekly body weight in the E_2 group was not statistically different from that of control rats. Feeding control and E_2 rats with chow containing resveratrol for 2 weeks did not produce any change in body weight. The average body weight over the entire period of observation (Fig. 4-16B) was significantly higher in the E_2 group (297.4±2.7) when compared to control rats (287.3±1.6; $p<0.01$). Feeding resveratrol did not alter the average body weight in control and E_2-treated rats. Body weight in E_2-treated rats fed chow containing resveratrol (299.6±0.4) was significantly higher compared to control ($p<0.05$).

Chronic E_2 exposure had no effect on heart weight and heart weight and body weight ratio

There heart weight or the ratio of heart weight to body weight was not different between the different treatment groups (Fig. 4-17).

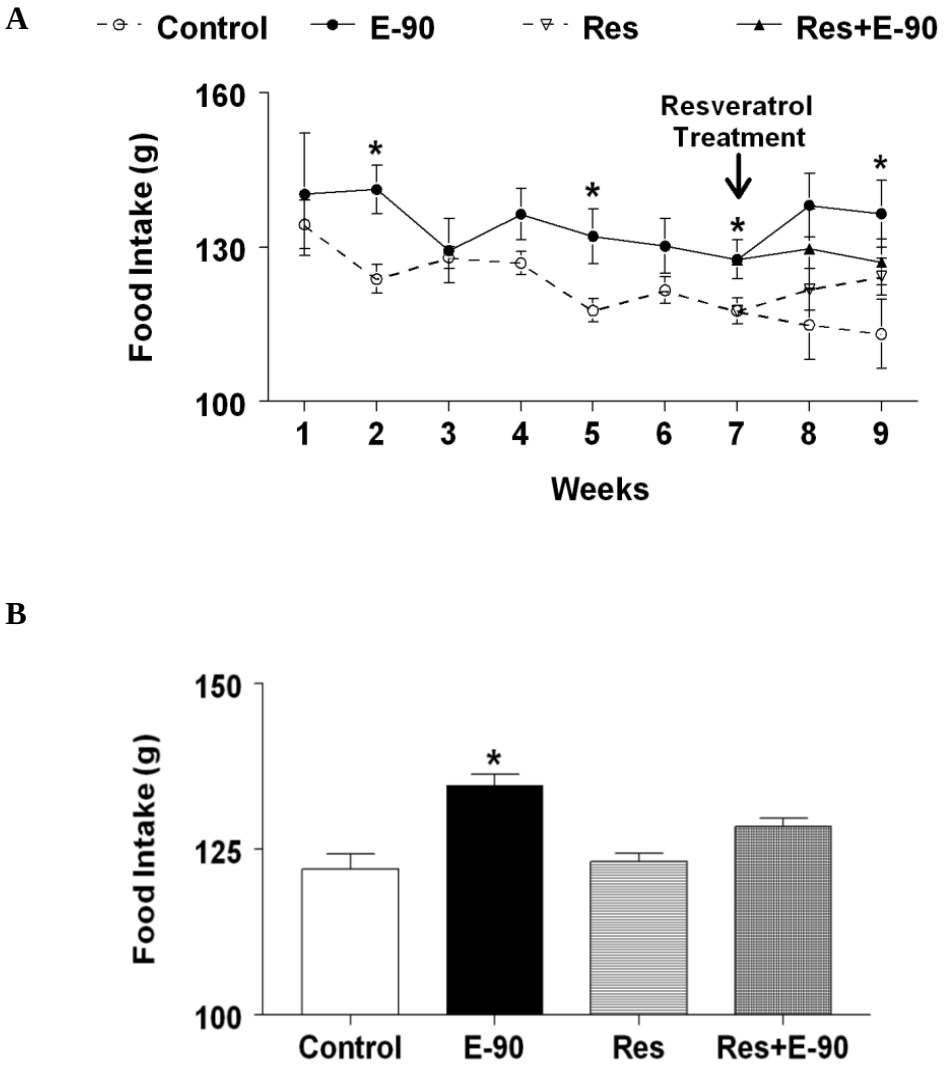


Fig. 4-14 Effect of chronic E_2 exposure and resveratrol on food intake

A: Line Graphs showing food intake (g) between different groups of female SD rats (n=6 per group): open circles shows control, closed circle shows E_2 pellet implanted (E-90), open inverted triangle shows resveratrol (Res) treatment alone, and closed triangle shows resveratrol treatment on E_2 implanted rats (Res+E-90). * indicates significant difference from control ($p<0.05$). B: Bar graph showing the average values of food intake shown in A. Fig B * denotes significant difference ($p<0.05$) from control and resveratrol treated groups.

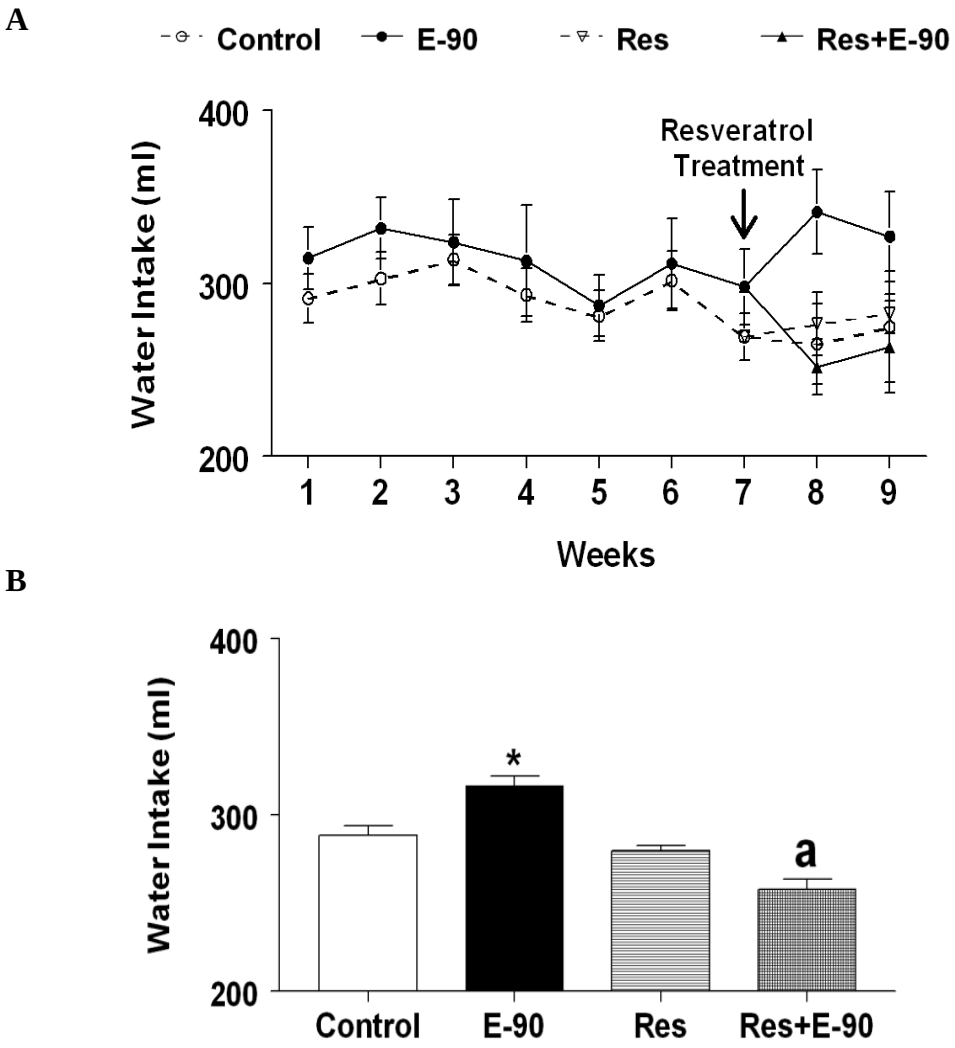
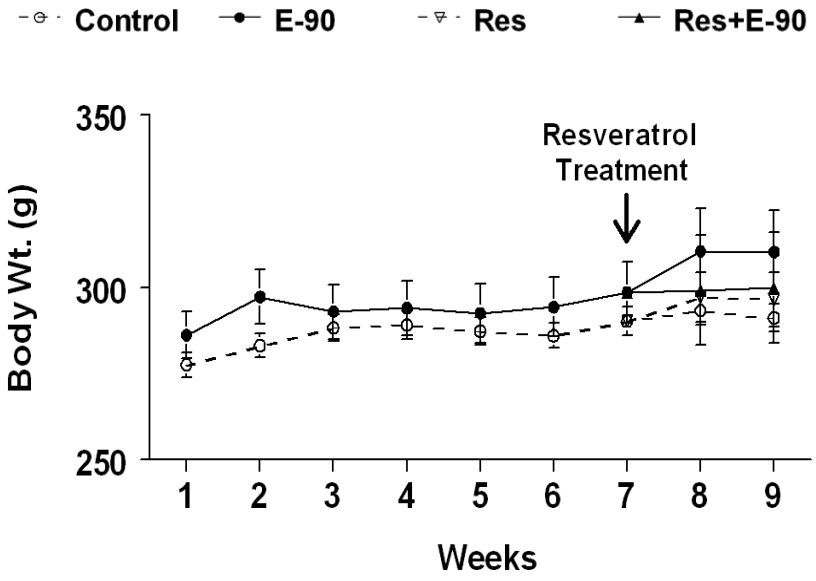


Fig. 4-15 Effect of chronic E_2 exposure and resveratrol on water intake

A: Line Graphs showing water intake (ml) between different groups of female SD rats (n=6 per group): open circles shows control, closed circle shows E_2 pellet implanted (E-90), open inverted triangle shows resveratrol (Res) treatment alone, and closed triangle shows resveratrol treatment on E_2 implanted rats (Res+E-90). B: Bar graph showing the average values of water intake shown in A. * denotes significant difference ($p<0.01$) from the rest of the groups. 'a' denotes significant difference from control ($p<0.05$).

A



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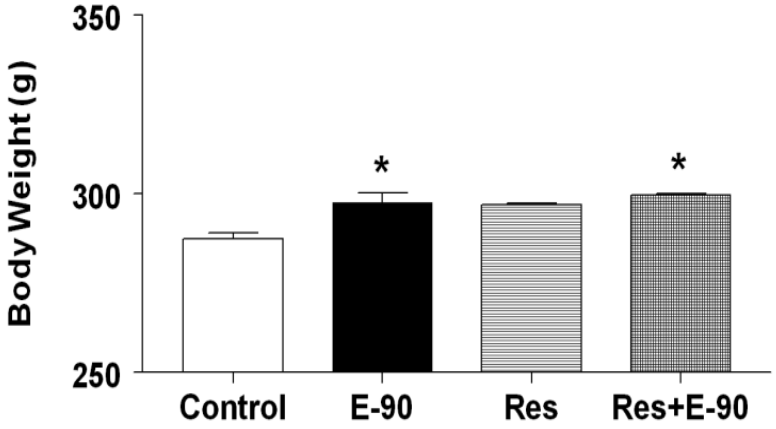


Fig. 4-16 Effect of chronic E_2 exposure and resveratrol on body weight

A: Line Graphs showing body weight (g) between different groups of female SD rats (n=6 per group): open circles shows control, closed circle shows E_2 pellet implanted (E-90), open inverted triangle shows resveratrol (Res) treatment alone, and closed triangle shows resveratrol treatment on E_2 implanted rats (Res+E-90). B: Bar graph showing the average values of body weight shown in A. * denotes significant difference ($p<0.05$) from control.

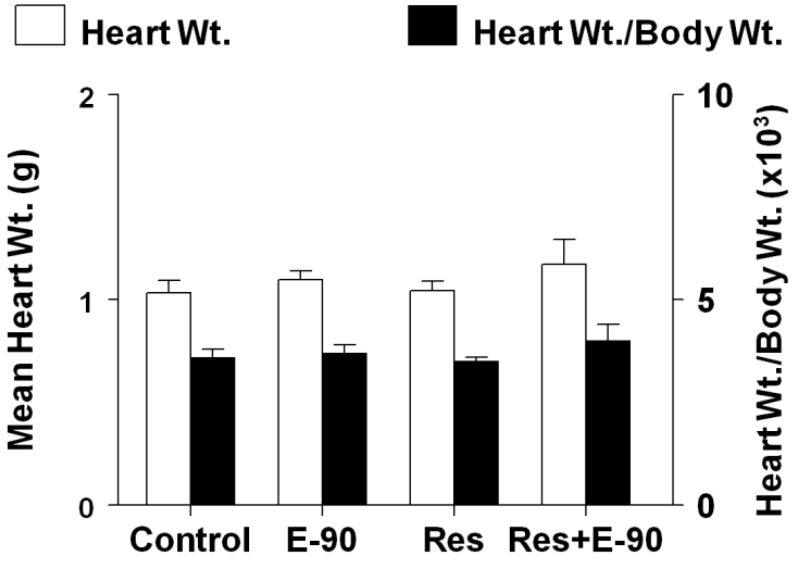


Fig. 4-17 Effect of chronic E_2 exposure and resveratrol on heart weight and heart weight to body weight ratio.

Bar graph showing the average values of heart weight (g) and heart: body weight ratio in the different treatment groups (n=6 per group).

Serum E_2

Serum E_2 levels (mean±SE, pg/ml) at the end of 90 days of E_2 exposure and resveratrol treatment are shown in Fig. 4-18. Exposure to E_2 resulted in significant increases in serum E_2 of animals that are treated with E_2 alone (68.2±4.07) or treated with E_2 and resveratrol (59.4±1.5) when compared to control rats (46.7±2.9) or rats treated with resveratrol alone (47.4±4.4, $p<0.001$). There was no difference in E_2 levels between the E_2 treated groups (with or without resveratrol).

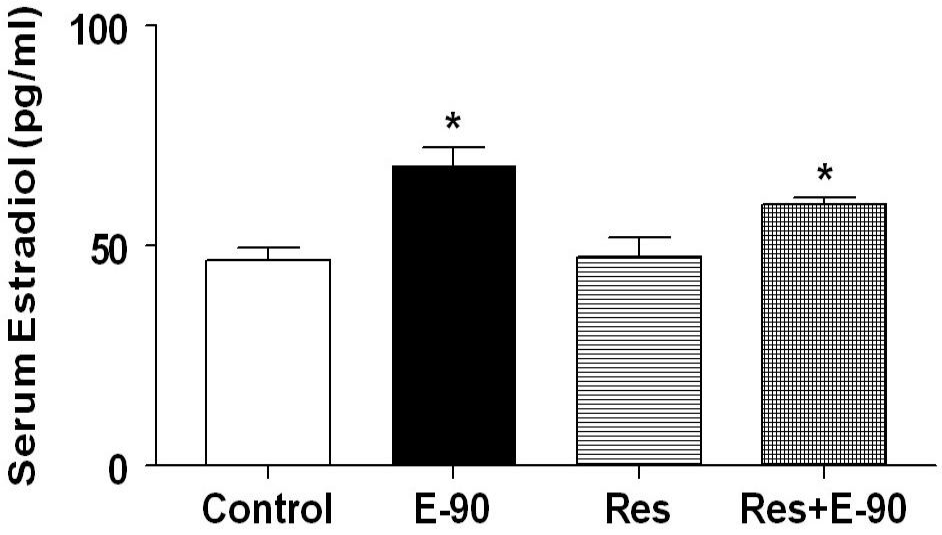


Fig. 4-18 Effect of chronic E_2 exposure on serum E_2 levels

Bar graphs showing serum E_2 levels in all the groups (n=5-7 per group). * denotes significant difference ($p<0.05$) from control and the group treated with resveratrol alone.

Resveratrol reverses chronic E_2-induced hypertension

The daily average profiles and the average mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) during the entire period of observation in control, E_2, resveratrol and resveratrol+ E_2-treated rats are shown in Fig. 4-19 to 4-22. Similar to what was observed in Fig.3-2 to 3-5, exposure to E_2 for 90 days significantly elevated MAP, HR, SBP and DBP compared to control rats. Feeding chow containing resveratrol to sham-implanted control rats did not alter any of the cardiovascular parameters. In contrast, feeding chow containing resveratrol to E-90 rats completely reversed E_2-induced increase in MAP, HR, SBP and DBP in E_2 implanted rats ($p<0.01$).

