THE ROLE OF ESTROGEN RECEPTORS IN THE CONTRIBUTION OF CONSTRICTOR PROSTANOIDS TO AORTIC COARCTATION-INDUCED HYPERTENSION

A Dissertation

by

MINGA MIOWN SELLERS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Veterinary Physiology

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Approved by:

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ABSTRACT

The Role of Estrogen Receptors in the Contribution of Constrictor Prostanoids to Aortic Coarctation-Induced Hypertension.

(December 2008)

Minga Miown Sellers, B.S., Texas A&M University

Chair of Advisory Committee: Dr. John N. Stallone

This study investigated the effects of selective estrogen receptor (ER) agonists on constrictor prostanoid (CP) function and on the development of mean arterial pressure (MAP) in aortic coarctation-induced hypertension (ACIH). Female Sprague-Dawley rats were divided randomly into four groups: intact (INT), ovariectomized (OVX), OVX + ERα selective agonist (4, 4', 4"-(4-Propyl-[1H]-pyrazole-1, 3, 5-triyl)*tris*phenol; OVX+PPT), or OVX + ERβ selective agonist (2,3-*bis*(4-Hydroxyphenyl)-propionitrile; OVX+DPN). Rats were then subjected to abdominal aortic coarctation (hypertensive, HT) or sham surgery (normotensive, NT). PPT, DPN or vehicle treatments were given daily as a subcutaneous injection. MAP was measured every other day at 2-14 days after coarctation. Mesenteric arterioles were harvested 12-14 days after coarctation for isometric tension studies to examine concentration-responses to VP. Basal and VP-stimulated prostanoid release and mRNA and protein levels of ERα and ERβ (using real time RT-PCR and immunoblotting) were measured in separate groups of arterioles. MAP was higher in INT-HT, OVX+PPT-HT and OVX+DPN-HT than in OVX-HT after 12 days. Vascular reactivity to VP was greater in OVX+PPT-NT rats than in other groups.

There were no significant differences in vascular reactivity to VP in HT groups. Blockade of thromboxane receptor (TP) with SQ 29,548 (TP receptor antagonist) did not have a significant effect in any groups. Inhibition of intracellular calcium release with simvastatin (blocker of IP_3 mediated calcium release) was greater in NT than in HT groups, and greater in OVX- and DPN-treated groups than in INT and PPT-treated groups. VP-stimulated release of thromboxane (TXA_2) and prostacyclin (PGI_2) were highest in INT-HT and OVX+PPT-HT rats. Neither mRNA nor protein expression of ERs changed significantly in response to selective ER agonist treatment or during hypertension. Selective ERα stimulation with PPT during development of ACIH resulted in similar effects to those seen in INT rats for CP release, VP reactivity of mesenteric arterioles and MAP, while selective stimulation of ERβ only increased MAP. While ERα is capable of modulating most of the effects of estrogen on the vasculature, ERβ has stimulatory effects on MAP during the development of ACIH that merit further investigation. Further studies of the vascular actions of ERα and ERβ may lead to better hormonal therapies that successfully prevent and/or treat cardiovascular disease in post-menopausal women.

DEDICATION

To my husband, Ryan Sellers, for supporting me both intellectually and financially in my quest for higher education. To Mary Wheat, for inspiring me to work hard and to be the best I can be in every situation and for teaching me that nothing can keep me down. To my family, who made me strive for more.

ACKNOWLEDGEMENTS

I owe a debt of gratitude to my doctoral advisor Dr. John N. Stallone for his support and encouragement throughout this project. His mentorship made it possible for me to endure, even though at times I wanted to quit. I would also like to thank the other members of my committee, Dr. Janet Parker, Dr. Farida Sohrabji and Dr. Cris Heaps for their guidance and clarification throughout this project. Very special thanks goes to Millie Mattox for teaching me how to use a myograph and answering my many, many questions. Very special thanks goes to Dr. Emily Wilson for the use of her lab space for my molecular biology studies, and to her students and technicians for teaching me the delicate procedures involved in molecular biology. I would like to thank Dr. Wendy Baltzer for teaching me surgical methods and for her friendship and encouragement. I would also like to thank Jennifer Crawford and Stephanie Hampe for their help with many experiments and company in the lab. The help of Feng Xu in many of my experiments is greatly appreciated; I don't know what I would have done without her. I would like to thank my dogs, Bruiser, Bailey, Bennie, Tia and Bling Bling and my cats, Bandito, Federali, Ho Chi Minh, Leon and Bunnicat and my birds Peanut Butter and Rocko for giving me something furry and feathery to love and hug when I got home after a long, hard day, and for putting a smile on my face and making me laugh. Finally my thanks goes to the Department of Veterinary Physiology and Pharmacology for their support and for never making me feel inferior. Thank you so much for this opportunity.

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CHAPTER I

INTRODUCTION AND HISTORICAL REVIEW

1.1 Introduction

Men are from Mars, women are from Venus. Well, maybe that is a bit extreme. Although men and women do hail from the same planet, the fact remains that there are many differences between the sexes, ranging from behavior to body function to aging. And the culprit for these differences? We suspect the sex steroid hormones: estrogens and androgens. Perhaps once thought to affect only reproductive function, it is now well known that these hormones affect nearly every facet of the body, from brain development, to skeletal growth, to cardiovascular function.

With respect to the cardiovascular system, considerable dogma has developed surrounding the effects of estrogens on cardiac and vascular functions. For many years it was thought that estrogens, particularly 17β-estradiol, exerted beneficial effects on the cardiovascular system and when estrogens vanished at menopause, so did their beneficial effects, resulting in increased incidences of cardiovascular diseases. This would certainly explain why premenopausal women experience much less cardiovascular disease (CVD) than men, and why after menopause, the incidences of CVD increase rapidly in women and attain levels virtually identical with those of men. This dogmatic view was based largely on epidemiological studies of coronary artery disease and hypertension in men versus women; however more recent clinical and epidemiological studies of other CVD

This dissertation follows the style and format of the *American Journal of Physiology – Heart and Circulatory Physiology.*

strongly suggest that estrogens exert deleterious effects on the vasculature.

Indeed, diseases of vascular origin such as Raynaud's disease and primary pulmonary hypertension occur more frequently in young premenopausal women than in men and appear to be estrogen dependent. Thus this doctoral dissertation will focus on cellular and

molecular mechanisms underlying the deleterious effects of estrogen on vascular function in the female.

1.2 Classical Effects of Estrogens

1.2.1 Estrogens in the Reproductive System

The estrogens are naturally occurring C_{18}-steroids characterized by the presence of an aromatic A-ring, a phenolic hydroxyl group at C-3, and either a hydroxyl group (estradiol) or a ketone group (estrone). As with all major classes of steroid hormones, the estrogens are derived from the common precursor molecule cholesterol (Fig. 1). The main estrogen secreted by the ovary is 17β-estradiol (E_2), which is derived from its immediate precursor, testosterone, by the action of the enzyme aromatase. It is the most potent and important of the estrogens. In addition, the weaker androgen androstenedione is converted to estrone (E_1) in the ovary, the secondary bioactive form of estrogen, also by the action of aromatase. E_1 is also secreted from the ovary, and while less potent than E_2, can exert many similar effects. While less E_1 is secreted from the ovary, it remains in equilibrium in the circulation with E_2, due to 17β-hydoxysteroid dehydrogenase activity, which reversibly converts E_2 to E_1. Then, E_1 and E_2 are converted to the primary estrogen

metabolite estriol (E_3) by the liver; E_3 is also secreted in small amounts by the ovary. Additionally, E_1 is the major remaining estrogen in the circulation after menopause (see Table 1 for production rates in pre-menopausal women).

The main roles of the estrogens are the development and maintenance of the female reproductive system and include: growth and hypertrophy of the uterus and vagina, development of the secondary sex characteristics, and development of the ductal system of the breast. Estrogens also play a central role in the regulation of the menstrual cycle and the process of ovulation. Production and secretion of E_2 by the ovarian follicle, stimulated by follicle stimulating hormone (FSH), causes circulating plasma E_2 levels to rise 5- to 9-fold (Table 1). The sharp increase in E_2 release from the dominant follicle triggers a surge in gonadotropins (FSH and luteinizing hormone, LH), resulting in ovulation. Estrogens also inhibit the growth of cohort follicles and prepare the fallopian tubes for transport of the ovum and zygote (146).

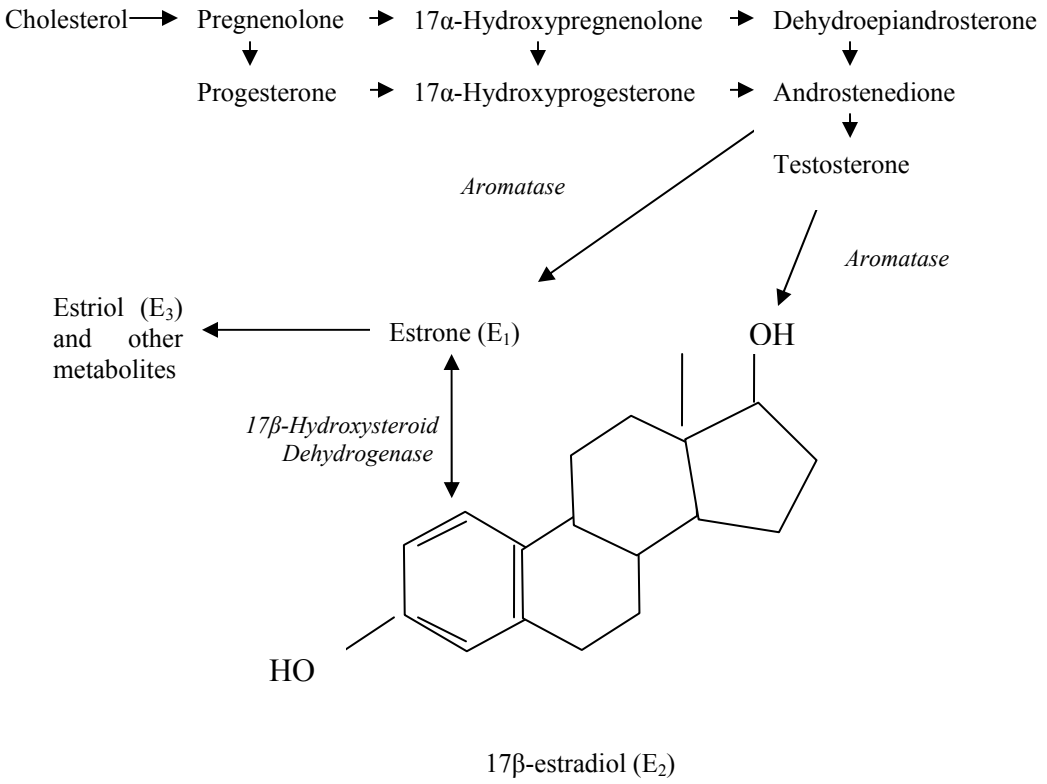

Figure 1: Synthesis of estrogens from cholesterol. Adapted from Ganong, *Review of Medical Physiology* 22nd edition (45).