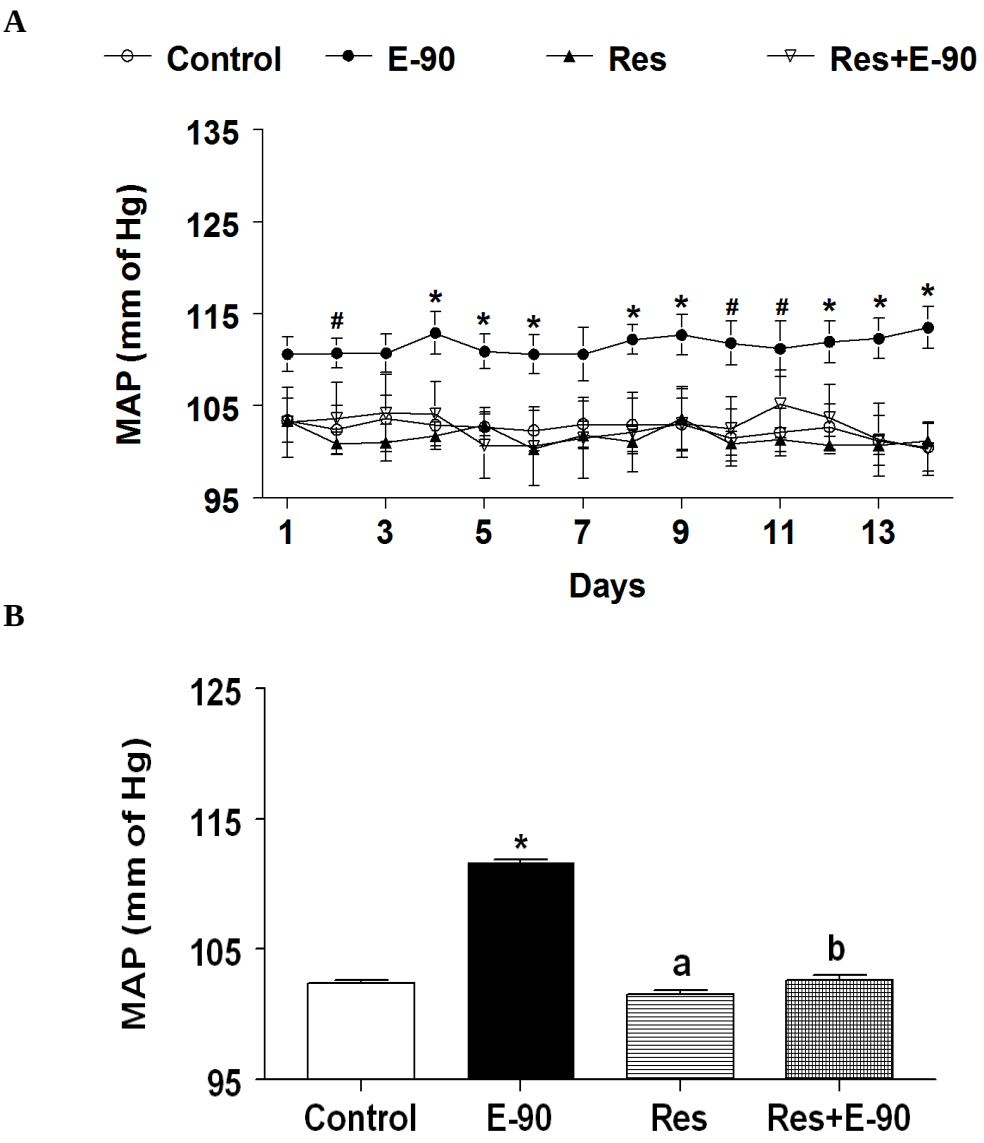


Fig. 4-19 Effect of resveratrol on chronic E_2-induced increase in MAP

A: Line graphs depicting MAP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control female SD rats, filled triangles represent rats fed with chow containing 0.84g/Kg resveratrol only and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats fed with chow containing 0.84g/Kg resveratrol. # denotes significant difference from control, * denotes significant difference from all the other groups. B: Bar graphs showing the average values of the BP parameters shown in A, * denotes significant difference ($p<0.05$) from all the other groups; 'a' represents significant difference from control and Res+E-90; 'b' represents significant difference from control.

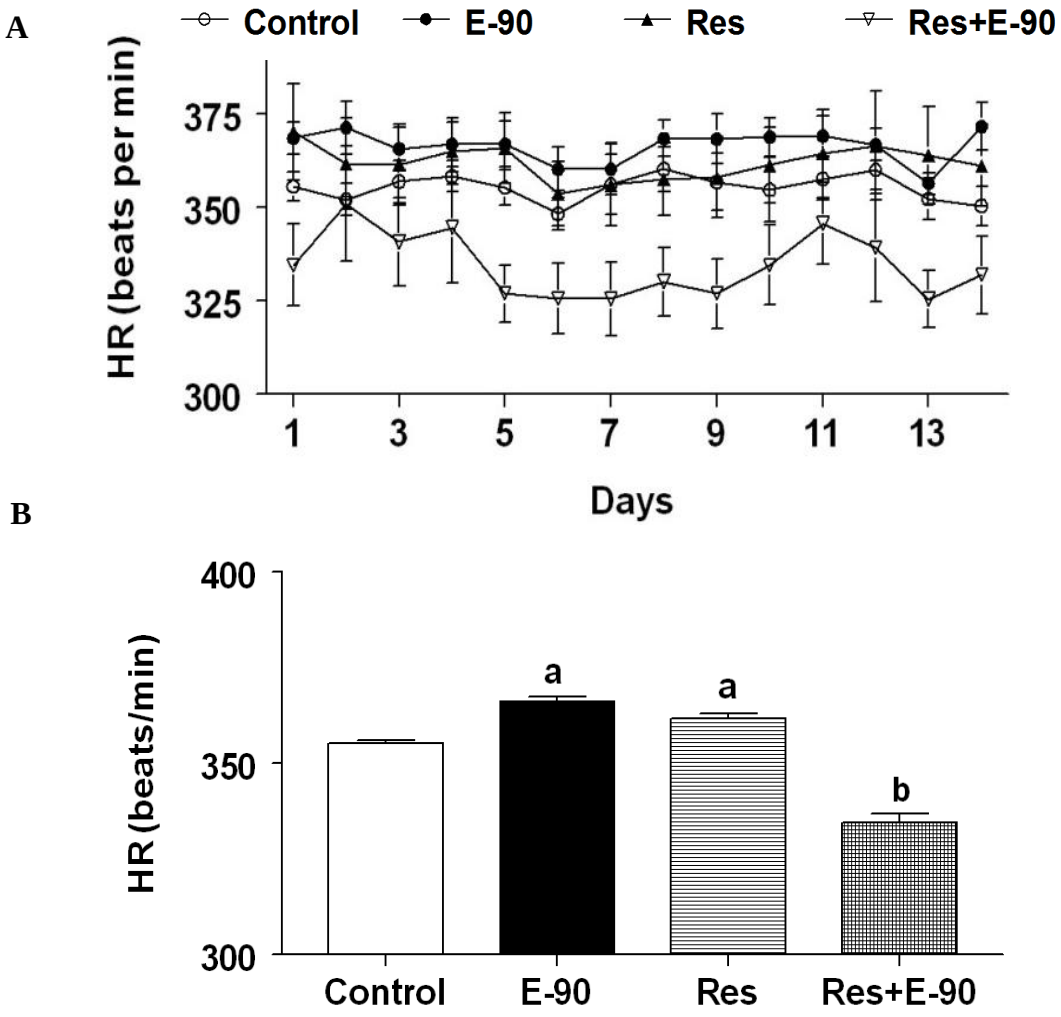


Fig. 4-20 Effect of resveratrol on chronic E_2-induced changes in HR

A: Line graphs depicting HR (beats/min): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control female SD rats, filled triangles represent rats fed with chow containing 0.84g/Kg resveratrol only and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats fed with chow containing 0.84g/Kg resveratrol. B: Bar graphs showing the average values of the BP parameters shown in A, 'a' represents significant difference from control and Res+E-90; 'b' represents significant difference from control.

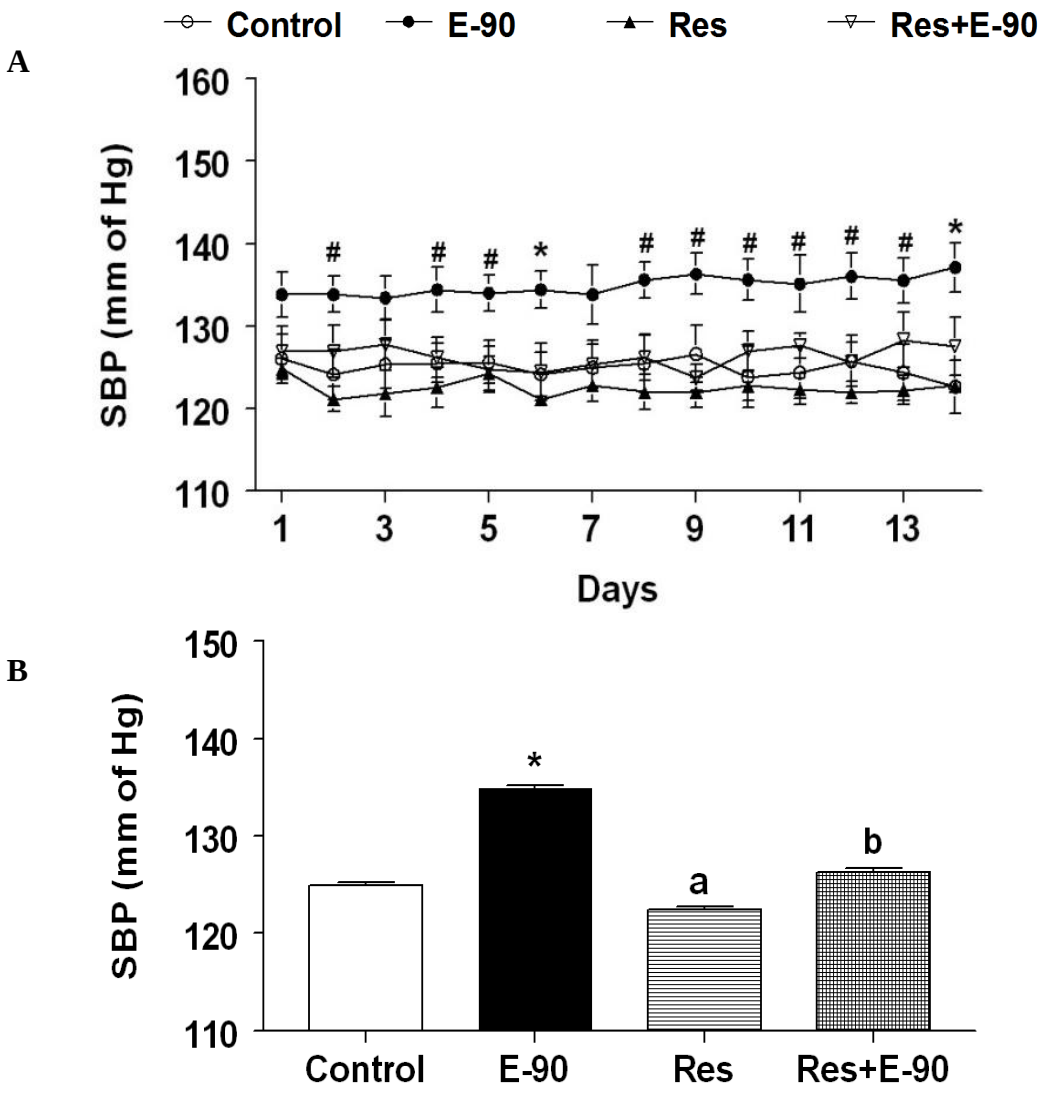


Fig. 4-21 Effect of resveratrol on chronic E_2-induced increase in SBP

A: Line graphs depicting SBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control female SD rats, filled triangles represent rats fed with chow containing 0.84g/Kg resveratrol only and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats fed with chow containing 0.84g/Kg resveratrol. # denotes significant difference from control, * denotes significant difference from all the other groups. B: Bar graphs showing the average values of the BP parameters shown in A, * denotes significant difference ($p<0.05$) from all the other groups; 'a' represents significant difference from control and Res+E-90; 'b' represents significant difference from control.

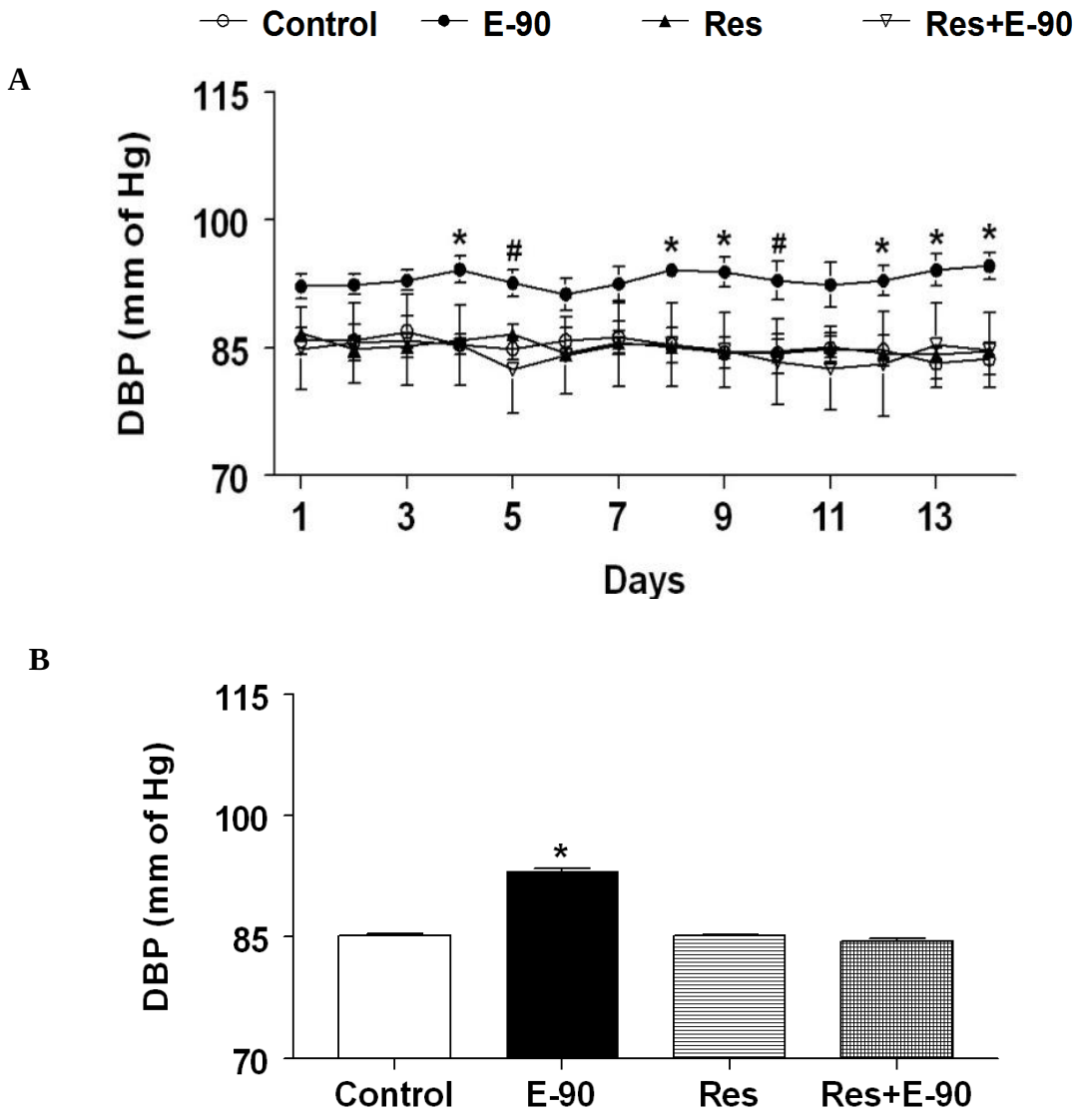


Fig. 4-22 Effect of resveratrol on chronic E_2-induced increase in DBP

A: Line graphs depicting DBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control female SD rats, filled triangles represent rats fed with chow containing 0.84g/Kg resveratrol only and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats fed with chow containing 0.84g/Kg resveratrol. # denotes significant difference from control, * denotes significant difference from all the other groups. B: Bar graphs showing the average values of the BP parameters shown in A, * denotes significant difference ($p<0.05$) from all the other groups.

Resveratrol attenuates oxidative stress in RVLM

Changes in O_2^- levels (mean±SE, nmol/min*mg) in the RVLM of control, E_2, resveratrol and resveratrol+E_2 groups are shown in Fig. 4-23. As seen in Fig. 4-2, O_2^- levels increased significantly in E-90 rats (0.61±0.05) compared to control rats (0.45±0.01; $p<0.01$). When sham-implanted control rats were fed chow containing resveratrol alone, it did not alter O_2^- levels (0.43±0.01) compared to control rats (0.45±0.01). In contrast, feeding E_2-treated rats with chow containing resveratrol completely reversed E_2-induced increase in O_2^- levels in the RVLM (0.43±0.01 vs. 0.61±0.05; $p<0.001$).

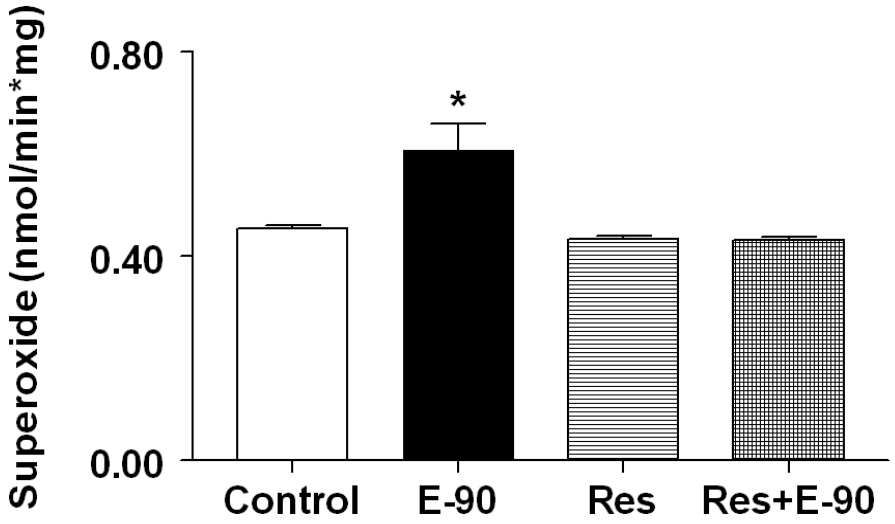


Fig. 4-23 Effect of resveratrol on chronic E_2-induced oxidative stress in RVLM

Bar graph showing O_2^- levels measured in RVLM punches from control, E_2 treated (20 ng/day, 90-day slow-release pellets), rats fed with chow containing 0.84g/kg resveratrol only and rats implanted with E_2 pellet (20ng/day, 90-day slow-release pellets) fed with chow containing 0.84g/kg resveratrol (n=4-6 per group). * denotes significant difference ($p<0.05$) from all the other groups**.**

5. Discussion

The results from the present study provide evidence that chronic exposure to low levels of E_2 for 3 months increases O_2^- levels in RVLM and results in hypertension. Further, findings from this study also provide evidence that E_2 exposure increased the gene expression of several subunits of NADPH oxidase, an enzyme that generates O_2^- and significantly decreased the gene expression of CuZnSOD, an enzyme that scavenges O_2^- in the RVLM. Further, the present study also provides mechanistic evidence for the involvement of O_2^- in the RVLM for estradiol-induced increase in BP. Treatment with an antioxidant, resveratrol, reversed E_2-induced increases in O_2^- levels in the RVLM and reversed the increase in BP indicating that chronic exposure to low levels of E_2 is capable of causing hypertension possibly by increasing O_2^- generation in the RVLM.

Several brain sites such as the PVN of the hypothalamus, the SFO, RVLM and NTS are known to be involved in the regulation of BP [149]. We chose to focus specifically on the PVN and RVLM, because they are known to be involved in the maintenance of vasomotor tone via regulation of SNA [150]. Moreover, the neurons in the PVN and RVLM express estrogen receptors [151-154] indicating that these are potential sites of E_2 action. Also, stimulation of the PVN and RVLM are known to activate sympathetic preganglionic neurons in the IML cell column of the spinal cord and increase SNA resulting in hypertension [15]. E_2-induced increase in O_2^- levels in the RVLM observed in the present study may act through similar mechanisms. Other studies have shown an association between O_2^- in the RVLM and hypertension in

multiple animal models [39, 40, 80, 144]. Moreover, reversal of hypertension by increasing the expression of SOD using gene transfer and adenoviral vectors or decreasing O_2^- in the RVLM substantiates the role of oxidative stress in the pathogenesis of hypertension [155-158]. Recently, data from our lab showed an increase in glial fibrillary acidic protein (GFAP) and IL-1β levels in the arcuate nucleus on chronic E_2 exposure in female SD rats [159]. Hence, it is highly possible that glial cells mediate the oxidative stress related effects of chronic E_2 exposure in the RVLM. The presence of estrogen receptors in the glial cells [160, 161] further supports this notion. However further studies are needed to explore this possibility.

In order to identify the source of O_2^- produced in the RVLM, we measured the gene expression levels of the NADPH oxidase subunits, which is the major source of ROS in the cardiovascular system [162]. In the present study, we observed significant increase in the gene expression of NADPH oxidase subunits NOX1 and 2 in the RVLM. In concordance with our results, NADPH oxidase-mediated increase in O_2^- levels have been reported in Ang II hypertension model where inhibition of NADPH oxidase attenuated Ang II induced O_2^- production in the RVLM [39]. In addition to increase in NOX subunits, we also observed a decrease in the expression of CuZnSOD levels in the RVLM, which is an enzyme that scavenges O_2^- and functions as an important anti-oxidant mechanism in the brain. It is possible that an imbalance between pro-oxidant and anti-oxidant mechanisms in the RVLM might mediate chronic estradiol-induced increase in O_2^- production. However, additional mechanistic studies are needed to further prove the role of NADPH oxidase and CuZnSOD in chronic estrogen induced oxidative stress in RVLM.

Several studies suggest that pro-inflammatory cytokines and NO from iNOS could stimulate O_2^- production in the PVN and RVLM. Hence, we wanted to investigate whether chronic estradiol-induced O_2^- production is associated with increases in IL-1β and NO levels in these regions. Interestingly, in the present study, chronic estradiol-induced increase in O_2^- levels were not associated with increases in both the protein and mRNA levels of IL-1β and iNOS in the RVLM or in the PVN. It is possible that E_2 induced oxidative stress in the RVLM is independent of pro-inflammatory changes.

In contrast to our findings, studies have portrayed E_2 in both ways: pro-inflammatory as well as an anti-inflammatory agent depending on the dose, duration and site of action. Calippe et al demonstrated increase in IL-1β, IL-6 and iNOS expression in the peritoneal macrophages of ovariectomized female mice after chronic E_2 exposure (20ug/day for 4 weeks) [163]. Conversely, studies have also portrayed E_2 as a neuroprotective agent where it acts on the glial cells to reduce inflammation [164]. Interestingly those studies that suggest the anti-inflammatory effects of E_2 are mostly *in vitro* and acute studies [165, 166]. These divergent effects of estrogen were clearly demonstrated by Calippe et al where short-term *in vitro* exposure of E_2 reduced cytokine release from mouse peritoneal macrophages and chronic E_2 treatment in mice increased the expression of cytokines and iNOS by resident peritoneal macrophages in response to LPS *ex vivo* [163].

Apart from cytokines and iNOS, ET-1 and Ang II have also been shown to increase O_2^- production both centrally and peripherally. Centrally administered Ang II increased ROS

production in the RVLM [167] and other brain regions regulating BP [168] and inhibition of NADPH oxidase attenuated Ang II induced O_2^- production in the RVLM [39]. In addition, ET-1 has also been implicated in the generation of O_2^- through NADPH oxidase peripherally [169-171]. It is possible that E_2-induced O_2^- production is mediated through ET-1 or Ang II. These possibilities are explored in the future studies.

Some of the other mechanisms proposed for oral contraceptive-induced hypertension include impaired renal handling of water resulting in volume-dependent hypertension [172] and hyperactivity of the renin-angiotensin system [138, 139]. Increased water intake as observed in the present study could be another contributing factor to volume expansion. However, it is not clear if this is associated with the increased food intake and body weight observed in E_2-treated animals. Interestingly, there were no measureable increases in heart weight, or the ratio of heart weight to body weight, in E_2 treated rats. This is perhaps due to the rather mild increase in BP caused by E_2, and to the known protective effect of E_2 against pressure-induced cardiac hypertrophy [173].

Our results clearly indicated that E_2 treatment increases O_2^- production in the RVLM, we wanted to explore the possibility of reversing this effect using the antioxidant, resveratrol. Resveratrol is a red wine polyphenol which is known to possess antioxidant and O_2^- scavenging properties [174] and exerts strong antioxidant effects in the brain [175]. In the current study, treatment with resveratrol reduced MAP, HR, SBP and DBP induced by E_2 treatment with complete quenching of O_2^- in the RVLM. Resveratrol has been shown to reduce

SBP in obese Zucker rats by increasing eNOS expression in the aorta [176]. Resveratrol treatment also prevented hypertension caused by high fat diet in female rats [177]. In the present study, treatment of E_2-treated rats with resveratrol reversed E_2-induced increase in O_2^{-} in the RVLM and also decreased BP raising the possibility that E_2's effects on BP could be mediated through increased production of $O_2^{-\cdot}$.

In conclusion, our results suggest that the hypertensive effect of E_2 may be related to increased O_2^{-} production in the RVLM. Although, this study examines the effect of E_2 on O_2^{-} generation in the RVLM, the involvement of other brain sites and peripheral tissues such as the vasculature and kidney should also be considered in the future.

CHAPTER 5

CHRONIC ESTRADIOL-17β INCREASES ENDOTHELIN-1 EXPRESSION IN THE RVLM AND PVN

1. Introduction

The PVN of the hypothalamus and RVLM are two important regions that are involved in central cardiovascular regulation [15, 40, 178, 179]. Recently, we have demonstrated that chronic low levels of estradiol exposure increases arterial pressure in adult young female SD rats and this hypertensive effect of E_2 was associated with increase in O_2^- production in the RVLM and these changes could be reversed using an antioxidant, resveratrol [180] (Chapter 3) . Several studies have provided evidence that central RAS and endothelin system play a role in the development of neurogenic hypertension directly or indirectly through oxidative stress related mechanisms [181-184].

Ang II is a high molecular weight (1046.18) circulating peptide that is well known for its vasoconstrictive properties [185]. The actions of Ang II on the central nervous system (CNS) were questioned for a longer period of time, since it does not cross the blood brain barrier. Later it was found that there is a separate RAS [46] present within the CNS [73]. Ang II receptor, type I or AT1 is distributed in important centers of the brain and brainstem that are involved in cardiovascular regulation such as the PVN, RVLM and NTS [70-72]. Studies in SHR have shown that there is increased Ang II activity in the brain areas that are involved in BP regulation [186]. ICV and microinfusion studies using Ang II also confirmed its central role in increasing BP [187, 188]. The hypertensive effect of Ang II is believed to be mediated by AT1 receptors as blocking these receptors in hypertensive rats reversed the increase in BP confirming its role in mediating BP [76, 77]. Studies have also demonstrated that increase in ROS in the RVLM and PVN as an important contributor for Ang II-mediated increase in BP, and blocking O_2^- production reverses the increase in arterial pressure seen in Ang II hypertensive rats [38, 80, 81]. Thus, several studies have provided evidence that central RAS plays a role in the development of

hypertension. Similarly another peptide that is known to play an important role in the development of hypertension is ET-1.

ET-1 is a vasoconstrictor peptide and is known to contribute to the pathogenesis of hypertension in several models of hypertension including the deoxycorticosterone acetate (DOCA)-salt and salt-sensitive hypertension [189]. ET-1 was first identified in the endothelial cells of the vasculature [190]. Later the distribution of ET-1 system including the protein and mRNA of ET-1, its receptors ET-1A, ET-1B, and endothelin converting enzyme (ECE) have been identified in the regions of the brain [84] and the central ET-1 system was implicated in cardiovascular regulation [84, 191]. Central administration of ET-1 (both ICV and directly into RVLM) increased mean arterial pressure and SNA in several models of hypertension like SHR, spontaneously hypertensive stroke prone rat (SHR-SP) and DOCA-salt hypertensive rats and blockade of ET-1A but not ET-1B receptors reversed ET-1 induced increases in BP [85-87]. Although, peripherally oxidative stress has been demonstrated as an important mediator in ET-1 induced hypertension, the mechanisms behind ET-1's central actions remains unclear.

The present study will test the hypothesis that oxidative stress associated chronic estradiol-induced hypertension involves central activation of RAS or ET-1 system in the RVLM and PVN (Fig 5-1).

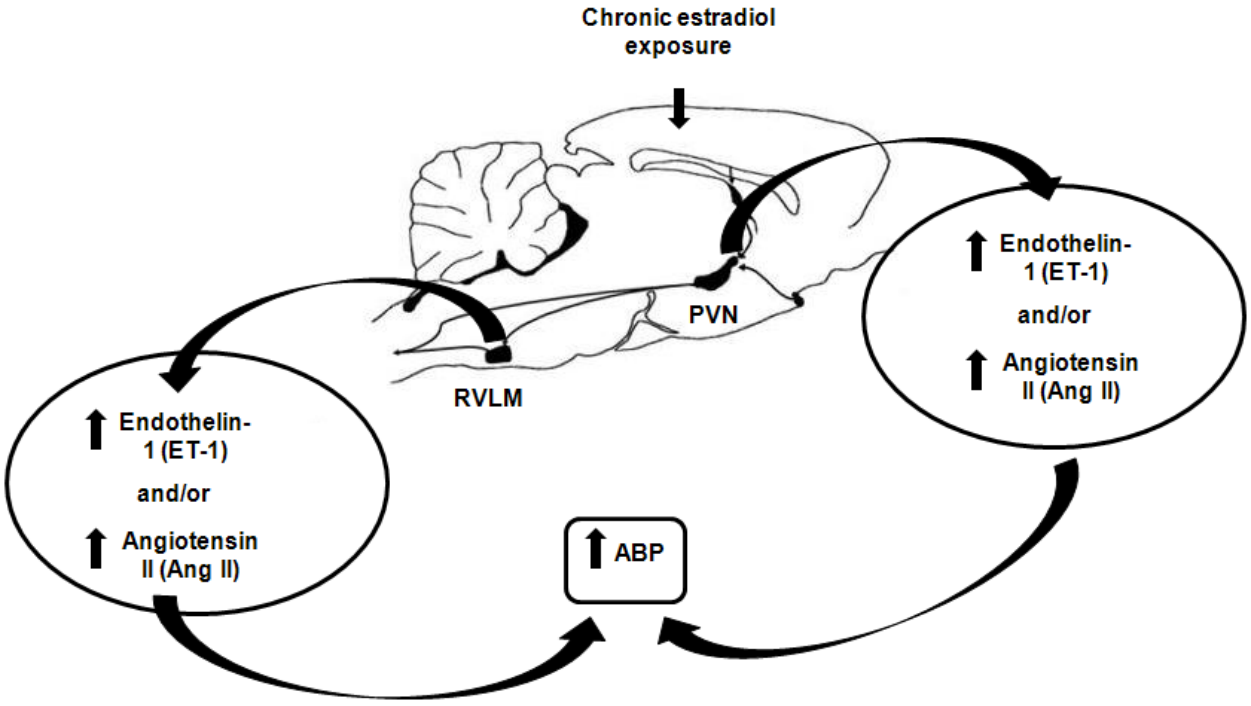


Fig. 5-1 Hypothesis 3 – Chronic exposure to low levels of estrogen increases arterial blood pressure (ABP) by increasing ET-1 and/or Ang II with in rostral ventrolateral medulla (RVLM) and paraventricular nucleus (PVN).

2. Experimental Design

In experiment 1, the role of ET-1and Ang II in chronic estradiol-induced hypertension was assessed. The animals were divided into 2 groups (n=6/group); sham-implanted (control); or implanted with E_2 (20 ng/day, 90-day slow-release pellet; Innovative Research America, Sarasota, FL). Animals were 3-4 months old at the beginning of the experiment. After 90 days of exposure to E_2, all the animals were sacrificed at noon on the day of estrous. Most of the E_2-treated animals were in persistent estrous after 90 days treatment. The control animals were sacrificed on the day of estrous after 90 days of sham implantation for comparison to the treatment group. The brains were removed, frozen on dry ice and stored at -70°C. The trunk blood was collected and serum was separated and stored at -70°C until processed.