Table 1: Hormone production and turnover in adult women (adapted from Berne and Levy eds. *Physiology,* 4th edition) (11).

Steroid	Plasma Concentration (ng/dl)	Production Rate (μg/day)	Metabolic Clearance Rate (L/day)
Estradiol	**6-50**	**80-700**	**1400**
Estrone	**5-20**	**100-500**	**2200**
Progesterone	**100-1000**	**2000-25000**	**2200**

1.2.2 Estrogens in Other Systems

Estrogen receptors (ERs) are found in nearly every tissue type in the body and thus, the effects of estrogens are widespread and have been observed in nearly every organ system. Estrogens act directly and indirectly on bone osteoclasts and resorptive cytokines, respectively, to inhibit bone resorption. E_2 stimulates reabsorption of sodium from the renal tubules. In addition, a number of proteins synthesized in the liver are increased by estrogens; these include angiotensinogen, the hormone-binding globulins, very-low-density lipoproteins and high-density lipoproteins.

Estrogens also exert effects in the brain and nervous system. Estrogens exert actions on several regions of the nervous system, including those involved with higher cognitive function, pain mechanisms, mood, susceptibility to seizures and even fine motor skills (83). There is also evidence that 17β-estradiol has neuroprotective effects in relation to stroke (9, 143) and Alzheimer's disease (125) in pre-menopausal women. In addition, there are nonnuclear ERs located in glial cells, dendrites, and presynaptic terminals. These nonnuclear ERs may couple with various second messenger systems to

regulate various cellular events (83). These neuroprotective effects of estrogens in the brain are attributed largely to increases in endothelial nitric oxide synthase (eNOS) and the resultant formation of nitric oxide (NO) in the brain vasculature and the possible maintenance of the integrity of the blood brain barrier (25, 42, 46, 47, 125).

1.2.3 Estrogens in the Vascular System

The well known effects of estrogens (especially E_2) on the vasculature are generally viewed as beneficial. E_2 has effects on both the endothelium and vascular smooth muscle (VSM), and usually acts as a vasodilator. It increases the release of vasodilators such as nitric oxide (NO), prostaglandin E_2, and prostacyclin, while decreasing production and activity of endothelin-1, a potent vasoconstrictor. In addition, E_2 stimulates the growth and migration of endothelial cells, while simultaneously inhibiting the growth of VSM cells. E_2 also enhances angiogenesis by facilitation of basic fibroblastic growth factor (bFGF) (112) and vascular endothelial growth factor (61, 77).

1.2.4 Hormone Replacement Therapy

If the loss of estrogens lead to increases in CVD in women, then it follows that replacement of estrogens will prevent or decrease the incidence of CVD. A number of earlier studies demonstrated beneficial effects of estrogens on CV health and disease. For example, *in vitro* treatment with E_2 inhibits Ca^{2+} influx in VSM cells, causing relaxation of arterial strips from male rats, and this effect was shown to be endothelium independent (50). In studies of human coronary arteries *in vitro*, E_2 produced relaxation by increasing VSM levels of cAMP and cGMP (91). In one clinical study, infusion of physiological

levels of E_2 potentiated endothelium-dependent vasodilation in healthy post-menopausal women and endothelium-dependent and –independent vasodilation in post-menopausal women with impaired vascular function and risk factors for atherosclerosis (48). Another study in primates with surgical menopause (OVX) and diet-induced coronary artery atherosclerosis showed that coronary arteries constricted in response to acetylcholine, but 20 minutes after infusion of ethinyl estradiol, the coronary arteries relaxed in response to acetylcholine (145). However, several recent clinical trials involving various types of hormone replacement therapy (HRT) reported that HRT was not protective against a variety of CVD, including atherosclerosis, pulmonary embolism (PE), myocardial infarction, stroke and venous thrombosis (51, 58, 111). Indeed, the incidences of CVD (nonfatal myocardial infarction and coronary heart disease death) and breast cancer were increased with HRT (111). The Heart and Estrogen/Progestin Replacement Study (HERS) studied women who had previous CHD, but no previous venothrombosis. The primary outcome for this study was increased incidence of coronary heart disease (CHD), either nonfatal myocardial infarction or CHD-related death in healthy postmenopausal women (no CHD prior to the trial) and a three-fold increase in risk for thromboembolic events in the HRT group (51). The Women's Health Initiative (WHI) trial was terminated early because it was clear that the risks of HRT significantly outweighed the benefits (111). The primary outcome of the WHI study was increased incidence of CHD, either nonfatal myocardial infarction or CHD death, in women that were postmenopausal and healthy (no CHD prior to the trial). Both of these studies used conjugated equine estrogens (CEE) combined with medroxyprogesterone acetate (MPA). There is some controversy concerning the use of CEE as the source of estrogen replacement, because it

is not the endogenous hormone 17-β estradiol. Arguments have been made that replacement of the missing hormone with the identical endogenous compound rather than CEE would result in the return of the protective effects on CV health. The Women's Estrogen-Progestin Lipid Lowering Hormone Atherosclerosis Regression Trial (WELL-HART) looked at the effects of E_2 either alone or with MPA on the progression of coronary-artery atherosclerosis and found that neither estrogen alone nor estrogen with sequentially administered MPA had any effect to reverse established atherosclerosis in women 20 years post-menopause, but that E_2 treatment could reduce the extent of atherosclerosis if HRT is initiated at the *beginning* of menopause (58). It was recently found that MPA can disrupt estrogen metabolism and abrogate the inhibitory effects of E_2 on the VSM cells (35). The findings of Dubey *et al.* (35) concerning the actions of progesterone on E_2 should come as no surprise, as progesterone modulates the actions of estrogen, both by decreasing ER levels and reducing nuclear bound estrogen-receptor complexes (146).

These recent studies on HRT only seem to add to the confusion surrounding the mysterious effects of estrogen. What are the pathways affected by estrogens that lead to the beneficial versus detrimental effects on the vasculature?

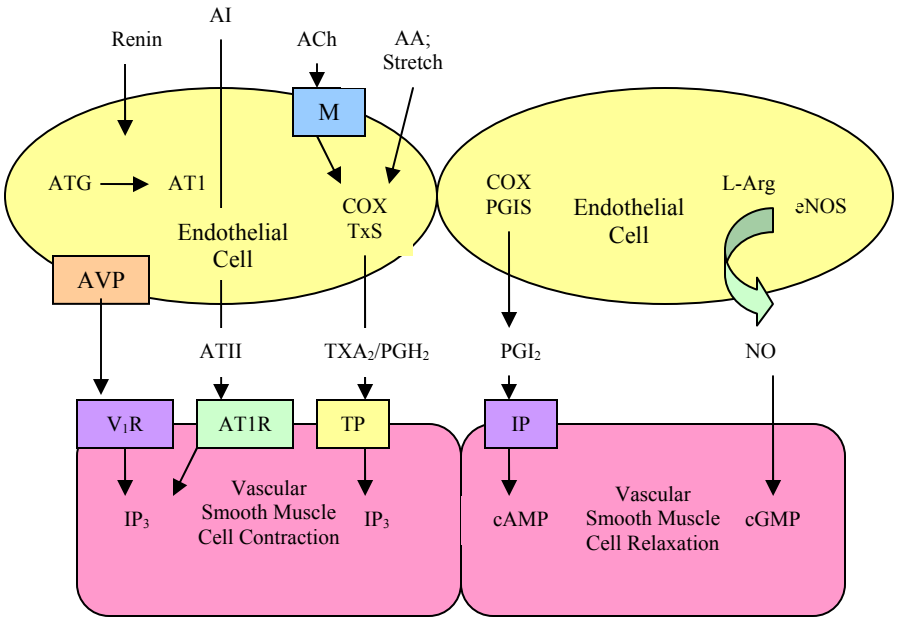

Figure 2: Vasoconstrictor and vasodilator mechanisms in the endothelium and vascular smooth muscle. (Adapted from Lüscher, 1994) (78) The endothelium releases both relaxing and contracting factors. The relaxing factors are prostacyclin (PGI_2) and nitric oxide (NO). The contracting factors are thromboxane/endoperoxide (TXA_2/PGH_2), angiotensin II (AII) and arginine vasopressin (AVP), which originates from the posterior pituitary. Estrogens can act on the AVP, ANG II, COX, TXA_2/PGH_2 and PGI_2 pathways. ATG, angiotensinogen; AT1, angiotensin I; Ach, acetylcholine; A, AII receptor; M, muscarinic receptor; AA, arachidonic acid; COX, cyclo-oxygenase; PGIS, prostacyclin synthase; L-Arg, L-arginine; eNOS, endothelial nitric oxide synthase; boxed AT1R, AT1 receptor; TP, TXA_2/PGH_2 receptor; IP_3, inositol triphosphate; IP, prostacyclin receptor; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; V_1R, arginine vasopressin receptor.

1.3 The Actions of E_2 on the Vasculature

E_2 exerts effects on both the endothelium and VSM, involving function and growth of the vascular wall (Fig. 2). Perhaps the most well known vascular effect of E_2 is on the nitric oxide (NO) pathway. An abundance of research in this area shows that E_2 enhances NO not only by its genomic action to increase the message for the enzyme that catalyzes formation of NO, endothelial nitric oxide synthase (eNOS), but also by enhancing the level of eNOS activation (20). The effects on eNOS levels occur at the level of gene transcription via ERs, while the enhanced activation of eNOS is a rapid, non-genomic effect. There is a subpopulation of ERα that localizes to caveolae (lipid ordered domains on the plasma membrane of endothelial cells that contain signal transduction molecules) and is coupled with eNOS. E_2 stimulates the tyrosine kinase/MAPK and PI3 kinase pathways and phosphorylates eNOS, resulting in its rapid activation (20). More recently it was discovered that ERβ also has nongenomic actions on eNOS in endothelial cell caveolae and rapidly increases eNOS activity in the presence of E_2 (21).

Numerous studies have established that E_2 increases prostacyclin (PGI_2) synthesis and activity of the cyclo-oxygenase (COX) pathway in various tissues (47, 62, 117). E_2 also appears to be involved in a number of thromboxane (TXA_2)-dependent vascular diseases such as Raynaud's disease (101, 139), primary pulmonary hypertension (109) and pre-eclampsia, all of which involve excessive vasoconstriction and elevated vascular production of TXA_2. The incidence of Raynaud's disease is up to seven-fold higher in women than in men (139), while that of primary pulmonary hypertension is about four-fold higher in women than men (68, 140). Similarly, in pre-eclampsia there is an

unfavorable shift in the balance between PGI_2 and TXA_2 (148), resulting in a greater prominence of TXA_2 during pregnancy. The balance between these two prostanoids is crucial, not only for the regulation of vascular tone, but also for hemostasis (16, 39).

The effects of E_2 on the prostanoid pathway have been elucidated further in more recent studies. Reactivity of the rat thoracic aorta to VP is substantially greater in females than in males or ovariectomized females, and estrogen replacement therapy (ERT) of OVX females restores reactivity to VP to that of the intact female. Selective blockade of various prostanoid pathway enzymes and/or receptors revealed that constitutive forms of COX-2 and thromboxane synthase (TXS) exist in the vasculature, and the greater production constrictor prostanoids (CP), mainly TXA_2, are responsible for the enhanced responsiveness to VP in females (72, 73). Estrogen enhances the release of and reactivity to CP in the female vascular wall and blockade of the pathway reduces reactivity to VP by 25-30%. These effects of E_2 are mediated by increased expression of COX-2, TXS, and TP receptor (73).

E_2 also is known to increase intracellular calcium (IC) release in the endothelium but to decrease extracellular Ca^{2+} entry into VSM (50, 89, 93, 113, 124). Reactivity of the female rat aorta to VP was reported to be more dependent on the release of IC, while that of the male aorta is more dependent on extracellular calcium entry (36).

In addition to the effects of E_2 on the vasculature, E_1, the less potent estrogen secreted by the ovary, exerts both genomic and non-genomic effects on the vascular wall via ERα and ERβ (108, 118), including increasing NO synthesis (via activation of mitogen activating protein kinase – MAPK; a non-genomic effect) and enhancing protein kinase C (PKC) activity and VSM cell proliferation (a genomic effect), as well as

increasing COX, thromboxane, and prostacyclin activity via the phospholipase C – IP_3-diacylglycerol – PKC signal transduction pathway (a non-genomic effect) (108, 118).

1.3.1 Regulation of Estrogen Receptors

E_2 exerts most of its long-term (genomic) effects through the two known cytosolic steroid estrogen receptors (ER), alpha (ERα) and beta (ERβ). Rapid (non-genomic) effects occur via cell membrane-associated steroid receptors and can occur through either ER, as both have been shown to localize to the plasma membrane (21) and are coupled to rapid-acting signal transduction pathways. The expression of ERs by both the VSM and endothelium of the vascular wall is well documented (1, 3, 10, 21, 28, 57, 60), however, the extent of their actions on the vascular wall is a subject that has barely been scratched on the surface.

ERs appear to be highly regulated and their expression is variable among different blood vessels. For example, E_2 treatment decreases expression of ERα in vena cava of OVX rats, but has no effect on ERα expression in thoracic and abdominal aorta (86). In contrast, in cerebral arteries, ERT with E_2 increases ERα gene expression while OVX decreases its expression (86). There is a significant decrease in the expression of cerebrovascular ERβ receptors with OVX, and while ERT with E_2 treatment upregulates ERα in the cerebrovasculature, it has no effect on ERβ gene expression (86).

The mechanisms underlying the effects of E_2 on ER expression appear to involve two distinct mechanisms. First, ERs can be altered by autologous downregulation, where an activated ER interacts with its own gene sequence to decrease its mRNA by suppressing transcription (86). Second, ERs can also be targeted by ubiquitin, a small

protein which marks the protein to which it attaches for degradation by the 26*S* proteasome (86). In this manner, ER proteins are decreased without altering their mRNA. Additionally, the ERα gene can be methylated and inactivated; thus, ERs may be affected by growth factors and hormone signals (86).

1.3.2 Mechanisms of Estrogen Receptor Actions

Most of the actions that estrogens exert throughout the body can be linked to their receptors, ERα and ERβ. The ERs are classical cytosolic steroid receptors, which require that estrogens bind and activate the receptors. The activated receptor-ligand complexes then translocate to the nucleus, and then form either homodimers or heterodimers and bind to estrogen response elements (ERE) located within the regulatory region of the target genes. Binding of the EREs activates transcription (formation of messenger RNA), then translation, resulting in increases or decreases in protein synthesis, which mediate the biological actions of the estrogens. ERs can also interact with other transcription factors, such as activator protein-1 (AP-1) or Sp1 transcription factor instead of the classical ERE; however, not all of the actions of estrogens can be attributed to genomic activity (Fig. 3). More recently, membrane-bound ERs have been identified, which are coupled to G-proteins, as well as so called estrogen receptor-related receptors (ERRs), some of which do not actually bind estrogen, but can actually activate transcription and even interact with traditional ERs (14, 53). The membrane-ERs can rapidly exert their actions without inducing transcription, and are reported to modulate ion channels, G protein-coupled receptors, tyrosine kinases, and mitogen-activated protein kinases (56,

86, 93). Membrane-bound ERs also activate phospholipase C and adenylyl cyclase signal transduction pathways (86).

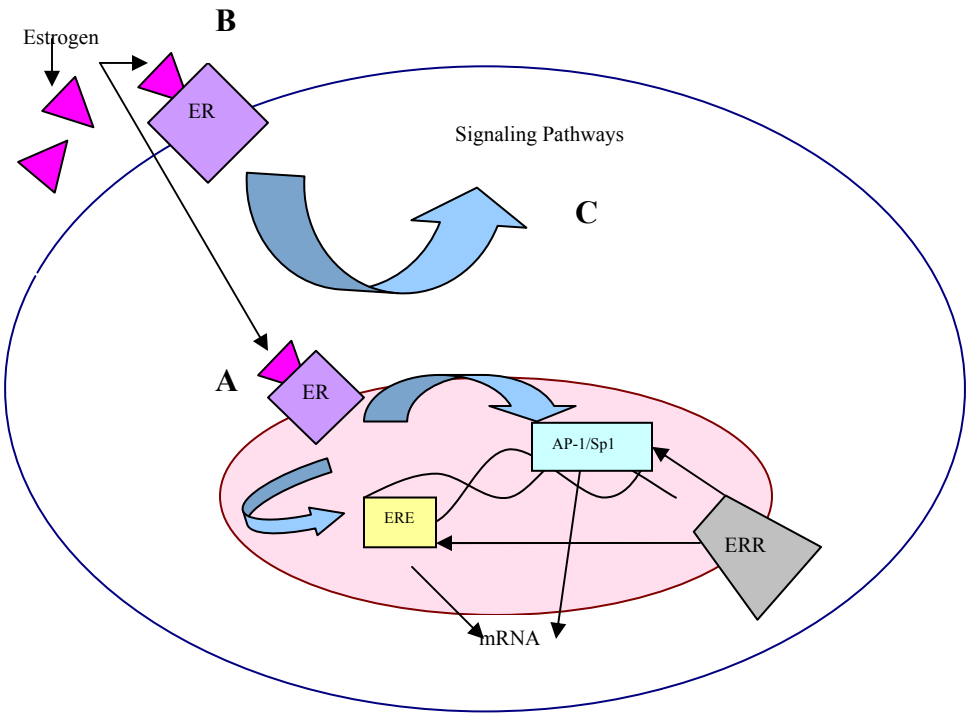

Figure 3: Mechanisms of estrogen action. Adapted from Hewitt *et al.*, 2005 (56). (A) Estrogen binds a nuclear receptor and activates transcription, either through ERE (classical mechanism) or AP-1/Sp1 (nonclassical mechanism). (B) Estrogen binds a plasma membrane bound receptor and activates any one of multiple signaling pathways (C). ER = estrogen receptor, ERE = estrogen response element, ERR = estrogen receptor-related receptor (also in the nucleus), AP-1 =

1.3.3 The Effects of E_2 on Intracellular Calcium

E_2 exerts effects on intracellular calcium (IC) release as well as extracellular Ca^{2+} entry via various calcium (Ca^{2+}) channels in a variety of tissues (113, 124, 135, 151). In the vasculature, the findings are rather conflicting. Some studies report that acute E_2 treatment increases IC release (113), while others show that acute E_2 decreases cytosolic Ca^{2+} levels (104). These conflicting findings may reflect differences in the tissues studied and/or the experimental preparations employed, for example, as some studies have utilized cultured cells (113, 124, 135), while others studied intact vessels (36). Most studies observe rapid (nongenomic) effects of E_2 to increase IC in endothelial cells (89) or genomic effects to down regulate L-type Ca^{2+} channel expression in VSM (93). At present, it appears that only very few studies have investigated the individual effects of ERs on the various Ca^{2+} channels (151).

E_2 exerts its effects on cytosolic Ca^{2+} via the vascular wall using one of two mechanisms. Rapid non-genomic effects occur when E_2 interacts with membrane ERα or ERβ or with another receptor capable of binding E_2, and then activating rapid cell signaling pathways and causing changes in cytosolic [Ca^{2+}] (increasing or decreasing, depending on the cell type) within seconds to minutes. In contrast, the "classical" genomic effect occurs when E_2 binds to its cytosolic receptor(s) and mediates mRNA transcription for vascular Ca^{2+} ion channels, such as decreases in expression of the L-type Ca^{2+} channel in VSM (136). Indeed, E_2 has differing effects on calcium handling in different cells within the same tissue. For instance, E_2 acutely inhibits L-type Ca^{2+} ion channel fluctuation in VSM cells (93), while increasing release from intracellular stores in endothelial cells (89, 113).