The brains collected from the above experiment were sectioned (300µm thick) and RVLM and PVN were microdissected using Palkovit's microdissection technique. The protein levels of ET-1 receptor (ET-1A) were analyzed using western blot. Further the gene expression of ET-1 and its receptors and AT1 receptor were measured in both the regions using quantitative RT-PCR. In order to get sufficient tissues for the above experiment, a separate batch of animals were reared using the same experimental design.

In experiment 2, the role of ET-1A receptor in mediating chronic estradiol-induced hypertension was assessed. The animals were initially divided in to two groups (n=7-8/group)

Group 1: sham-implanted (control)

Group 2: implanted with E_2 slow-release pellets (E-90; 20 ng/day for 90 days; [120])

Animals were implanted with telemeters one week before the beginning of the experiment to measure cardiovascular parameters. After 5 days of control measurement the animals were

implanted with E_2 pellets. After 75 days of E_2 treatment the animals divided in to four groups (n=3-4/group):

Group 1: control + artificial cerebrospinal fluid (aCSF)

Group 2: E_2-treated (E-90) + aCSF

Group 3: control + BQ-123 (400 pmol/hr) and

Group 4: E_2-treated + BQ-123 (400 pmol/hr)

All the rats were implanted with intracerebroventricular (ICV) cannulae for continous infusion (0.5 µl/hr) of the ET-1A receptor antagonist, BQ-123 or artificial cerebrospinal fluid (aCSF) by Alzet Osmotic minipump for 2 weeks. The doses used in this study are from recent studies by Rossi et al. 2011 [192]. After 2 weeks i.e. at the end of 90 days of E_2 implantation, the rats were sacrificed while in the state of estrous.

3. Results

Chronic E_2 exposure increased the mRNA expression of ET-1 levels in the RVLM

The level of ET-1 gene expression in the RVLM of E_2-treated rats (2.25±0.29) was significantly higher compared to the control (1.12±0.27, $p<0.05$) (Fig. 5-2).

Chronic E_2 exposure increased the mRNA expression of ET-1 in the PVN

Similar to RVLM, also in the PVN the gene expression of ET-1 is upregulated in the E_2-treated rats (2.29±0.37) significantly compared to the control (0.88±0.28, $p<0.05$) (Fig. 5-3).

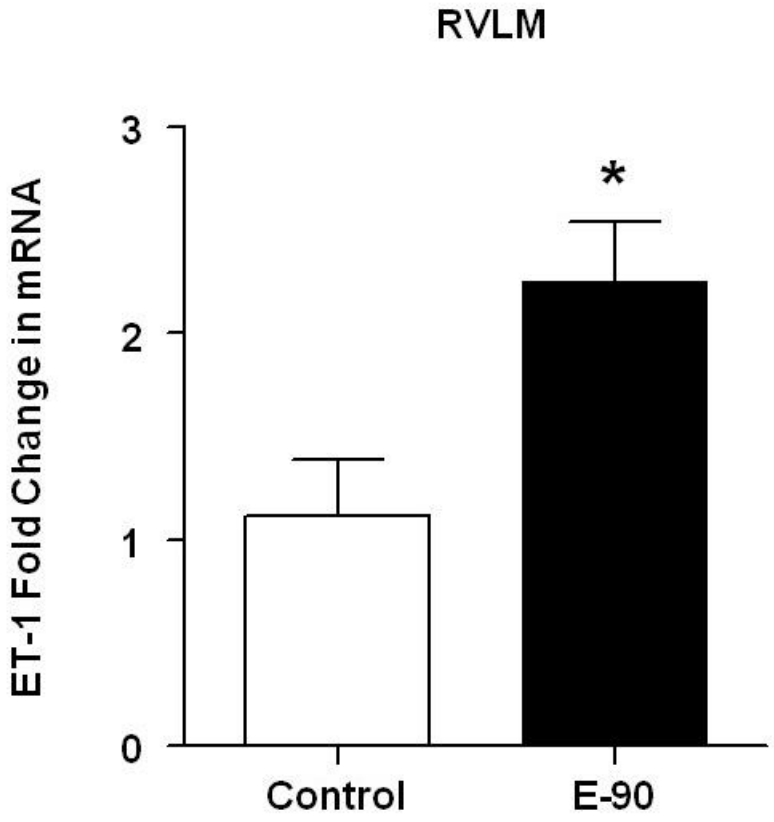


Fig. 5-2 Effect of chronic E_2 exposure on the gene expression of ET-1 in the RVLM

The mRNA expression levels of ET-1 in the control and E_2 treated rats. The fold change was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$. The C_t values of all the groups were normalized to control rats (n=4 per group). * denotes significant difference ($p<0.05$) from control group.

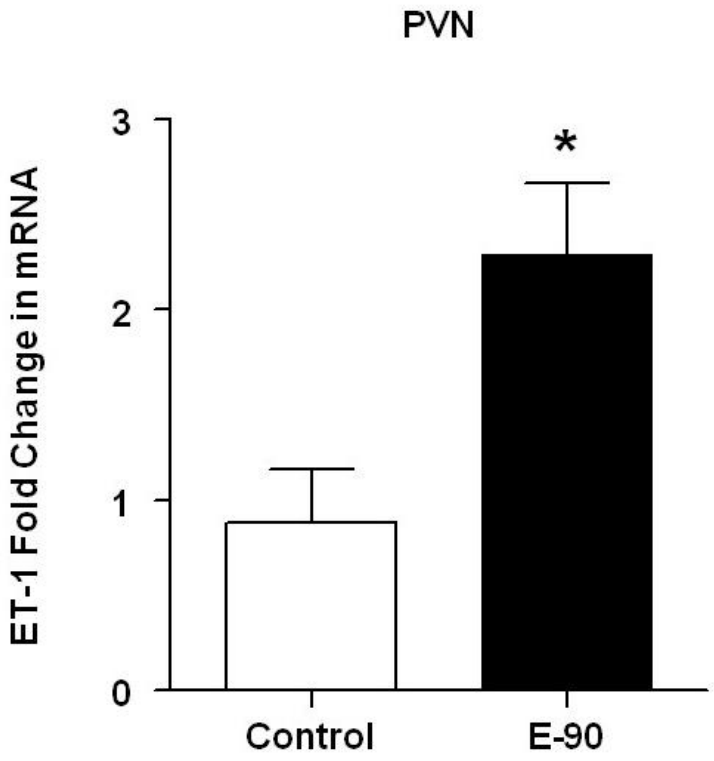


Fig. 5-3 Effect of chronic E_2 exposure on the gene expression of ET-1 in the PVN

The mRNA expression of ET-1 in the control and E_2 treated rats. The fold change was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$. The C_t values of all the groups were normalized to control rats (n=4 per group). * denotes significant difference ($p<0.05$) from control group.

Chronic E_2 exposure increased the mRNA expression of ET-1A in the RVLM

ET-1A receptor gene expression was upregulated in the E_2-treated (4.43±1.2) compared to the control rats (1.03±0.12) (Fig. 5-4).

Chronic E_2 exposure increased the mRNA expression of ET-1A in the PVN

The gene expression of ET-1A receptor was upregulated in the PVN of E_2-treated (1.92±0.21) compared to the control rats (1.08±0.21, $p<0.05$) (Fig. 5-5).

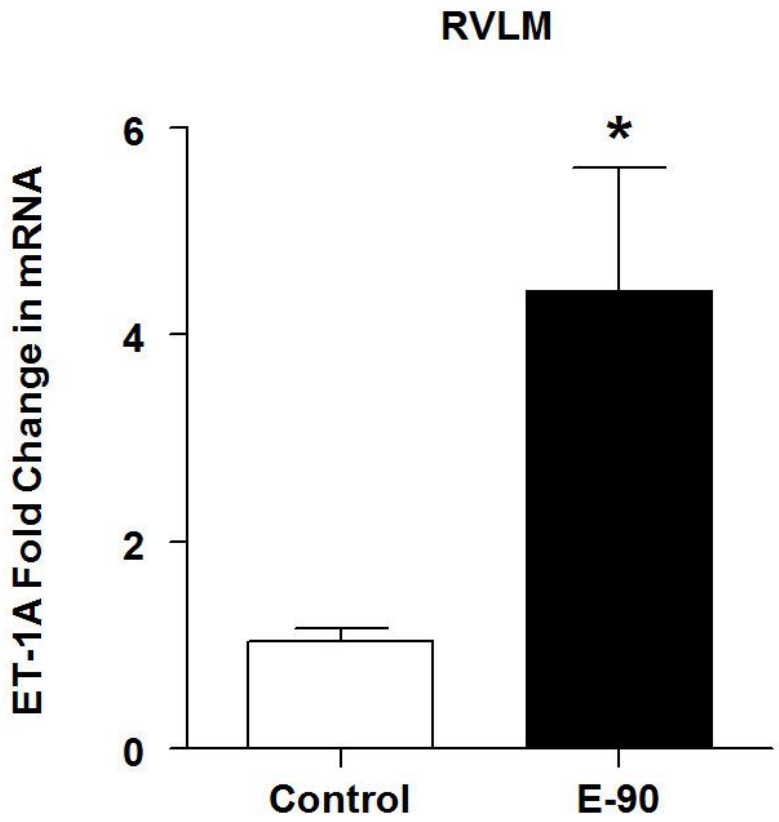


Fig. 5-4 Effect of chronic E_2 exposure on the gene expression of ET-1A in the RVLM

The mRNA expression levels of ET-1A between the control and E_2-treated rats (n=5/group). The fold change for each mRNA was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$. * denotes significant difference ($p<0.05$) from control group.

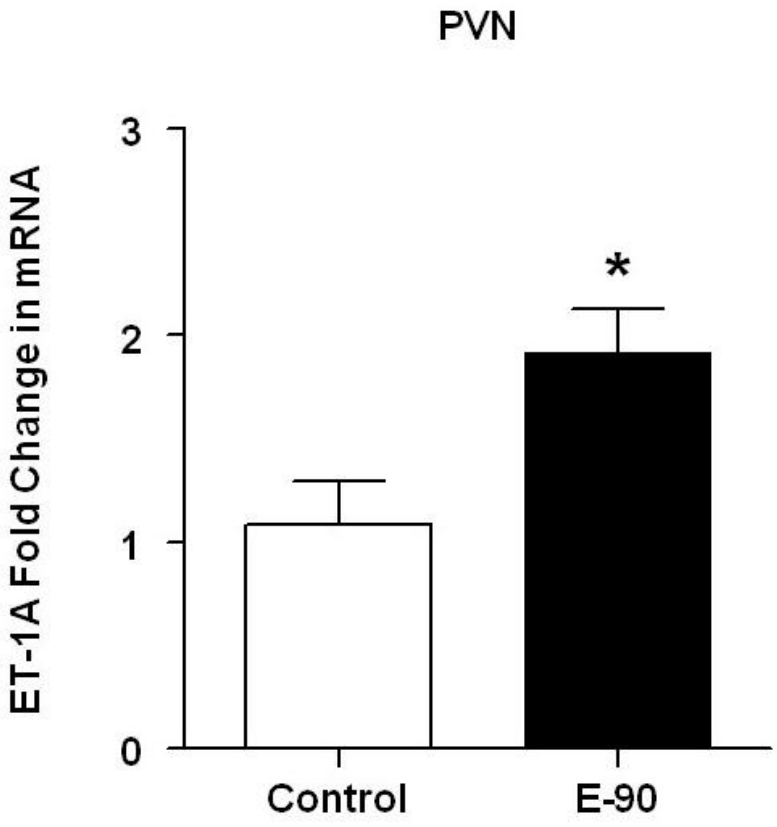


Fig. 5-5 Effect of chronic E_2 exposure on the gene expression of ET-1A in the PVN

The mRNA expression levels of ET-1A between the control and E_2-treated rats. The fold change in the gene expression was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$.

Chronic exposure to E_2 had no effect in the mRNA levels of ET-1B in RVLM

There was no difference in the level of ET-1B gene expression between the control (1.06±0.21) and E_2-treated rats (0.6±0.1) (Fig. 5-6).

Chronic exposure to E_2 had no effect in mRNA levels of ET-1B in PVN

The gene expression of ET-1B in the E_2-treated group (0.78±0.24) was not significantly different compared to the control (0.72±0.16) (Fig. 5-7).

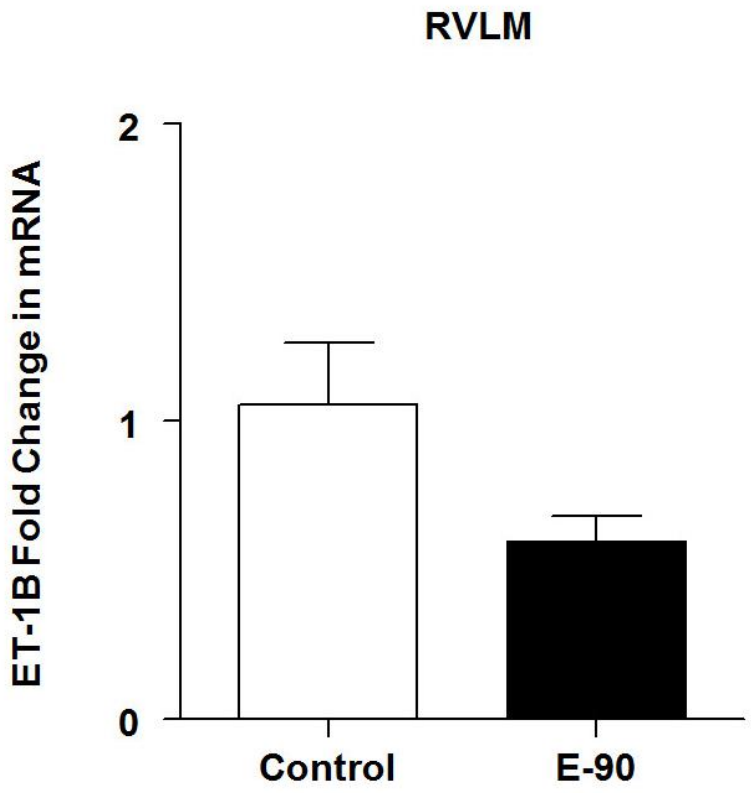


Fig. 5-6 Effect of chronic E_2 exposure on the gene expression of ET-1B in the RVLM

The mRNA expression levels of ET-1B between the control and E_2-treated rats (n=5/group). The fold change for each mRNA was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$.

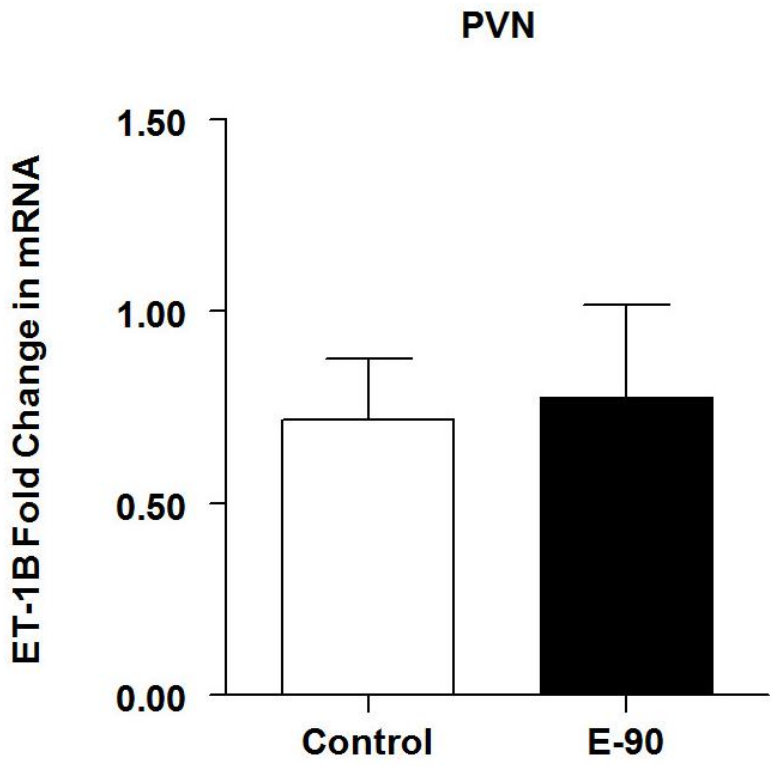


Fig. 5-7 Effect of chronic E_2 exposure on the gene expression of ET-1B in the PVN

The mRNA expression levels of ET-1B between the control and E_2-treated rats (n=5/group). The fold change for each mRNA was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$.