The cytosolic Ca^{2+} concentration is very important to the mechanism of contraction in VSM. An increase in Ca^{2+} ion can occur by release from intracellular stores, namely, the sarcoplasmic reticulum (SR) and/or by extracellular entry through membrane Ca^{2+} channels (Fig. 4). Once in the cytosol, the free Ca^{2+} ion binds to calmodulin and the calcium-calmodulin complex in turn activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chains in the presence of ATP, and this phosphorylation allows the myosin heads to form a cross bridge with the actin filaments, resulting in shortening of the VSM cell, and thereby producing contraction (Fig. 5). In VSM, contractile force is graded so that the higher the cytosolic $[Ca^{2+}]$, the greater VSM contraction that results.

In the endothelium, activation of eNOS is dependent upon intracellular Ca^{2+} concentration (66). Similarly, prostanoid synthesis by the endothelium is also a Ca^{2+} dependent process, due to sensitivity of phospholipase A_2 to Ca^{2+} (23). Therefore, the effects of E_2 on the IC pathway could be crucial to the understanding of the multifaceted and complicated effects of E_2 on the vascular wall.

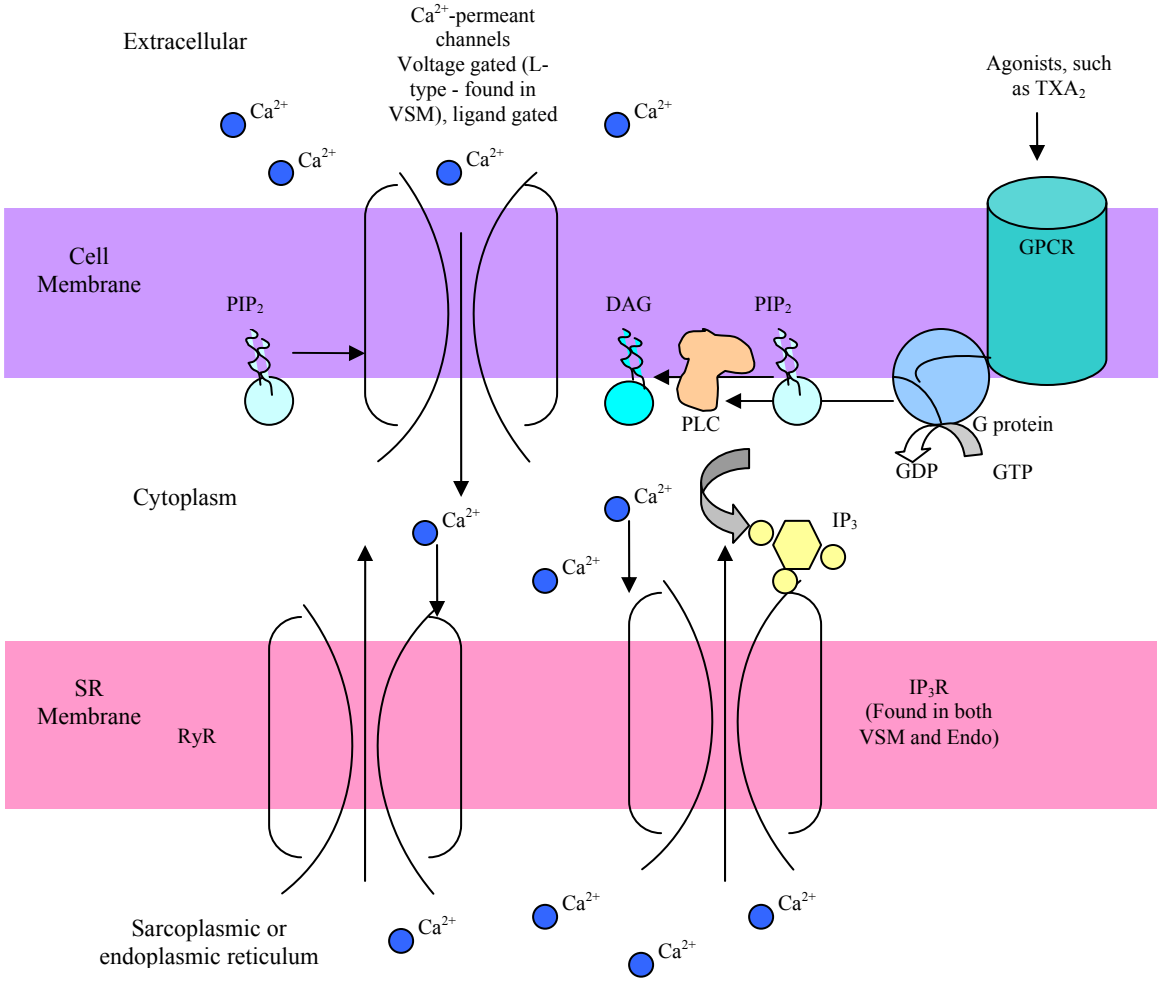

Figure 4: Calcium signaling mechanisms. (adapted from Clapham 2007) (26). Plasma membrane calcium channels are opened by changes in voltage or ligand binding. Ca^{2+} ions rush into the cytoplasm and this triggers additional Ca^{2+} release from RyR and IP_3R. GPCR activates PLC to form IP_3 and DAG from PIP_2. IP_3 activates IP_3R to trigger Ca^{2+} release from SR. PIP_2, phosphatidylinositol 4,5 bisphosphate; DAG, diacyl glycerol; PLC, phospholipase C; GPCR, G protein coupled receptor; GTP, guanosine triphosphate; GDP, guanosine diphosphate; IP_3, inositol triphosphate; IP_3R, inositol triphosphate receptor; RyR, ryanodine receptor.

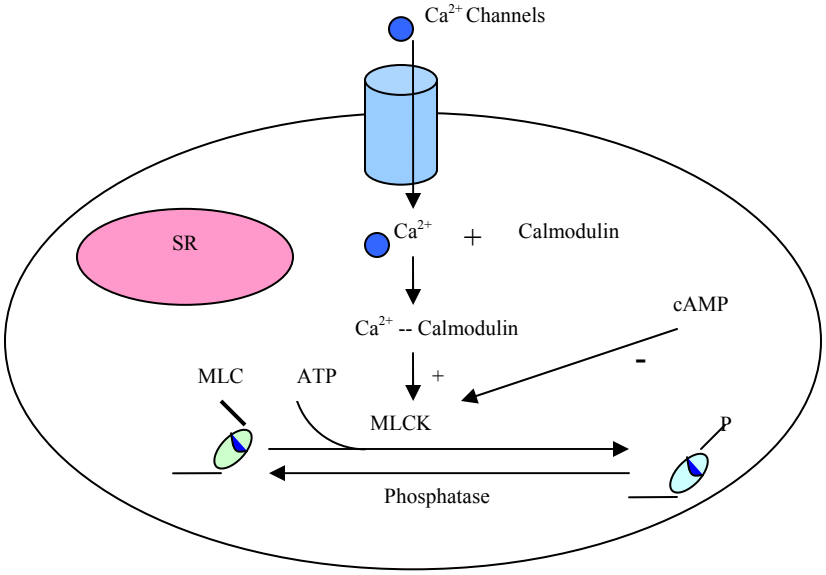

Figure 5: Mechanism of vascular smooth muscle contraction. (Adapted from *Cardiovascular Physiology Concepts,* Klabunde, 2004) (67). Calcium ions enter the smooth muscle cell and combine with calmodulin. The calcium-calmodulin complex along with ATP activates myosin light chain kinase, which phosphorylates the myosin head and enables it to bind with actin. Ca^{2+}, calcium ion; SR, sarcoplasmic reticulum; MLC, myosin light chain; MLCK, myosin light chain kinase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; P, phosphorylation.

1.3.4 The Effects of E_2 on Arachidonic Acid Metabolism

The initial step in arachidonic acid (AA) metabolism is its liberation from membrane glycerophospholipids through the action of phospholipase A_2 (PLA_2) or diacylglyceride (DAG) lipase. The liberated AA is then metabolized to prostaglandin

endoperoxide G_2 (PGG_2) and then to prostaglandin endoperoxide H_2 (PGH_2) by the enzyme cyclo-oxygenase. PGH_2 is then converted to a variety of bioactive prostaglandins (PGs) by their respective terminal PG synthases to form PGI_2 (prostacyclin synthase; PGIS), PGD_2, PGE_2 and PGF2α (mixed isomerases) and to TXA_2 (thromboxane synthase; TXS). The PG synthases exhibit cell- and tissue-specific distributions, resulting in specific profiles of PG production. The liberation of AA from the cell membrane is the rate-limiting step in prostanoid biosynthesis, but much of the research on AA metabolism is focused on the cyclo-oxygenase (COX) enzymes, because they are expressed in several different isoforms and the fact that these enzymes are the primary targets for pharmacological inhibition. COX-1 is localized to the endoplasmic reticulum and is constitutively expressed in many tissues, including the vascular wall, and it is the only isoform expressed in platelets. The prostanoids derived from COX-1 are involved in the maintenance of normal physiological homeostasis ("housekeeping" functions). COX-2 is localized mainly in the nuclear envelope, and is traditionally considered the "inducible" form, as its expression is increased in response to mitogens, endotoxins and proinflammatory cytokines. Recent research has identified a constitutive form of COX-2 in the kidney and aorta (73, 97, 102).

Similar to other areas of research on the effects of E_2, there is conflicting evidence concerning the effects of E_2 on the PG pathway. The effects of E_2 on COX-1, COX-2, and PGI_2 have been studied extensively in various tissues, while the effects of E_2 on TXA_2 and TP receptor have been studied only to a limited extent. It is possible that the apparent conflicts in the evidence for the actions of E_2 may in fact reflect differential effects of E_2 in different tissues. For example, E_2 upregulates COX-1 expression in rat

cerebral vessels (47) and in ovine fetal pulmonary arterial endothelium (62), but does not affect COX-1 expression in endothelium or VSM in rat aorta (73). Since PGIS is the most abundant prostaglandin synthase enzyme in most vascular beds, COX-1 is the enzyme traditionally linked with increases in PGI_2 production in the vascular wall. However, recent studies have shown that COX-2, PGI_2 and TXA_2 are upregulated in parallel in intact female rats, compared to male or ovariectomized female rats, whereas COX-1 is unchanged (73). This recent finding suggests not only the presence of a constitutive form of COX-2, but the possibility that PGI_2 can also formed by COX-2. In light of this more recent evidence regarding regulation of the COX-2 enzyme and its association with TXA_2, function of the prostanoid biosynthesis pathway and its regulation by E_2 must be reconsidered. Because both PGI_2 and TXA_2 share a common precursor (PGH_2; see Fig. 6) and their respective enzyme (PGIS and TxS) as well as COX-2 are all upregulated by E_2, the vascular actions of E2 are more complex than previously recognized, since they involve simultaneous upregulation of both PGI_2 and TXA_2 production by the vascular wall. Thus, it is becoming increasingly apparent that the effects of E_2 on the vasculature are dynamic and complicated.

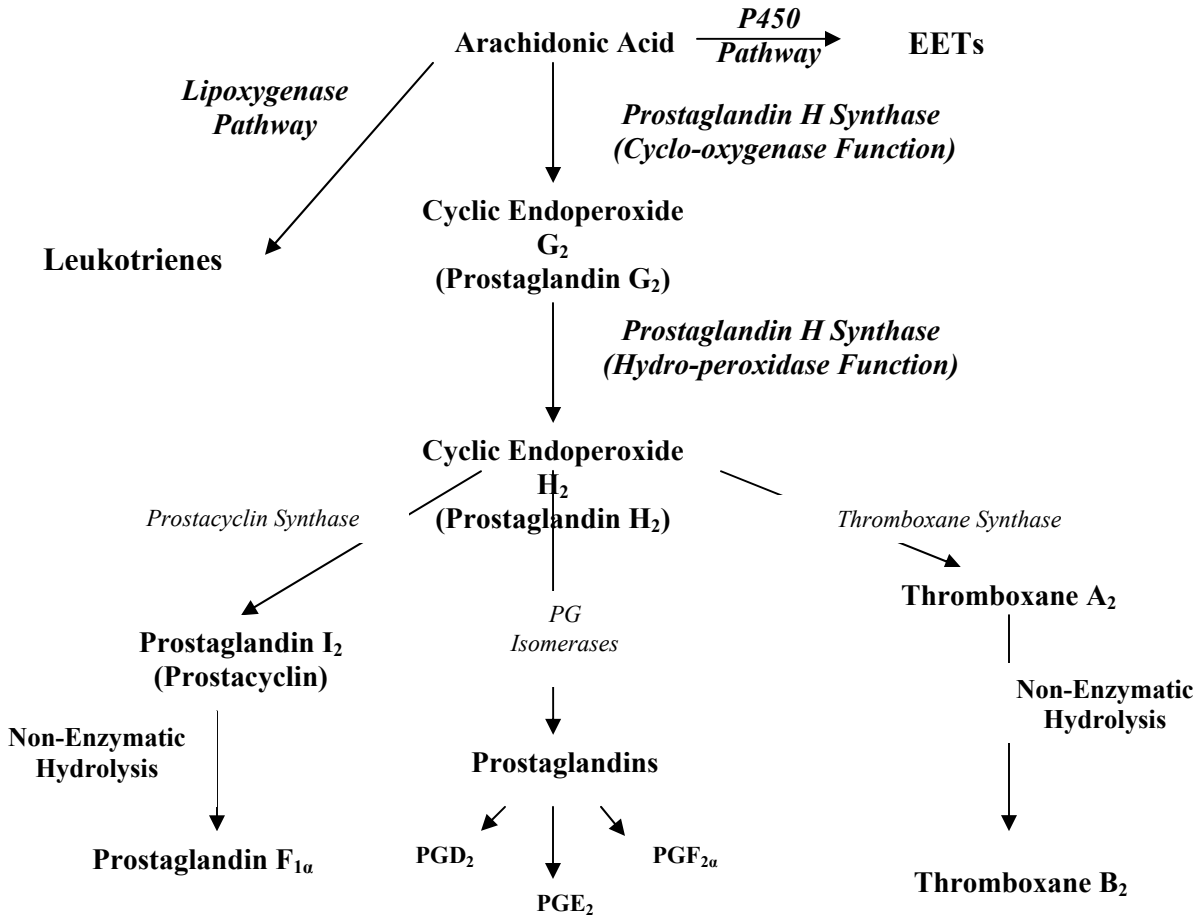

Figure 6: Arachidonic acid (AA) metabolites. Adapted from Moncada and Higgs, 1986 (90).

1.3.5 Mechanism of Action for TXA_2 and PGI_2

The prostanoid receptors as a group belong to the heterotrimeric G-protein-coupled rhodopsin-type superfamily (15). The TXA_2 receptor (TP) is a G_q-type G-protein, which when occupied by its ligand, activates phospholipase C (PLC-β), which in turn cleaves phosphatidylinositol 4,5 bisphosphate (PIP_2) to form 1,4,5-inositol triphosphate (IP_3) and diacylglycerol (DAG) (15). IP_3 then binds to IP_3 receptors on the sarcoplasmic reticulum to stimulate the release of intracellular Ca^{2+} into the cytosol (see

Fig. 4). DAG acts as an additional second messenger, which remains in the cellular membrane and activates one of several isoforms of protein kinase C (PKC). In turn, PKC phosphorylates target proteins leading to the various cellular effects of TXA_2. Mitogen-activated protein (MAP) kinases such as extracellular signal-related kinase 1 and 2 (ERK1/2) and p38 kinase also have been linked to TP stimulation (13, 15). There is also some evidence that p38 mitogen-activated protein kinase (p38 MAP kinase) is required for TXA_2 induced contraction in rat resistance arteries (12).

The PGI_2 receptor (IP) is a $G\alpha_s$-type G-protein. IP stimulation leads to activation of adenylyl cyclase and accumulation of the second messenger cyclic AMP (cAMP), and this in turn leads to activation of cAMP-dependent protein kinase (PKA). PGI_2 is the predominant AA metabolite produced by endothelial cells and there is conflicting evidence about which COX enzyme is responsible for its production (COX-1 or -2) (39, 41, 64, 82).

In 1983, Moncada *et al.* proposed that the balance between PGI_2 and TXA_2 was important to maintaining hemostasis in the circulation (90). More recently it was shown that IP knock-out mice show enhanced platelet activation and vascular proliferation in response to vascular injury (24). TXA_2 biosynthesis is increased in wild-type mice after vascular injury, and this response is further enhanced in the IP knock-out mice (24). There is also growing evidence of cross talk between IP- and TP-dependent signaling pathways: TP agonists stimulate PGI_2 release from the endothelium, TP desensitization causes sensitization to IP agonists and IP activation evokes TP desensitization (24). It is becoming more evident that the proper balance between TXA_2, PGI_2 and possibly other

metabolites of the AA pathway is complex and crucial to the maintenance of normal vascular homeostasis.

1.4 Recent Findings – Deleterious Effects of Estrogens

Recently it was determined that the non-selective COX inhibitor indomethacin (Indo) attenuates the contractile responses to vasopressin (VP) and to the α-adrenergic agonist phenylephrine (PE) in female but not male rat aorta (43). Contractile responses to VP and PE in the female rat aorta also are attenuated by the PGH_2/TXA_2 receptor antagonist SQ 29,548, suggesting that CP play a significant role in the regulation of tone in the normal female vasculature, but play little or no role in the normal male vasculature. Sex differences in constrictor responses to the thromboxane mimetic U-46619 also occur in the rat aorta (72). The contractile responses of the female aorta to U-46619 are significantly higher than those of male aorta, and OVX markedly attenuates while E_2 replacement restores responses to U-46619, revealing that E_2 enhances reactivity to TP agonists (72).

The prominent sex differences in reactivity to VP in the rat aorta are clearly estrogen dependent, since INT and E_2 replaced (ER)-OVX female Sprague-Dawley rats are more reactive to VP than OVX females or males (43, 72). Reactivity to VP in the female rat aorta is enhanced by CP and it appears that COX-2 is the isoform responsible for CP synthesis in the aorta, as COX-2 selective inhibitors such as NS-398 and niflumic acid attenuate reactivity to VP to the same extent as the non-selective COX inhibitor Indo (41% and 47% vs. 43% respectively) (72).

The greater effects of CP in the female vasculature are associated with estrogen-enhanced expression of COX-2, thromboxane synthase (TXS) and TP receptor mRNA in both Endo and VSM of the female aorta, whereas estrogen-dependent differences in the expression of COX-1 mRNA were not observed (73). Immunohistochemical (IHC) studies of the aorta revealed that expression of COX-1 protein in Endo and VSM did not differ among male, intact female, OVX female or OVX-ER females, whereas COX-2 and TXS staining were higher in Endo and VSM of intact and OVX-ER females, compared to males and OVX females (73).

The cellular and molecular effects of estrogen on the COX pathway in the female rat aorta are paralleled by functional changes in the release of TXA_2 and PGI_2, which are higher in INT and OVX-ER females, than in males or OVX females (73). In contrast, radio-immunoassay (RIA) of basal prostanoid release does not differ between sexes or in OVX vs. INT females (73). The prostanoid release data, coupled with the mRNA expression and IHC data clearly reveal that estrogen enhances both the production of and the reactivity to CP in the normotensive female rat aorta.

If the activity of the entire TXA_2 pathway is upregulated by E_2 in the normotensive female rat (compared to the male rat), what are the consequences of this sex difference in CP function during pathophysiological states such as hypertension? Of particular interest, what are the consequences of these sex differences in CP function in models of hypertension that are dependent on the TXA_2 pathway? Aortic coarctation-induced hypertension (ACIH) is just such a model of hypertension. Ligation of the abdominal aorta (see section 1.3) results in a markedly lower blood pressure in the left than the right kidney, enhancing the release of renin by the left kidney and the formation

of angiotensin II (ANG II) and CP. The acute phase of ACIH (12-14 days) exhibits marked increases in vascular tone due to increases in reactivity to ANG II and ANG II-stimulated CP release, mainly TXA_2 (75).

Recent studies on the sexual dimorphism of the CP pathway in ACIH reveal that INT female rats do indeed exhibit exacerbated hypertension in response to ACIH. During the acute phase of ACIH (12-14 days), the MAP of female hypertensive (F-HT) rats continues to increase over the entire time period to 186 ± 7 mmHg. In contrast, in male hypertensive (M-HT; 160 ± 4 mmHg) and ovariectomized female hypertensive (OVX-HT; 150 ± 8 mmHg) rats, MAP plateaus at lower levels between days 6 and 10 (6, 7). Estrogen replacement of OVX-F hypertensive rats (OVX+ER-HT) restores MAP (171 ± 3 mmHg) to levels comparable to those of INT females (6, 7). Further, during an acute i.v. infusion of SQ 29,548, F-HT and OVX+ER-HT exhibited marked decreases in MAP of 50-60 mmHg, while M-HT and OVX-HT decreased only 20-22 mmHg. Chronic daily oral treatment with Ridogrel (TxS and TP receptor antagonist) during the development of ACIH produces a 40-60 mmHg decline in MAP in F-HT and OVX+ER-HT, while M-HT and OVX-HT exhibited a minimal decline in MAP (6, 7). These data reveal that the CP pathway is largely responsible for the higher pressures in the F-HT and OVX+ER-HT rats, that the TXA_2 pathway is active in the resistance arterioles responsible for the regulation of MAP, and that E_2 plays a significant role in the regulation of CP pathway function and blood pressure in this model of hypertension.