Chronic exposure to E_2 had no effect in the gene expression of Ang II type I receptor (AT1) in RVLM

There was no difference in the level of AT1 mRNA levels between the control (0.59±0.26) and E_2-treated rats (0.92±0.16) (Fig. 5-8).

Chronic exposure to E_2 had no effect in gene expression of AT1 in PVN

The mRNA levels of AT1 in the E_2-treated group (1.54±0.38) were not significantly different compared to the control (1.18±0.28) (Fig. 5-9).

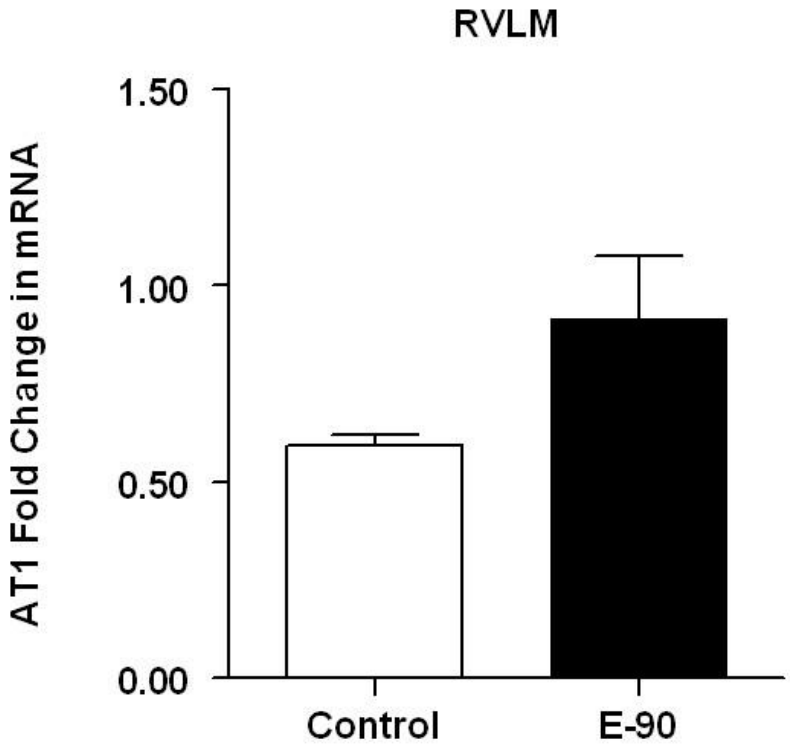


Fig. 5-8 Effect of chronic E_2 exposure on the gene expression of AT1 in the RVLM

The mRNA expression levels of AT1 between the control and E_2-treated rats (n=4/group). The fold change for each mRNA was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$.

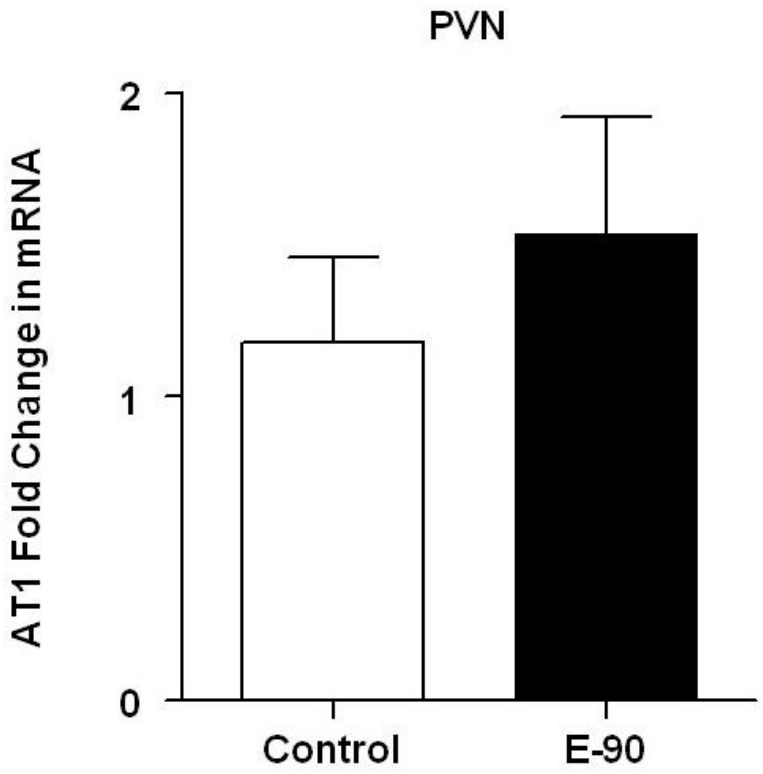


Fig. 5-9 Effect of chronic E_2 exposure on the gene expression of AT1 in the PVN

The mRNA expression levels of AT1 between the control and E_2-treated rats (n=5/group). The fold change for each mRNA was calculated relative to β-actin by the comparative C_t method using $2^{-\Delta\Delta Ct}$.

Chronic E2 exposure increased the ET-1A receptor protein levels in the PVN

To quantify the changes in the ET-1A expression, protein was isolated from the PVN after 90 days E_2 treatment and analyzed using western blot. There was a significantly higher protein expression of ET-1A receptor in the PVN of E_2-treated rats compared with control rats (Fig. 5-10). To ensure that equal amount of protein was added in all the wells, β-tubulin was used and all the wells are compared. No difference was observed in β-tubulin expression between the different treatment groups.

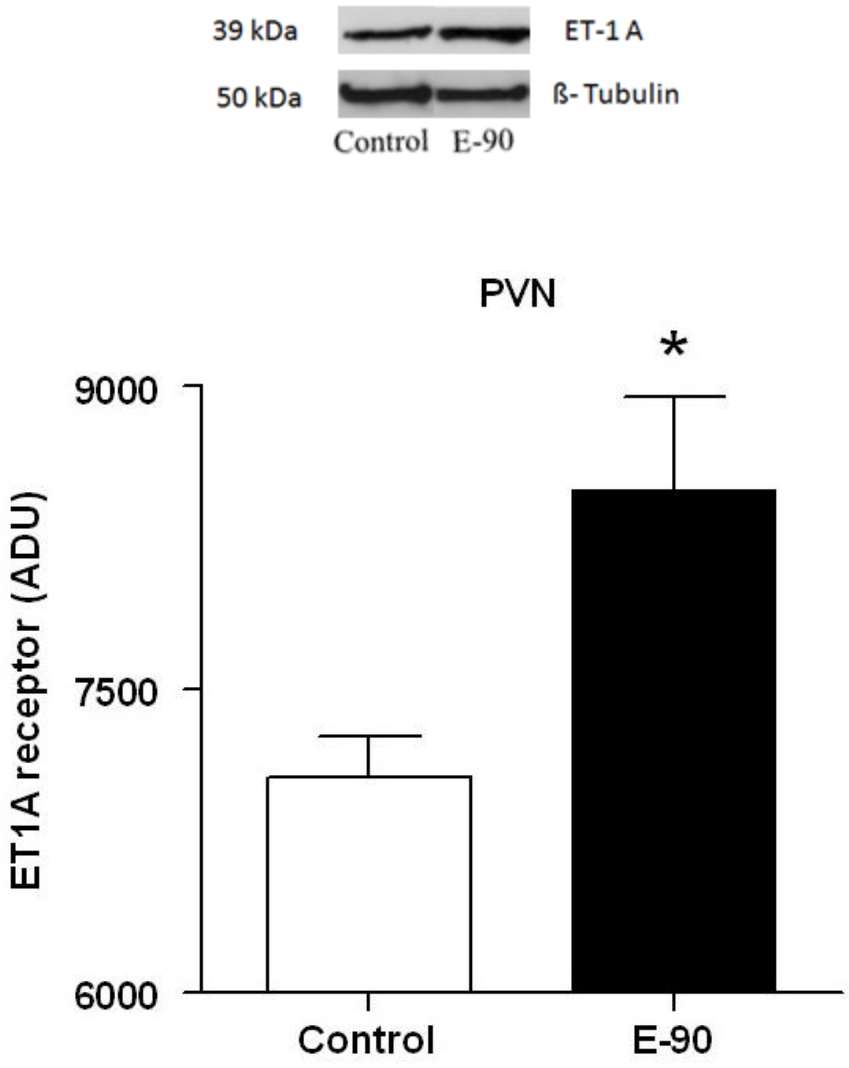


Fig 5-10 Effect of chronic E_2 exposure on the protein levels of ET-1A in the PVN

Sample blots and densitometry results from western blot analysis of ET-1A in the PVN of control and E_2-treated rats. Bar graphs are the mean±SEM for 4-5 animals. * indicates significant difference from control animals.

Chronic exposure to estradiol-17β causes hypertension

The daily average profiles and the average mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) starting from the beginning of treatment in control and E_2-treated rats are shown in Fig. 5-11 to 5-14. The MAP in control animals remained steady over the entire period of observation. In contrast, E_2 exposure increased MAP significantly ($p<0.05$; Fig. 5-11A) starting around three weeks of treatment. The average MAP (mean±SE, mmHg) measured during the 1^{st}-11^{th} week of observation in control rats was 99±0.1. In contrast, E_2 exposure increased MAP significantly to 104±0.1 ($p<0.05$; Fig. 5-11B). The HR profile of E_2-treated rats had a tendency to increase around 11^{th} week compared to control rats (Fig. 5-12A). The average HR (mean±SE, beats/min) during the 1^{st}-11^{th} week of treatment in E_2-treated rats (375.2±0.4) was significantly elevated compared to control rats (372.7±0.5; $p<0.05$; Fig. 5-12B). Similarly, the SBP and DBP profiles in E_2-treated were significantly elevated in E_2-treated rats compared to control rats ($p<0.05$; Figs. 5-13A and 5-14A). E_2 exposure also increased the average SBP and DBP (mean±SE, mmHg; 124±0.2 and 87.4±0.1 respectively) significantly compared to control rats (119±0.2 and 82.8±0.1 respectively; $p<0.05$; Figs. 5-13B and 5-14B).

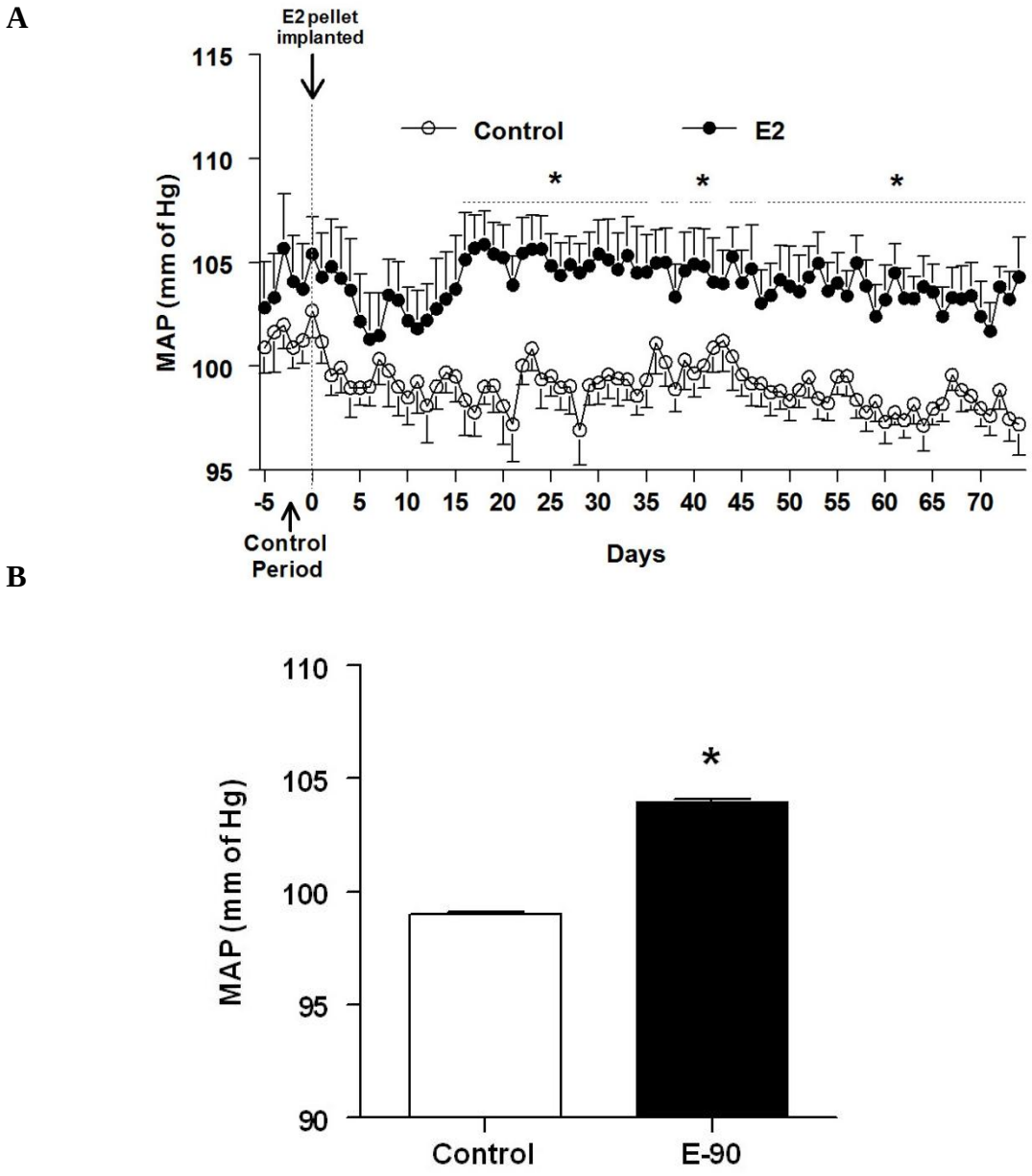
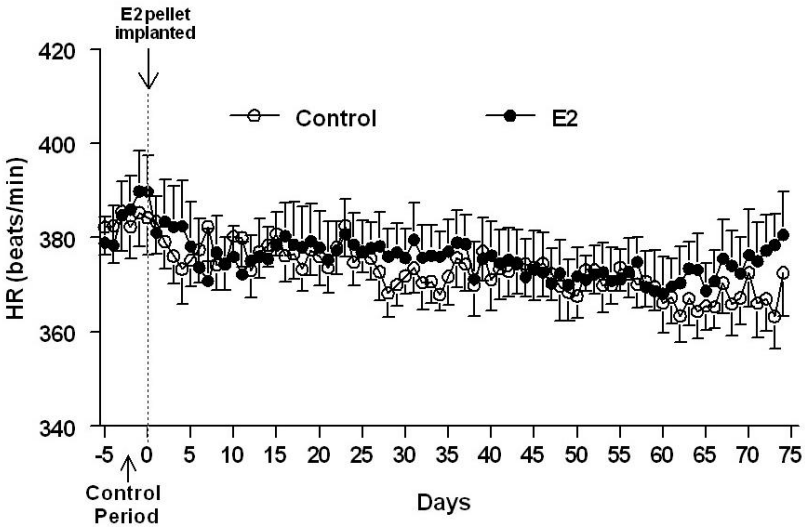


Fig. 5-11 Effect of chronic E_2 exposure on Mean Arterial Pressure (MAP).

A: Line graph depicting MAP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats (n=7-8 /group). B: Bar graph showing the average MAP values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

A



B

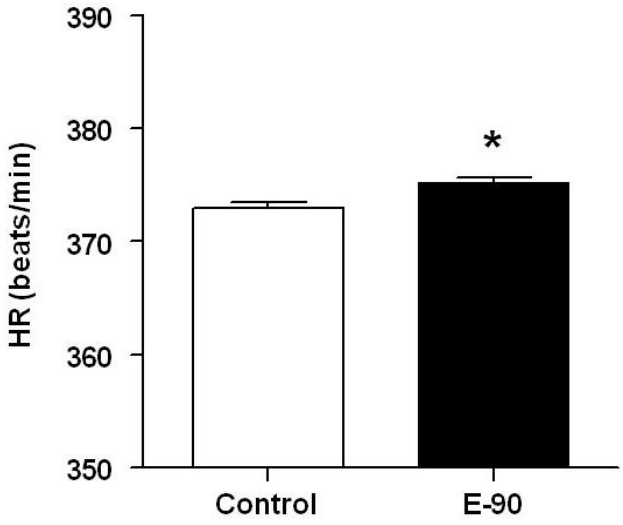


Fig. 5-12 Effect of chronic E_2 exposure on Heart Rate (HR).

A: Line graph depicting HR (beats/min): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats (n=7-8 /group). B: Bar graph showing the average HR values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

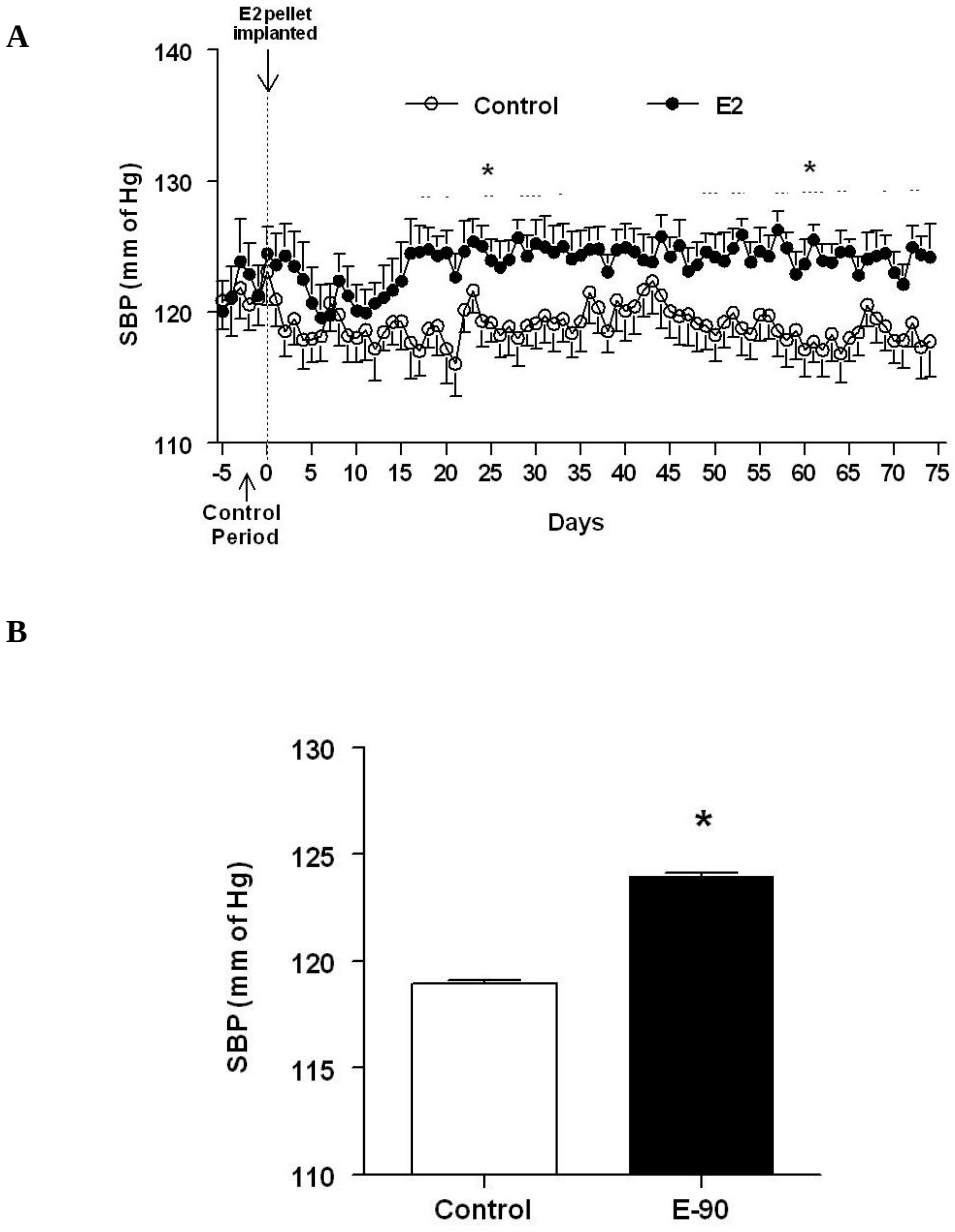


Fig. 5-13 Effect of chronic E_2 exposure on Systolic Blood Pressure (SBP).

A: Line graph depicting SBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats (n=7-8 /group). B: Bar graph showing the average HR values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

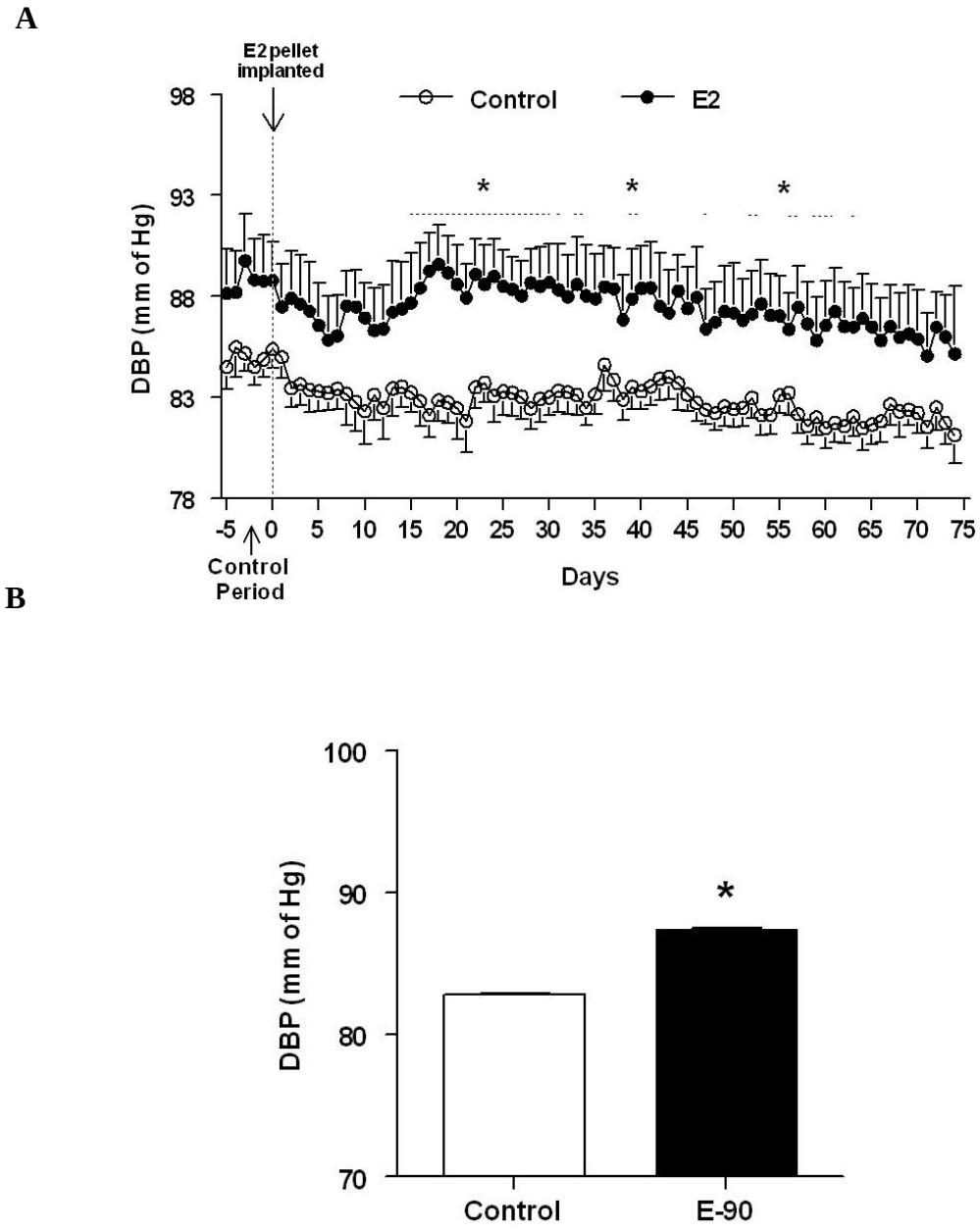


Fig. 5-14 Effect of chronic E_2 exposure on Diastolic Blood Pressure (DBP).

A: Line graph depicting DBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) and open circles represent control rats (n=7-8 /group). B: Bar graph showing the average HR values shown in A, between E_2 implanted and control rats. * denotes significant difference ($p<0.05$) from control rats.

ET-1A receptor antagonist (BQ-123) reverses chronic E_2-induced hypertension

The daily average profiles and the average mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) during the 10^{th}-13^{th} week of E_2 treatment for control+aCSF, E_2+aCSF, Control+BQ-123 and E_2+BQ-123 rats are shown in Fig. 5-15 to 5-18. BQ-123 when administered to control or E_2-treated animals reduced MAP and SBP. Heart rate (HR) and DBP were reduced only in control animals treated with BQ-123 but not in E_2-treated animals injected with BQ-123.

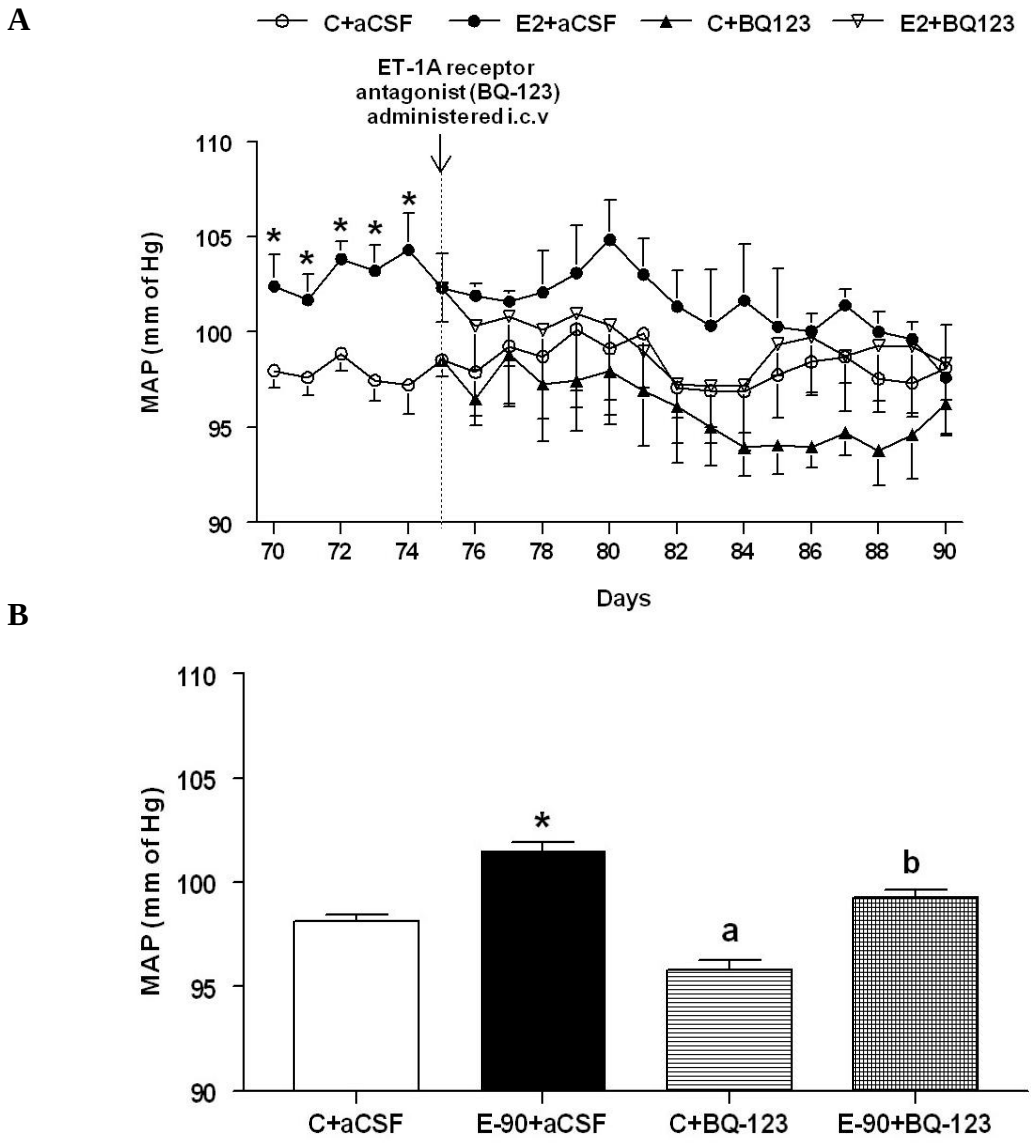


Fig. 5-15 Effect of ICV ET-1A antagonist on chronic E_2-induced increase in MAP

A: Line graphs depicting MAP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) treated with aCSF and open circles represent control female SD rats treated with aCSF, filled triangles represent rats control rats treated with ET-1A antagonist (BQ-123) and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats treated with BQ-123.* denotes significant difference from control group. B: Bar graphs showing the average values of the BP parameters shown in A. * denotes significant difference ($p<0.05$) from all the other groups; 'a' represents significant difference from c+aCSF and E-90+BQ-123; 'b' represents significant difference from control+aCSF.

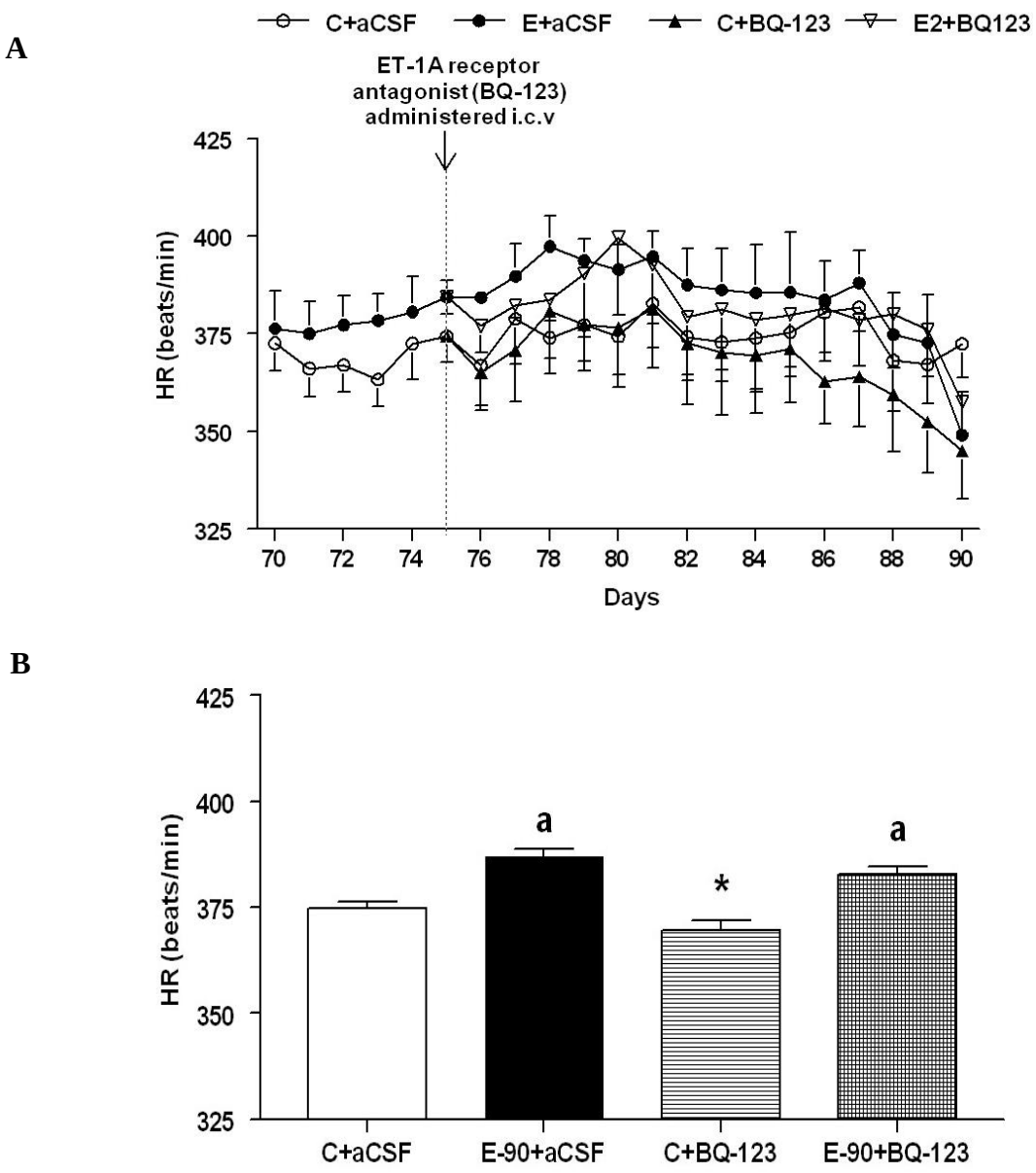


Fig. 5-16 Effect of ICV ET-1A antagonist on chronic E_2-induced changes in HR

A: Line graphs depicting HR (beats/min): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) treated with aCSF and open circles represent control female SD rats treated with aCSF, filled triangles represent rats control rats treated with ET-1A antagonist (BQ-123) and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats treated with BQ-123. B: Bar graphs showing the average values of the BP parameters shown in A, * represents significant difference from all the groups; 'a' represents significant difference from control.