In vitro prostanoid release studies in the thoracic aorta reveal that basal TXA_2 release does not differ between M and F during NT; however, in response to ANG II, TXA_2 release is nearly 2-fold higher in F than in M aortas. During the acute phase of

ACIH, there is a 7-fold increase in basal TXA_2 release in F compared to NT, while M only exhibits a 4-fold increase (6, 7). Therefore, basal release of TXA_2 during hypertension is nearly 2-fold higher in F than in M aorta, and ANG II stimulated release remains 28% higher in F than in M aorta.

PGI_2 release in ACIH is slightly more variable than TXA_2. There were no differences in basal release between F and M in NT controls; however, ANG II stimulation of the aorta did not increase PGI_2 release, in contrast to TXA_2 release. During the acute phase of ACIH, basal release of PGI_2 is 1.5-fold higher in F than in M, and ANG II stimulation produces a small increase in PGI_2 release in F, while there is no change in M (6).

While it is clear that E_2-dependent CP function is enhanced to a much greater extent in F than in M during the development of ACIH, several important issues regarding the relationship between E_2, CP function, and blood pressure remain unanswered. Specifically, what are the effects of the individual ERs on the CP pathway, the development of ACIH, and HT-associated changes in vascular function? It is also not known whether arterioles (100-200 μm) show similar trends of TXA_2 and PGI_2 release in response to VP, and whether the individual ERs have differing effects on arteriolar function or the development of hypertension.

1.5 Aortic Coarctation-Induced Hypertension

The model of hypertension known as aortic coarctation-induced hypertension was chosen for the present studies for two reasons. First, it is well known that ACIH is a renin-dependent and CP-dependent form of hypertension (5, 70, 74). To produce this

model, the aorta is completely ligated between the renal arteries, resulting in decreased arterial pressure in the left kidney, and subsequently a dramatic increase in renin release. This renin release results in downstream increases in angiotensin II (ANG II). ANG II acts directly on the vasculature to cause vasoconstriction; in addition, it stimulates generation of TXA_2 by the vascular wall, increasing local and plasma levels of TXA_2 (87, 144). Second, it is a form of hypertension in which female rats develop markedly higher MAP than males (7). The higher MAP results from the effects of estrogen on the CP pathway, vascular function and structure. When females are OVX, the MAP and TXA_2 production are much lower and is similar to that seen in male rats. Thus, ACIH is a relevant model to study the vascular actions of E_2 and CP involved in the development of hypertension.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals

Female Sprague-Dawley rats (14-16 weeks old at time of study, Harlan; Houston, TX) were used in all studies. Animals were housed in vivarium facilities at the College of Veterinary Medicine and Biomedical Sciences with controlled temperature (22-24°C), relative humidity (~50%), and lighting (12:12-h light-dark cycle). Animals were housed in pairs in standard plastic laboratory rat cages. Tap water and rat chow (HarlanTek-lad Global Diet; 16% protein) were provided ad libitum. This special diet is free of phytoestrogens, which are common in standard rat chow and have been reported to confound the effects of ovariectomy on vascular reactivity (28, 50). All experiments were reviewed and approved by the Texas A&M University Small Animal Care Committee (SACC).

NT and HT rats were divided randomly into ten experimental groups: intact normotensive females (F-NT), ovariectomized normotensive females (OVX-NT), estrogen receptor alpha agonist-treated OVX normotensive females (OVX+PPT-NT), estrogen receptor beta agonist-treated normotensive females (OVX+DPN-NT), ovariectomized normotensive females with 17-β estradiol replacement pellets (OVX+ER-NT), intact hypertensive females (F-HT), ovariectomized hypertensive females (OVX-HT), estrogen receptor alpha agonist-treated hypertensive females (OVX+PPT-HT), estrogen receptor beta agonist-treated hypertensive females (OVX+DPN-HT) and ovariectomized hypertensive females with 17-β estradiol replacement pellets (OVX+ER-HT). Previous studies established that contractile responses to vasopressin and

phenylephrine did not vary during various stages of the estrous cycle; therefore, intact female vessels were studied without regard to phase of the estrous cycle (2).

2.1.1 Animal Preparation

At 6 weeks of age, half of the female rats were subjected to bilateral OVX using standard surgical methods (see section 2.0.2 for anesthetic procedure). OVX rats remained untreated, or received estrogen replacement therapy (ER) with 17-β estradiol time-release pellets (2 pellets/rat; 0.05 mg/60-day pellet, 17-β estradiol), or with an ERα selective agonist (4, 4', 4"-(4-Propyl-[1H]-pyrazole-1, 3, 5-triyl)*tris*phenol; PPT, 410-fold selectivity of ERα over ERβ) (129a), or with an ERβ selective agonist (2,3-*bis*(4-Hydroxyphenyl)-propionitrile; DPN, 70-fold selectivity of ERβ over ERα) (86a), or vehicle treatment. 17β-estradiol pellets were placed approximately 1 week prior to coarctation or sham surgery and no sooner that 4 weeks following OVX, although for most rats, there was a 6-8 week interval from OVX to pellet implantation. PPT and DPN treatments were started 1-2 days prior to coarctation or sham surgery, both to acclimatize the rats to the injection and to ensure stable circulating plasma levels at the time of surgery. PPT and DPN were dissolved in DMSO, and then diluted 1:10 in corn oil for daily subcutaneous injection. Vehicle alone (1:10 DMSO in corn oil) was used in the vehicle control-treated groups (INT-F and OVX-F). The final concentration of PPT and DPN in solution was 0.5 mg/ml. Each rat received 0.10 mg/day of PPT or DPN (0.20 ml) injection each day for 13-15 days. Vehicle treated rats received 0.2 ml of vehicle alone. Treatment times were similar in all groups receiving PPT or DPN.

2.1.1.1 Blood Samples for Estradiol and Estrone Measurement

Blood samples were taken on the day of (but before) pellet implantation and one week after pellet implantation on the day of ACIH (or sham surgery) in OVX and OVX+ER as well as days 4, 7 and 10 after ACIH or sham surgery. In INT, surgeries were performed and the day of diestrus, and blood samples were taken on the subsequent days of diestrus (approx. every 4 days). Plasma 17β-estradiol and estrone levels were measured in these groups using specific RIA kits for 17β-estradiol and estrone.

2.1.1.2 Vaginal Smears

Vaginal smears were performed each day on INT-F to determine phase of the estrous cycle.

2.1.2 Preparation for Aortic Coarctation-Induced Hypertension

Aortic coarctation or sham surgery was performed at 12-14 weeks age. Rats were given a subcutaneous injection of atropine (25 μg/ml) as a pre-anesthetic at a dose of 0.05 mg/kg. Rats were then anesthetized with an IP injection of a solution of Ketamine (55 mg/kg) and Chloral Hydrate (180 mg/kg). After the animals achieved a stable plane of surgical anesthesia, the abdominal aorta between the renal arteries was exposed and ligated with silk 4-0 suture using sterile surgical procedures. In sham coarcted animals, the aorta was dissected free but not ligated. A chronic indwelling catheter (Micro-Renathane-033 tubing) was placed in the right carotid artery in some groups of animals for measurement of mean arterial pressure. Since rats were prone to catheter tampering,

custom vests were placed around the chest of the animal to secure the catheters and protect them from damage from chewing.

2.2 Measurement of Mean Arterial Pressures

Rats used in BP studies were trained in small restrainer boxes (9 x 3.5 x 3.5 in.) that allowed for free movement but prevented the rats from tampering with the catheters. The rats were trained for approximately 2 weeks prior to surgery for the measurement of arterial blood pressure (MAP). Using the chronically implanted carotid artery catheters, MAP was measured every other day for twelve days post-coarctation or sham surgery. The catheters were connected to a pressure transducer (Model DTX, Becton-Dickinson) and pressures recorded continuously on a Gould Model 2400S chart recorder. The rats were allowed a 10 minute adjustment period in the restraint box before the pressures were recorded. After the adjustment period, MAP and heart rate (HR) were recorded for 30 minutes and averaged.

2.3 Preparation of Isolated Mesenteric Arterioles

Rats were euthanized via rapid decapitation at 12-14 days post-coarctation. The entire mesentery was removed and immediately placed in chilled, (4°C) gassed (95% O_2/5% CO_2) KHB solution for function studies or in chilled RNALater for molecular studies. Vessels were immediately dissected free of surrounding fat and connective tissue prior to being used in vessel function (myograph) or PG release, or placed in RNALater at -20°C (molecular studies) as described below. First and second order arterioles (approx. 150-200 μm) branching off the superior mesenteric artery were used in all

myograph and PG release studies. The entire mesenteric arcade was used in all molecular studies.

2.4 Vascular Function

Rats were euthanized by rapid decapitation and the entire mesentery and small intestines were then removed rapidly *en bloc* and placed in chilled, (4°C) gassed (95% O_2/5% CO_2) KHB. The intestines and mesentery were then placed in fresh buffer in a Petri dish, along with a frozen cube of KHB to ensure that the solution remained at 4°C during the entire dissection procedure. First and second order mesenteric arteries were cleaned of fat and connective tissue under a dissecting binocular microscope (Olympus SZ), taking care not to stretch or damage the vessels in any way. Once cleaned of connective tissue and fat, the arteries were then cut away from the mesenteric arcade and placed in fresh, cold KHB. Using a Filar eyepiece micrometer, the arteries were then cut into four segments approximately 1.50 mm long each. The vessels were allowed to rest in cold, gassed KHB for 45 minutes to an hour prior to mounting on the myograph.

After resting, each vessel segment was mounted on a wire myograph (Danish Myotechnology) for force-tension studies. Each 1.50 mm vessel segment was placed on the foot of a myograph chamber and a 40 μm diameter piece of stainless steel wire was passed through the lumen of the vessel, taking care not to tear or damage the smooth muscle or endothelium. The wire was then secured under a screw at the top of the left foot of the myograph. The wire was then stretched straight across the left foot of the myograph and then secured at the bottom screw of that same foot, thereby positioning the vessel vertically. A second wire was passed through the lumen of the vessel and then

secured at the top and bottom screws of the right foot. The feet were then adjusted so that the two wires were parallel throughout the length of the vessel. The wires were then stretched just to the point of touching the endothelium without stretching the vessel. Measurements were then made for vessel segment length, internal diameter and wall thickness using an ocular micrometer. The myograph chambers were then placed on the main block of the myograph for recording tension and heating the bath to 37°C. 95% O_2/5% CO_2 was bubbled into the myograph chambers continuously throughout the experiment. Once all four chambers were connected, the chambers were then drained and refilled with 6 mL of fresh KHB, gassed and warmed to 37°C. The resting tension-internal circumference relationship was determined for each vessel using progressive stretches (50 μm per stretch) and measurements of passive tension and internal vessel circumference (L) at each level of stretch. L was calculated by using the following formula: $L = 2f + 4\ D/2 + 2(\pi D/2)$, which reduces to $L = 2f + D\ (2 + \pi)$, where D is wire diameter and f is the distance between the wires as measured by the ocular micrometer (92). Once L was determined, the vessels were stretched to $L_{95,}$ where L_{95} is 0.95 L_{100} and L_{100} is the internal circumference the artery would have under a transmural pressure of 100 mmHg (92). For the hypertensive groups, the transmural pressures were set at 0.95 L_{150} for OVX females, and 0.95 L_{180} INT, PPT or DPN females, respectively. The L values were based on the average MAP measured in those groups of rats at 12 days post aortic coarctation prior to sacrifice. A contractile response to phenylephrine (PE; $2x10^{-6}$M) was used to determine smooth muscle viability and a subsequent dilation response to acetylcholine (ACh; 10^{-6}M) was used to determine endothelial integrity. Myograph chambers were then drained and refilled with fresh, warmed, gassed KHB.

The vessels were then allowed to re-equilibrate in the baths for 20 minutes prior to pretreatment with inhibitors and/or vehicle-controls added to the baths. Following a 20 minute pretreatment, a cumulative concentration-response to vasopressin (VP; 10^{-12} – 10^{-8}) was obtained.

2.5 Evaluation of Basal and Agonist-stimulated PGI_2 and TXA_2 Production

Paired adjacent segments of mesenteric feed arteries (2-3 mm long; 150-200 μm) were cleaned of all connective tissue and fat, and were then placed into chilled, (4°C) gassed (95% O_2/5% CO_2) KHB solution, allowing the arteries to rest for at least 30-45 minutes. The arteries were then transferred into 1.5 ml polyethylene microcentrifuge tubes with 450 μl KHB, gassed continuously, and gradually warmed up to 37°C. After pre-incubation for 30 minutes, the KHB solution was carefully aspirated, then 300 μl of either KHB alone (basal) or KHB with VP (10^{-8} M) was added to the tissues and incubated for 45 minutes at 37°C and gassed continuously. After incubation, the KHB was collected and stored in -80°C until RIA of the stable metabolites of PGI_2 (6-keto-prostaglandin $F_{1\alpha}$; 6-keto-$PGF_{1\alpha}$) and TXA_2 (TXB_2). RIA is performed in duplicate for each sample.

2.6 RNA and Protein Extraction from Rat Mesenteric Arterioles

Mesenteries were quickly removed and the arteries, veins and fat were cut away from the small intestine. The mesenteric arcade was then placed in ice cold RNALater (Ambion) and stored at -20°C for a minimum of three days prior to further dissection. Tissues were submerged in cold RNALater (4°C) in a Petri dish that had been cleaned

previously with RNaseZap (Ambion) to destroy any RNase present. The Petri dish was then placed on a platform of ice during dissection. Arteries were dissected away from fat and connective tissue, then placed in fresh RNALater and stored at -80°C until RNA isolation.

Mesenteric arterial tissues were submerged in 1.0 mL of TRI reagent. Tissues were homogenized for 15 seconds and then placed on ice for 1 minute; homogenizing and icing steps were successively repeated three to four times. The homogenate was centrifuged at 14,000 RPM for 5 minutes to remove large cytoskeletal components. The supernatant was aspirated and transferred to a new RNase-free tube, 105 µl of 3M sodium acetate and 350 µl of bromochloropropane were added, and the contents were mixed and placed on ice for 15 minutes. The sample was centrifuged at 12,000 RPM for 5 minutes; the aqueous phase was aspirated and transferred to a new RNase-free tube for further RNA extraction. The organic phase was aspirated and transferred to a new RNase-free tube, 300 µl of 100% ethanol was added and the sample was stored at -20°C for later protein extraction. For each mL of the aqueous phase, 1 mL of phenol/chloroform/isoamyl alcohol (125:24:1) was added, and the tubes were mixed vigorously and centrifuged at 12,000 RPM for 5 minutes. The aqueous phase was again aspirated and transferred to a new RNase-free tube, with equal volume of phenol/chloroform/isoamyl alcohol (125:24:1), mixed and centrifuged at 12,000 RPM for 5 minutes. Total RNA was then precipitated by incubating the final aqueous extraction with an equal volume of 100% isopropanol at 4°C for 30 minutes. The isopropanol-extracted mixture was then centrifuged at 14,000 RPM for 30 minutes at 4°C to pellet the precipitated RNA. The RNA pellet was then washed with 70% ethanol, dried at room

temperature for 5 minutes, and dissolved in RNAStore (Ambion) at -80°C until real time RT-PCR was performed. Prior to performing real time RT-PCR, all samples were treated with Turbo DNase system (Ambion) to destroy any DNA present in the samples.

The organic phase samples (for protein analysis) were thawed and centrifuged at 6000 RPM for 5 minutes at 4°C. The supernatants were removed and incubated with 1.5 mL of isopropanol for 10 minutes at room temperature. The samples were then centrifuged again at 14,000 RPM for 10 minutes at 4°C to pellet the proteins. The pellets were then washed with 2 mL of 0.3 M guanidine hydrochloride/95% ethanol solution, incubated at room temperature for 20 minutes, and then centrifuged at 11,500 RPM for 5 minutes at 4°C. This process was repeated 3 times. After the final wash, the pellets were air dried for 5-10 minutes at room temperature. Total proteins were dissolved in 10M urea while incubating at 50°C. Lysis buffer was added to the samples prior to storage at -80°C. Lysis buffer consisted of: 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM Na-Orthovanadate (pH 10), 5 µM $ZnCl_2$, 100 mM NaF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride and Milli-Q water. Amount added depended on the size of the pellet.

A second method was used for protein extraction in the second half of the study when RNA was not required. In this method, freshly removed mesenteries were placed in RNALater and placed in the freezer for at least 72 hours prior to dissection. Mesenteric arteries were then dissected in fresh ice cold RNALater and the fat, veins and nerve bundles were stripped away, leaving the entire mesenteric arcade for protein extraction. Mesenteric arcades were stored in fresh RNALater at -80°C until ready for protein extraction.

Mesenteric arcades were then removed from RNALater and 250 µl of lysis buffer was added to the tissue in 4 mL tubes along with a magnetic steel bead. The tissues were then ground up in a Fisher high-throughput tissue homogenizer at speed setting 10 for approximately 10 minutes. Tubes were removed from the machine at the halfway point (5 minutes) and placed on ice for 5 minutes to ensure that the tissues did not overheat before completing the homogenization for an additional 5 minutes. The tissue homogenates were then transferred to 2 mL Eppendorf tubes and centrifuged at 14,000 RPM at 4°C for 30 minutes. The supernatant was aspirated and transferred to a fresh Eppendorf tube and placed in the freezer at -80°C until proteins were quantitated and immunoblots were performed.

2.7 Real Time RT-PCR

The Invitrogen SuperScript III Platinum qRT-PCR with SYBR Green Kit was used for all real time RT-PCR assays. Total mRNA was quantitated using the Agilent RNA Nano Kit.

2.7.1 First-Strand cDNA Synthesis (Reverse Transcriptase)

The kit instructions provided by the manufacturer were followed. All tubes were placed on ice throughout the preparation procedure. A mastermix of kit components was made, including: 2X RT Reaction Mix, RT Enzyme Mix and DEPC-treated water. 12 µl of mastermix was added to 300 ng of mRNA in each 200 µl Eppendorf tube. Additionally, a separate mastermix which excluded the RT Enzyme Mix was prepared and added to tubes with 150 ng mRNA as the external control (RT-). Samples were then

placed in a thermocycler and incubated at 25°C for 10 minutes and then incubated at 42°C for 50 minutes. The reaction was then terminated by incubation at 85°C for 5 minutes and then chilled on ice. 1 µl of E. coli RNase H was added to each tube and the samples were incubated at 37°C for 20 minutes. The samples were then stored at -20°C until the real time PCR assay was performed.

2.7.2 Real Time PCR

Following the protocol from the Invitrogen PCR kit, a mastermix of Platinum SYBR Green qPCR SuperMix-UDG, ROX Reference Dye, forward and reverse primers and autoclaved distilled water in a total volume of 45 µl was added to each reaction tube. 5 µl of cDNA from the RT reaction was added to the appropriate reaction tube. Duplicate tubes for each RT+ reaction were made. 18S RNA was used as an internal control. Due to high expression of 18S RNA, a 1:10 dilution of cDNA from the RT+ tubes was made into autoclaved distilled water. 5 µl of this dilution was used in the 18S PCR reaction.

Following PCR kit instructions, a 3-step cycling program was used for the PCR reaction: 50°C for 2 minutes hold, then 95°C for 2 minutes hold, then 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. PCR Reactions were run on ABI Prism 7700 and 7500Fast. The results were exported to a desktop computer and saved into an Excel spreadsheet for data analysis.

2.8 Immunoblot

Total protein was quantitated using the Pierce BCA Protein Assay Kit. After quantification, protein samples were combined with sample buffer (1.15 ml distilled H_2O,

0.42 ml 75% glycerol, 0.63 ml 0.5 M Tris (pH 6.8), 0.25 ml beta-mercaptoethanol (Sigma Chemical, St. Louis, MO), 0.05 ml 0.25% bromophenol blue and 0.125 g SDS) and then immersed in boiling water (100°C) for 5 minutes to denature the proteins. The sample buffer and protein mixture were then loaded onto the SDS-PAGE gels at a volume of 40 µl.