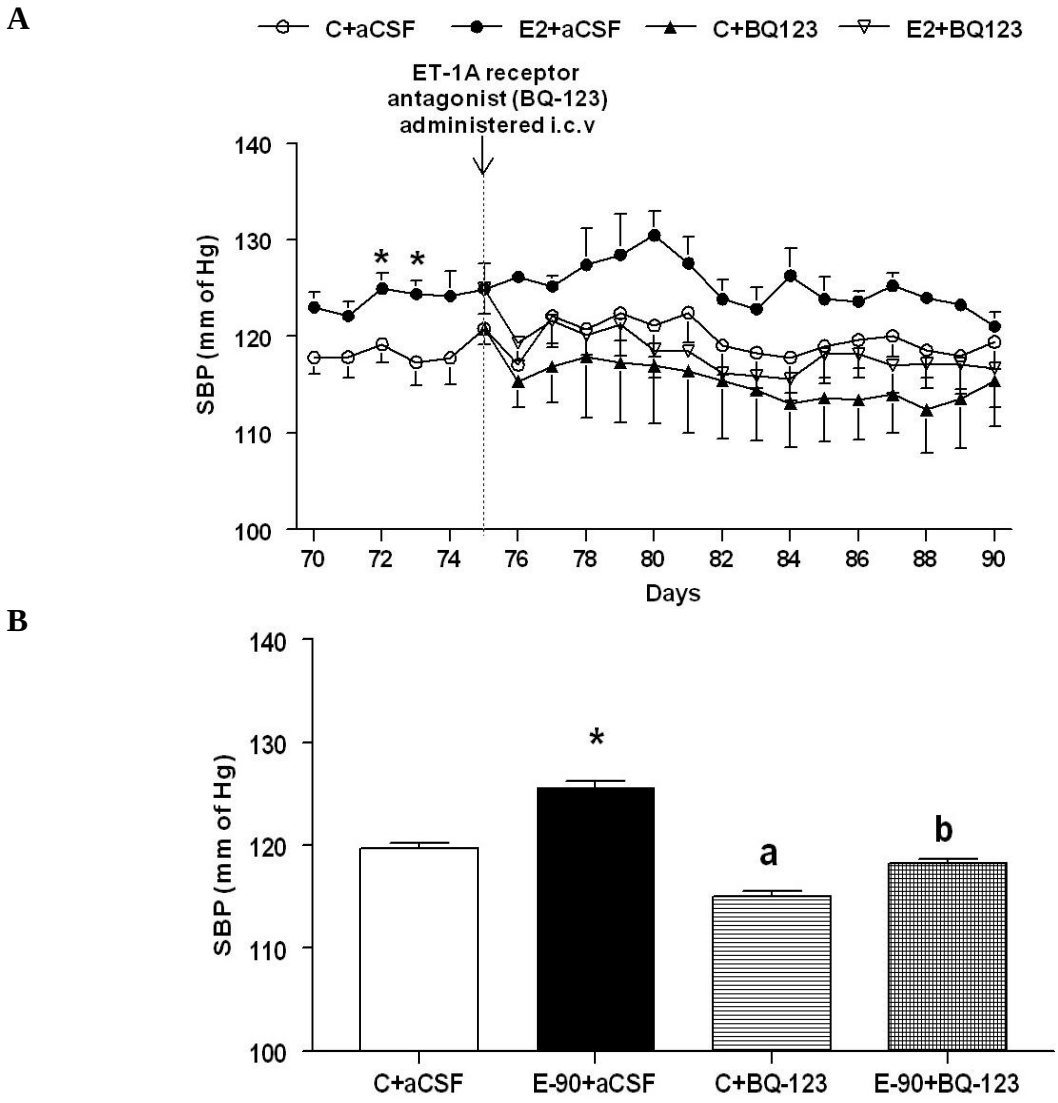


Fig. 5-17 Effect of ICV ET-1A antagonist on chronic E_2-induced increase in SBP

A: Line graphs depicting SBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) treated with aCSF and open circles represent control female SD rats treated with aCSF, filled triangles represent rats control rats treated with ET-1A antagonist (BQ-123) and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats treated with BQ-123. * denotes significant difference from all control group. B: Bar graphs showing the average values of the SBP parameters shown in A, * denotes significant difference ($p<0.05$) from all the other groups; 'a' represents significant difference from control+aCSF and E-90+BQ-123; 'b' represents significant difference from control+aCSF.

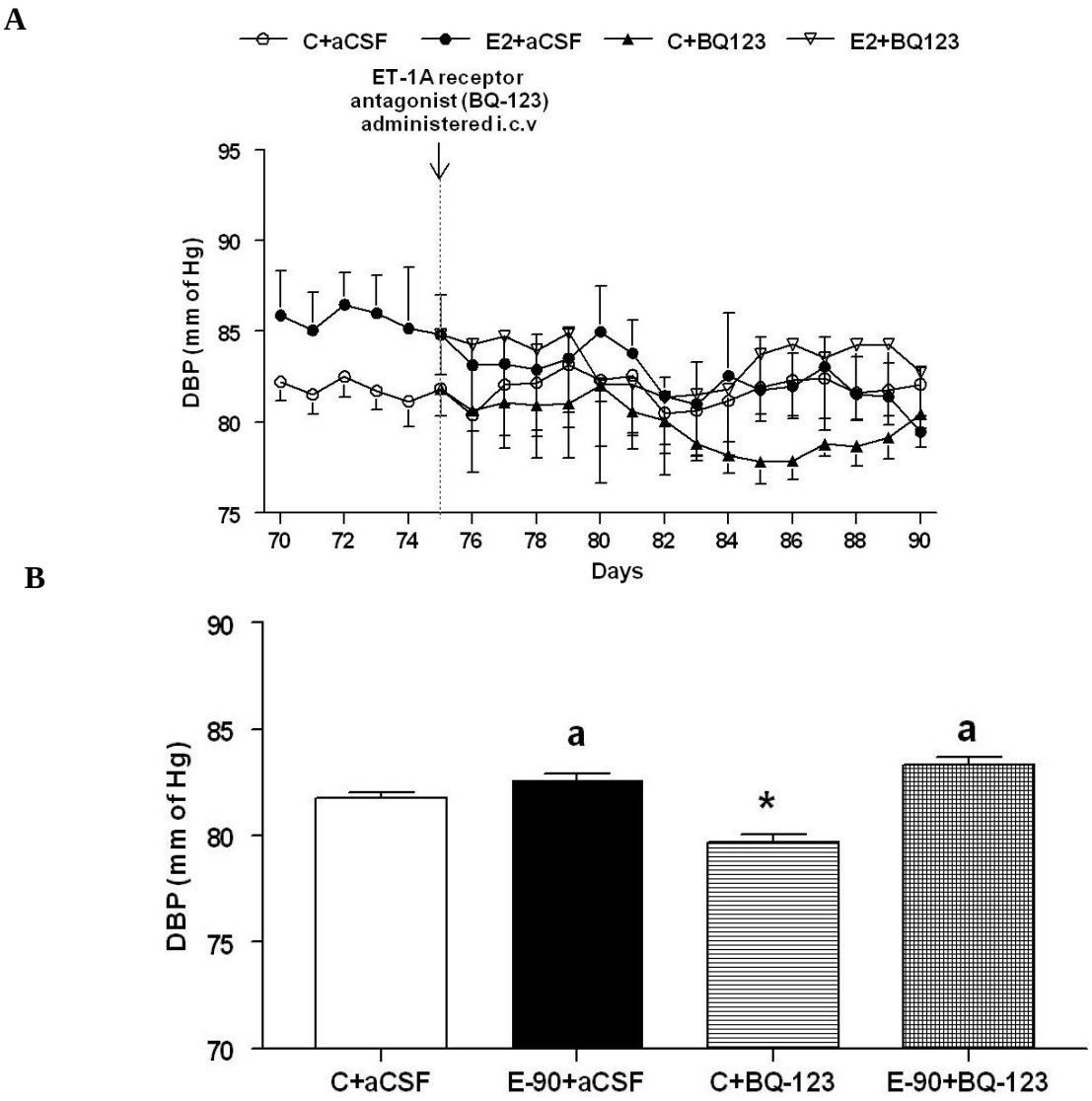


Fig. 5-18 Effect of ICV ET-1A antagonist on chronic E_2-induced increase in DBP

A: Line graphs depicting DBP (mmHg): closed circles represent E_2 pellet implanted (20 ng/day, 90-day slow-release pellets) treated with aCSF and open circles represent control female SD rats treated with aCSF, filled triangles represent rats control rats treated with ET-1A antagonist (BQ-123) and open inverted triangles represent E_2 pellet implanted (20ng/day, 90-day slow-release pellets) rats treated with BQ-123. B: Bar graphs showing the average values of the BP parameters shown in A, * denotes significant difference ($p<0.05$) from all the other groups; 'a' represents significant difference from control.

Discussion

Previously, we reported that chronic E_2-induced increase in MAP is associated with oxidative stress in the RVLM in young female SD rats. Recently, ET-1 and Ang II have been shown to increase O_2^- levels in the RVLM and PVN in various models of hypertension. Hence, we wanted to test the possibility of involvement of ET-1 and Ang II in the RVLM and PVN in chronic E_2-induced hypertension. Similar to our previous studies, chronic E_2 exposure increased MAP, HR, SBP and DBP. In contrast to our first study, we were able monitor cardiovascular parameters from the beginning of the experiment. We observed the hypertensive effects of estrogen treatment starting as early as 3 weeks after E_2 implantation. The results from the present study showed that there is increase in ET-1 gene expression in both the RVLM and PVN on chronic estradiol exposure. These changes were accompanied by increased expression of ET-1A receptor in both RVLM and PVN on chronic estradiol treatment. Further, we also found that intracerebroventricular (ICV) administration of an ET-1A receptor antagonist, BQ-123 reversed the estradiol-induced increase in MAP. Further, our results indicate that RAS in the PVN and RVLM may not be involved in chronic estradiol-induced hypertension. Taken together these results suggest that increased ET-1 activity and oxidative stress related changes may be involved in chronic estradiol-induced hypertension.

Several studies complement our findings. Increase in ET-1 levels in the brain paralleled increases in MAP in DOCA-salt hypertensive rats [86]. Also, Rossi et al. showed that ICV administration of ET-1 increased MAP in a dose-dependent manner in Long-Evans rats [193], while others have also demonstrated the same in SHR and SHR-SP rats [85]. In the present study, we hypothesized that ET-1A receptors may be involved in mediating ET-1's effects in the

PVN and RVLM, as ICV ET-1A blockade using BQ-123 reversed chronic E_2 induced hypertension. Our results are supported by other studies which have shown that ICV ET-1A but not ET-1B receptor blockade reversed ET-1 induced increases in BP [85, 193]. Interestingly, in the present study, ET-1A blockade decreased MAP in control animals also, suggesting a physiological role for ET-1 in regulating BP. Similar findings were reported in a study where ICV administration of ET-1 oligodeoxynucleotide targeted to prepro ET-1 reduced MAP in control animals [194].

Some of the speculated mechanisms involved in ET-1 mediated chronic E_2-induced hypertension are as follows: In the periphery, ET-1 has been reported to increase O_2^- production via an NADPH oxidase dependent mechanism in the vasculature of DOCA-salt hypertensive rats [195]. Based on our findings such as an increase in O_2^- production and NADPH oxidase expression in the RVLM and increased expression of ET-1 in both the RVLM and PVN in chronic E_2 treated animals; we surmise that the ET-1 might activate NADPH oxidase to produce O_2^- resulting in hypertension. On the other hand, O_2^- has also been shown to mediate Ang II-induced ET-1 release from fibroblasts, and NADPH oxidase inhibitors prevent this effect [196]. Hence, we still do not know if O_2^- increases ET-1 or vice versa. We believe that the principal source of ET-1 in the RVLM and PVN are glial cells. Presence of estrogen receptors in the glial cells allows us to hypothesize that chronic estrogen exposure activates glial cells to release ET-1, which in turn acts on the adjacent neurons and glial cells in a paracrine manner. Supporting this notion, ET-1 has been shown to be synthesized from the glial cells [197]. However further studies are needed to prove this hypothesis.

In vitro studies using isolated perfused rat mesenteric arteries showed increased release of Ang II upon ET-1 infusion [89]. Hence, it is possible that ET-1 activates RAS in the brain to cause O_2^- production. However, this possibility should be carefully considered as we did not observe changes with AT1 receptor expression in the PVN or RVLM. Though chronic E_2 effects in the RVLM and the PVN has been investigated in the present study, the possibility for the involvement of other regions in particular the circumventricular organs like SFO, OVLT and area postrema should also be considered. Afferent projections from the circumventricular organs reach RVLM and PVN and thus could integrate blood borne signals to affect SNA and BP.

In conclusion, our studies demonstrate that chronic E_2 exposure increases MAP as early as 3 weeks of treatment. This hypertensive effect of E_2 was associated with increases in ET-1 and ET-1A receptor expression in PVN and RVLM and administration of central ET-1A antagonist, BQ123 reverses chronic estradiol-induced hypertension.

CHAPTER 6

SUMMARY AND CONCLUSION

It is becoming increasingly evident that chronic use of estrogenic preparations in the form of hormonal replacement therapy and oral contraceptives does not confer cardioprotection as it was believed, instead increases the risk for cardiovascular diseases[7]. Despite this new awareness about the adverse effects of chronic estrogen use, a larger percentage of women still use them for other ailments. Understanding the mechanisms behind these adverse effects of chronic estrogen exposure is of paramount importance to minimize the risk associated with it. My thesis focuses on understanding the central mechanisms underlying the effects of chronic estrogen exposure on the cardiovascular system, in particular its role in the development of hypertension.

Estrogen is shown to have a number of beneficial effects on the cardio vascular system [128, 129]. Estrogen therapy has been shown to decrease the risk for coronary disease [198], improve endothelial dysfunction [199], reduce aortic stiffness [200] etc. Some of the possible mechanisms by which estrogen provides its cardio-protective are by increasing eNOS production in the aorta of rats and by causing vasodilation [200]. Similarly, estrogen has also been shown to increase the production of NO through nNOS in the vascular smooth muscle cells and cause relaxation of endothelium-denuded coronary arteries [201]. These findings show that the mechanisms behind the cardioprotective effects of estrogen might involve production of NO in the endothelial and vascular smooth muscle cells. Clinical studies using acute estrogen exposure in postmenopausal women with coronary artery disease showed symptomatic relief for myocardial ischemia [202, 203] and increased vasodilation [204]. In addition to promoting vasodilatation, estrogen replacement therapy may also reduce the incidence of cardiovascular disease by decreasing cholesterol levels, and/or improving glucose metabolism [205, 206]. Another means by which estrogen could provide cardioprotection is by its action on the brain.

Estrogen exposure increases baroreceptor reflex in response to an increase in BP [207, 208]. Also estrogen exposure is shown to reduce SNA and increase baroreflex sensitivity [209] suggesting the possibility that the central autonomic regulation of BP is affect by estrogen.

Contrary to the above findings, recent studies from WHI have provided evidence that postmenopausal women on HRT using estrogenic preparations were at increased the risk for coronary heart disease [7]. Similarly, clinical studies conducted on young women using oral contraceptives also showed an increase in BP [8, 34, 136]. One of the possible reasons for these contrasting results is that most of the above mentioned studies were acute studies or were performed under *in vitro* conditions, which make it increasingly difficult to interpret these results under *in vivo settings*. The contrasting results from the above studies necessitate the thorough understanding of the cardiovascular effects of chronic estrogen exposure.

In order to study the effects of chronic estrogen exposure on the cardiovascular system, we developed a model in which we exposed female SD rats with a low dose of estradiol pellets for a period of 90 days. We hypothesized that chronic estrogen exposure in adult young female SD rats will cause hypertension. In our first set of experiment, we found that chronic exposure to E_2 to young female SD rats increased mean arterial pressure (MAP), heart rate [114] (Chapter 3), systolic blood pressure (SBP) and diastolic blood pressure (DBP). The results from the present study are consistent from studies by other investigators in humans [137-139]. Chronic use of oral contraceptives is shown to slightly elevate the systemic arterial pressure by 3-6/2-5 mmHg with other adverse effects on the cardiovascular system [8]. Previous studies that reported the cardioprotective actions of estrogens are acute studies (3 weeks) with a high dose of estrogen (1.5mg/day). Moreover these studies were performed in ovariectomized transgenic hypertensive

rats [210]. In our study, we have exposed female intact SD rats with estrogen (20ng/day) for a period of 13 weeks. This makes the comparison between the results from the two studies difficult; however it is interesting to note that with such a low dose of estrogen, we were able to achieve an increase in arterial pressure.

It is highly possible that estrogen has dual effects in the cardiovascular system. Studies by Calippe et al. have clearly demonstrated the opposite effects of estrogen on the function of peritoneal macrophages [163]. Acute *in vitro* exposure of estrogen to peritoneal macrophages decreased the production of cytokines [163]. However, chronic *in vivo* administration of estrogen increased the expression of cytokines in the macrophages [163]. Similarly, studies by White et al. also reported the dual effects of estrogen on coronary arteries[211]. Estrogen caused relaxation or contraction of the coronary arteries based on the coupling or uncoupling of NO respectively. It was hypothesized that estrogen acts on the type I nNOS in the coronary arteries to cause a vasodilation through NO dependent mechanisms or vasoconstriction through O_2^- dependent mechanisms [211]. Thus, it is possible that estrogen when given for a shorter duration confers cardioprotection and when exposed for a longer period of time it has the opposite effects. Apart from the duration of exposure, the dose of estrogen might also play a critical role in precipitating its effects on the cardiovascular system. This could be one of the possible reasons why hormone replacement therapy was not cardioprotective for women as presumed. Hence the acute effects of estrogen alone should not be considered to arrive at conclusions on the effects of estrogen on cardiovascular system.

In chapter 4, we focused on understanding the central mechanisms behind chronic estradiol-induced increase in mean arterial pressure (chapter 3). The brain is an important center

for the control of BP regulation. [37-40]. The paraventricular nucleus (PVN) of the hypothalamus and rostral ventrolateral medulla (RVLM) of the brainstem are not just two important areas that are involved in the BP control but are also critical in development of hypertension through sympathetic activation [152]. Only recently the role of oxidative stress in mediating BP elevation in several models of hypertension came to limelight [38-40, 144]. Similarly, neuroinflammation marked by increases in brain proinflammatory cytokines has also been implicated in the development of hypertension [81, 116, 212]. In light of all these studies, we hypothesized that chronic estradiol-induced hypertension involves central mechanisms mediated by oxidative stress and proinflammatory pathway.

In order to test the hypothesis, whether chronic estradiol exposure increases ROS production in the RVLM and PVN, we measured the O_2^- levels in both these regions after estradiol treatment. We found that chronic estradiol exposure increased O_2^- production specifically in the RVLM but not in PVN. One of the possible mechanisms by which increased O_2^- generation in the RVLM could elevate the MAP is by activating the sympathetic preganglionic neurons which project to the IML cell column of the spinal cord resulting in increased SNA and hypertension [15]. In this study we investigated the involvement of O_2^- only in RVLM and PVN; however the role of other brain centers such as SFO, NTS that are known to be involved in O_2^- production in other models of hypertension is unknown. The role of these areas has to be examined in future studies.

In further exploring the source of O_2^- production in the RVLM, we investigated the role of NADPH oxidase and its subunits, as NADPH oxidase is believed to be one of the major

sources of ROS in the cardiovascular system [213]. In the present study, we measured the gene expression of different NADPH oxidase subunits (Nox1, Nox2, Nox4, $p47^{phox}$, $p22^{phox}$) in the RVLM. In order to produce O_2^- from NAPDH oxidase subunits a catalytic core need to be formed from one of the Nox isoforms and $p22^{phox}$ [214] However this core complex, gets activated only after the addition of $p47^{phox}$ or GTP*ase Rac* [215]. In our study we observed a significant increase in the gene expression of Nox1 and 2 in the RVLM on estrogen treatment, but there was no change in the other subunits like $p22^{phox}$ or $p47^{phox}$, so it is still unclear how the increased expression of Nox1 and 2 contributes to the production of O_2^- in our model. Also in our study the Nox4 isoform was significantly downregulated, so the possibility of Nox4-$p22^{phox}$ complex in increasing O_2^- is also highly unlikely. SOD is an enzyme that scavenges superoxide and acts as a major source of antioxidant defense. In our study we found that the gene expression of CuZnSOD is significantly downregulated on estrogen treatment. It is possible that the imbalance between the pro-oxidant and anti-oxidant defense mechanism characterized by increased NADPH oxidase and decreased CuZnSOD is responsible for the observed increase in superoxide levels in the RVLM of chronic estrogen-induced hypertensive rats. However further studies are needed to clearly delineate the mechanisms by which NADPH oxidase and CuZnSOD play a role in O_2^- production in our model.

The role of NO in the RVLM in mediating BP remains controversial. Recently overexpression studies by Kishi et al., in the RVLM of awake normotensive rats showed that iNOS increases arterial pressure through activation of sympathetic nervous system, which could

be mediated by oxidative stress in the RVLM [111]. There are other studies to support and dispute these findings [216, 217]. However when NO is produced in excess it could react with O_2^- to form peroxynitrite [218]. Previous studies from our lab have demonstrated increase in NO levels on chronic estradiol exposure resulting in nitration of tyrosine hydroxylase, a rate limiting enzyme that in involved in the synthesis of dopamine in one of the hypothalamic nuclei, the arcuate nucleus (AN) [219]. In light of these findings, we investigated the role of NO in both the RVLM and PVN in mediating chronic estradiol-induced hypertension. Interestingly there were no changes in protein levels of NO, measured in the form of nitrates and mRNA levels of iNOS in both the regions of our interest. These findings excluded the possible combined role of NO and O_2^- in mediating chronic estradiol-induced hypertension at least in RVLM and PVN.

In recent years the role of inflammatory pathway in mediating hypertension is gaining more attention. Central proinflammatory cytokines have been implicated in the production of O_2^- and in the development of hypertension [81, 212]. Recent studies by Shi et al. [118], clearly demonstrates that Ang II-induced hypertension is mediated by the production of PICs in the PVN. Studies by Kang et al [81] describes the role of transcription factor, nuclear factor-kappa B in increasing oxidative stress in the PVN and contributing to development of hypertension by sympathoexcitation. Previous studies from our lab have also demonstrated increased production of PIC, interleukin-1β in the AN upon chronic estradiol exposure. Taken together we investigated the role of IL-1β in PVN and RVLM in chronic estradiol-induced hypertension. To our surprise, we didn't see any changes in the protein and mRNA levels of IL-1β in both the PVN and RVLM. The findings have thus far excluded the role of IL-1β and NO in mediating chronic estradiol-induced hypertension and O_2^- production in the RVLM.

In order to further confirm the role of oxidative stress in chronic estradiol exposure-induced hypertension, we fed estradiol-treated rats with resveratrol, an antioxidant to see if it reverses both the BP and oxidative changes in RVLM. Resveratrol was able to completely reverse the chronic estradiol-induced hypertension and abolish the production of O_2^- in the RVLM in estradiol-treated rats. These findings clearly demonstrate that oxidative stress in the RVLM is associated with chronic estradiol-induced hypertension. However at this point the results are still correlative; it is not clear whether the increase in O_2^- levels in the RVLM are a cause or effect of the hypertension or if this change is due directly or indirectly to an action of estrogen in the RVLM.

In the series of experiments in chapters 3 and 4, we described that chronic low-dose estradiol exposure increases O_2^- production in the RVLM and this is clearly associated with chronic estradiol-induced hypertension. It is interesting to note that we did not see any changes in the IL-1β and NO levels in both the RVLM and PVN in chronic estradiol-induced hypertension. At this point the role of PVN in estradiol-induced hypertension is still unclear as the oxidative changes that we have seen so far are specific to the RVLM. One hypothesis is that the neuronal population in the PVN and RVLM varies. In addition to the sympathetic neurons, PVN also acts as a hub for other neuronal populations that are involved in the regulation of stress axis, energy metabolism and thyroid function. Thus, it is possible that differences in neuronal as well glial population between these two areas could be responsible for the observed differences. This needs further exploration in future studies.

Brain renin-angiotensin system and endothelin system have been previously reported to be associated with oxidative stress mediated increases in BP. It is highly likely that E_2-induced

O_2^- production is mediated through ET-1 or Ang II. In chapter 5, we addressed this possibility in our model by measuring the levels of ET-1 and its receptors and AT1 receptor expression in the PVN and the RVLM. We found that chronic estradiol exposure induced activation of ET-1 system characterized by increased expression of ET-1 and its receptor A in both the RVLM and PVN. However we did not see any changes in the expression of AT1 or ET-1B in both these regions.

We further investigated the role of ET-1 system in chronic estradiol-induced hypertension by using a mechanistic intervention. ICV administration of ET-1A receptor antagonist BQ-123 reversed the chronic estradiol-induced increase in MAP and SBP. However no changes were seen in the HR and DBP. Interestingly, ET-1A antagonism produced a fall in MAP in control animals suggesting a physiological role for ET-1 in BP regulation. Our studies have also provided evidence that (chapter 3 and 4) chronic estradiol-induced hypertension is associated with increased oxidative stress in the RVLM, though the exact mechanism by which oxidative stress modulates the activity of RVLM is unclear. The overall summary of our research is given in Fig. 6-1. Based on these results, one could hypothesize that ET-1 acting via its receptor ET-1A increases activation of NADPH oxidase, which in turn could enhance the production of O_2^- centrally in our model. Other possibility is that estrogen could directly produce O_2^- via NADPH oxidase-dependent mechanism resulting in increased oxidative stress which could have activated ET-1 to increase BP through activation of sympathetic nervous system. It is also noteworthy that chronic estradiol exposure may activate sympathetic nervous system which inturn can increase the risk for other cardiovascular diseases such as heart failure without the

involvement of brain. In the present study, the cause and effect role of ET-1 and oxidative stress are still unclear and need to be explored further in future experiments.

The presence of both the estrogen receptor subtypes (α and β) in the PVN and RVLM suggest that the hypertensive effects of estrogen might be mediated through these receptors. The central cardiovascular effects of estrogen have been shown to be mediated primarily but not exclusively through ERβ [6, 220]. Estrogen receptors α and β has been shown to be co-localized with tyrosine hydroxylase containing C1 neurons in the RVLM, suggesting that estrogen can directly act on these neurons to mediate its actions. Further patch clamp recordings from isolated C1 RVLM neurons indicate that estrogen can directly modulate the functions of these neurons by acting through ERβ [220]. These studies suggest the possibility that the hypertensive effects of estrogen seen in the present study could be mediated through estrogen receptors.

In conclusion, our studies provide evidence that chronic exposure to low levels of estradiol can increase BP in young SD female rats. Our studies also provide evidence that estradiol could act at the level of brainstem in the RVLM and at the level of hypothalamus in the PVN. Chronic estradiol exposure increased O_2^- production in the RVLM and activated the ET-1 system in both the RVLM and PVN. Taken together these findings suggest that interplay between ET-1 system and oxidative stress could play a role in chronic estradiol-induced increase in BP and provides valuable insights in understanding the central mechanisms behind chronic estradiol-induced hypertension.

Future Directions

Even though, our studies have provided valuable insights in understanding the central mechanisms behind chronic estrogen-induced increase in BP, there are still compelling questions

that remains to be answered. The involvement of SNA that would link the changes in PVN and RVLM with the increase in blood pressure needs to be evaluated. Direct sympathetic nerve recordings or NE spill over techniques could be employed to address this issue. Further, the cause and effect role of endothelin and oxidative stress needs to be determined. Direct microinjection studies using ET-1A antagonists or superoxide scavengers specifically in the PVN or RVLM will help answer this question. In addition to this, the contribution of neurons or glial cells in mediating the effects of estrogen in the PVN and RVLM still needs to be solved. The role of estrogen receptors, alpha and beta in mediating the actions of estrogen in the brain also needs to be determined. Estrogen receptor knock out can be used to address this issue. Though, we independently looked at the effects of estrogen in the PVN and RVLM, it is important to understand that these two regions are connected by afferent and efferent neuronal projections. Hence, the phenotype of neurons that signals between PVN and RVLM in chronic estrogen induced hypertension should be identified. Finally the effects of chronic estrogen exposure in the peripheral tissues such as the blood vessels, heart and kidney should also be evaluated. This will provide a global picture of the hypertensive effects of chronic low dose estrogen exposure.

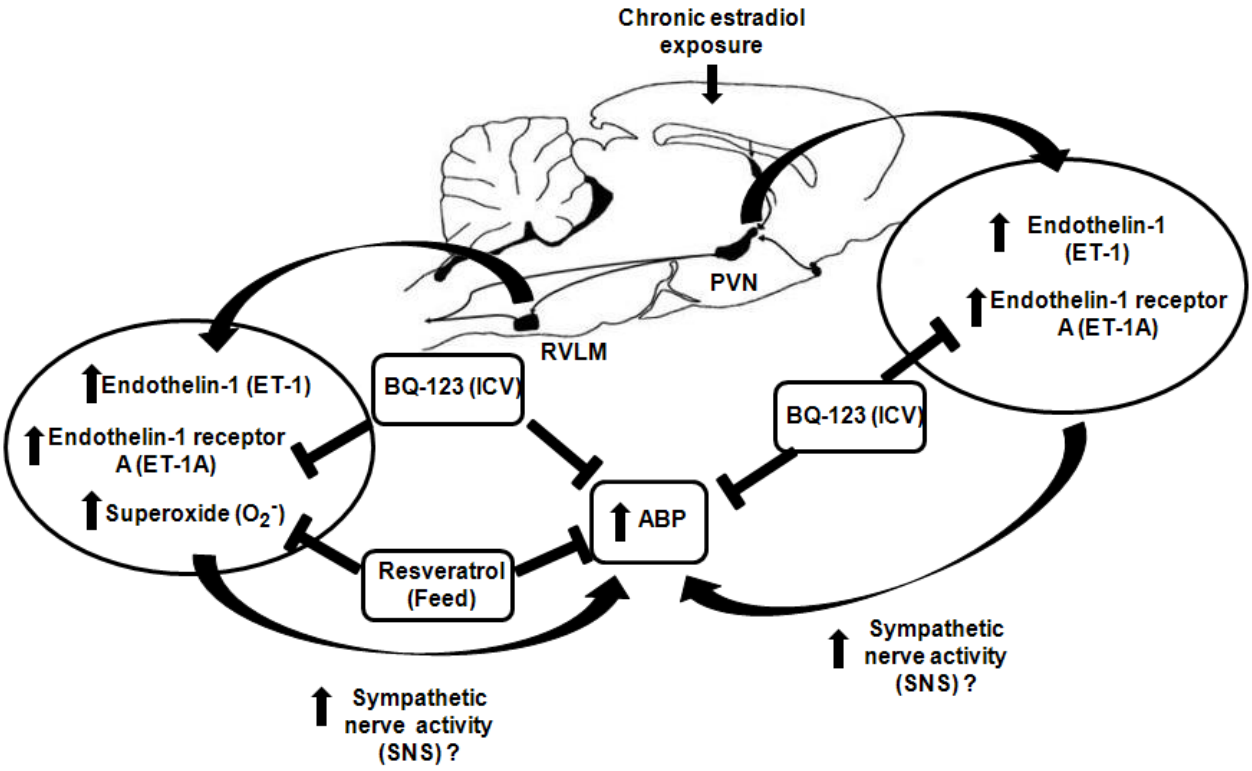


Fig. 6-1 Schematic of conclusions – Chronic exposure to low levels of estrogen increases arterial blood pressure (ABP) in female SD rats increasing superoxide at least in part via NADPH oxidase dependent mechanism in the RVLM and activation of ET-1 in both the RVLM and PVN via ET-1A dependent mechanism.

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