40-50 µg of total protein were required to visualize the ERs in the mesenteric arteries. The positive controls were human recombinant estrogen receptor β for ERβ and uterine protein or MCF-7 whole cell lysate for ERα. Beta actin was used as a loading control.

Proteins were combined with the sample buffer and loaded onto 7.5% Tris-HCL pre-cast gels (Bio-Rad). Electrophoresis was performed at 125 V for 75 minutes. After the electrophoresis, the gel was teased off into a 0.2% SDS transfer buffer solution, along with one piece of filter paper and one sponge. The PVDF membrane was first wet in methanol, then rinsed in Milli-Q water and finally placed in transfer buffer with no SDS, along with a second piece of filter paper and sponge to be used in the "sandwich". The transfer unit was assembled in the 0.2% SDS transfer buffer solution and transferred overnight at constant voltage (12 V). The next morning the transfer unit was disassembled and the membrane rinsed in Milli-Q water and dried at room temperature. The membranes were stored in a desiccation chamber until the immunoblots were performed.

Prior to incubation with the antibodies, PVDF membranes were soaked in methanol. The membranes were then washed in Tris buffered saline (TBS) for 5 minutes and then placed in blocking solution (5% non-fat milk in TBST; TBS + 0.1% Tween 20)

for a minimum of 2 hours at room temperature. The primary antibodies were then added to the milk solution at the following dilutions: ERα 1:200 (rabbit polyclonal, cat. no. sc-542; Santa Cruz), ERβ 1:500 (mouse monoclonal, cat. no. ab16813; Abcam), and beta actin 1:2500 (rabbit polyclonal, cat. no. ab8227; Abcam). The primary antibodies were then incubated with the membranes overnight at 4°C on a rocker. The membranes were removed from primary antibody solutions, rinsed in TBST for 15 minutes, and then rinsed in fresh TBST 2 times for 5 minutes each time prior to placing in the secondary antibody solutions. The secondary antibodies were also diluted in 5% non-fat milk in TBST at the following dilutions: ERα 1:10.000 (goat anti-rabbit, cat. no. ab6721; Abcam) ERβ 1:10,000 (rabbit anti-mouse, cat. no. ab5762; Abcam) and beta actin 1:5,000 (goat anti-rabbit, cat. no. ab6721; Abcam). The secondary antibodies were incubated for a minimum of 2 hours at room temperature, followed by one 15 minute wash with TBST, and then four to six 5 minute washes prior to addition of the substrate for visualization in the imaging equipment (Fuji; Model LAS 3000). Substrate (Millipore) was added to the blot for 5 minutes. All secondary antibodies were horseradish peroxidase (HRP) conjugated.

2.9 Radioimmunoassay (RIA) of 6-keto-$PGF_{1\alpha}$, TXB_2, Estradiol and Estrone

Basal and VP-stimulated release of prostacyclin and TXA_2 by mesenteric arterioles into the incubation medium were measured using specific RIAs for the stable metabolites 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 as reported previously (72, 126). Briefly, prostanoid standards (1.95-1,000 pg) or unknown samples were incubated with [^{3}H]-6-keto-$PGF_{1\alpha}$ or [^{3}H]-TXB_2 and with the appropriate

prostanoid antiserum overnight at 4°C. The charcoal-dextran method was used to separate bound and free fractions of [^{3}H]-6-keto-$PGF_{1\alpha}$ or [^{3}H]-TXB_2. Bound radioactivity was counted by liquid scintillation spectroscopy. The limit of detection of the RIAs is 1.95 pg/tube for TXB_2 and 3.90 pg/tube for 6-Keto-$PGF_{1\alpha}$; the cross-reactivity of the antiserum to other prostanoids is <0.1%, and the intra-assay and interassay coefficients of variation are 5.0% and 7.6% respectively (126).

17β-estradiol was measured using a double-antibody RIA. Blood samples taken to assess plasma estrogen levels were centrifuged and the plasma stored at -80°C until the assays were completed. Estrogen standards (0-500 pg/ml) or unknown samples were incubated with [I-125]-estradiol and antiserum at room temperature (25°C) for one hour. The bound and free fractions were separated using a secondary antibody and polyethylene glycol. The intra-assay and interassay coefficients of variation for 17β-estradiol are 7.1% and 4.6% respectively (Diagnostic Systems Laboratories, Inc.).

Estrone was measured using a double-antibody RIA. Estrone standards (0-2,000 pg/ml) or unknown samples were incubated with [I-125]-estrone and antiserum at room temperature (25°C) for one hour. The bound and free fractions were separated using a secondary antibody and polyethylene glycol. The intra-assay and interassay coefficients of variation for estrone are 6.5% and 9.1% respectively (Diagnostic Systems Laboratories, Inc.).

2.10 Data Analysis

All data were expressed as the mean ± standard error with "n" indicating the number of animals studied for each experimental treatment group. Prostanoid output is

expressed as picograms of 6-keto-$PGF_{1\alpha}$ or TXB_2 per mg dry weight of mesenteric arterioles. Plasma 17β-estradiol and estrone concentrations are expressed as picograms per mL. To detect significant differences among the means of the experimental groups, data groups were analyzed by 1-way or 2-way analysis of variance (ANOVA). Factors compared for the 2-way ANOVA were sex (F vs. OVX vs. OVX-DPN vs. OVX-PPT or F vs. OVX vs. OVX+ER) and experimental treatment (VEH vs. SQ and VEH vs. SIM or NT vs. HT). Unpaired t-tests were used to determine significant pair-wise differences among the means of the various experimental groups. The Bonferroni test was employed to correct for type I error associated with multiple comparisons and differences between means were accepted as significant if $P<0.05$.

2.11 Chemicals

DPN and PPT were purchased from Tocris Bioscience (Ellisville, MO). Phenylephrine hydrochloride and acetylcholine chloride (Sigma Chemical, St. Louis, MO) were prepared daily from 10^{-2} M stock stored at -20°C and diluted in KHB. Arginine vasopressin (Sigma Chemical, St. Louis, MO) was prepared daily from 10^{-3} M stock stored at -80°C and diluted in KHB. SQ 29,548 (Cayman Chemical, Ann Arbor, MI) was dissolved in 100% ethanol (1 mg/ml), stored at -20°C, and diluted daily with KHB. Simvastatin was a generous gift from Merck. ERβ and beta actin primary and secondary antibodies were purchased from Abcam (Cambridge, MA) and ERα primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All lysis buffer and immunoblot ingredients were purchased from Sigma Chemical or Fisher Scientific (Fair Lawn, NJ) and were of reagent grade quality. RNALater,

RNaseZap and TurboDNase were purchased from Ambion (Austin, TX). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA).

SuperScript III Platinum SYBR Green Real time RT-PCR kit and primers were purchased from Invitrogen (Carlsbad, CA). Primer sequences were as follows: ERα forward: CTG ACA ATC GAC GCC AGA A; ERα reverse: CAG CCT TCA CAG GAC CAG AC (GenBank Accession No. Y00102); ERβ forward: CTT GCC CAC TTG GAA ACA TC; ERβ reverse: CCA AAG GTT GAT TTT ATG GCC (GenBank Accession No. U57439); 18s RNA forward: AAA TGT GGC GTA CGG AAG AC; 18s RNA reverse: TTC ACG CCC TCT TGA ACT CT (GenBank Accession No. V01270).

CHAPTER III

OBJECTIVES AND RATIONALE

3.1 Objectives

It is apparent that more research needs to be done on the cardiovascular effects of estrogen in both males and females. Estrogen appears to exert detrimental effects in various states of pathophysiology, and to elucidate these deleterious effects of estrogen more clearly, this study will focus on the effects of estrogen and its receptors on the vascular wall, both in normotension and during the development of hypertension.

The central hypothesis to be tested is that:

In aortic coarctation-induced hypertension, estrogen upregulates the constrictor prostanoid pathway in the vascular wall via activation of ERβ, enhancing vascular tone and leading to enhanced development of hypertension in the female rat.

This hypothesis will be tested by addressing the following specific aims:

Specific Aim I: To determine the effects of hypertension on plasma estradiol levels and estradiol metabolism, and on the expression of estrogen receptors in the vascular wall of the rat mesenteric microvasculature.

Specific Aim II: To determine the mechanisms by which ERα and ERβ increase CP pathway function in vascular wall of the rat mesenteric microvasculature.

Specific Aim III: To determine the roles of ERα and ERβ in mediating the effects of estrogen on rat mesenteric arterial vascular function and on systemic arterial BP.

3.2 Experimental Design

3.2.1 Specific Aim I: To determine the effects of hypertension on plasma estradiol levels and estradiol metabolism, and on the expression of estrogen receptors in the VSM and endothelium in the rat mesenteric microvasculature.

3.2.1.1 Rationale

Recent studies reveal that the TxS enzyme is upregulated during aortic coarctation-induced hypertension in the aorta (6, 7). In addition, expression of COX-2 and TxS mRNA in both endothelium and VSM of aorta is higher in both F and OVX+ER than in M or OVX females in both normotension and hypertension, while expression of TP receptor mRNA is higher in VSM of F and OVX+ER (73). Studies also reveal that plasma levels of the sex hormones change during essential hypertension in humans (59, 65) and that ERβ mRNA is increased after vascular injury in M rat aorta (76), and that ERβ mRNA and protein are increased in primate carotid arteries after injury (1). However, it is not known whether the message or expression of ERs changes during this type of hypertension, or how the ERs are linked to the pathway of TXA_2. Studies have shown that estrogen regulates the expression of its receptors by up- or down-regulating either ER mRNA or protein synthesis (60, 86). There is a possibility that if estrogen secretion and/or metabolism are altered during the development of hypertension that the expression of ERs will increase or decrease and parallel changes in the TXA_2 pathway

could occur via this mechanism. Both secretion (in INT-F rats) and metabolism (in OVX+ER rats) of 17β-estradiol and estrone will be measured in this study. Finally, this specific aim will address the relationship between ER mRNA and protein (transcription and translation).

3.2.1.2 Specific Aim 1 Experiments

Protocol 1: Determine Plasma Estrogen Levels During Normotension and Hypertension

Blood samples were collected from rats on the day of the coarctation surgery through the carotid catheter or the jugular vein. Blood samples were then taken on the 4^{th}, 7^{th}, and 10^{th} days and then on the day of sacrifice (day 12-14) for all OVX and OVX+ER groups. For OVX+ER groups, blood samples were also taken 4 weeks post-OVX on the day of the E_2 pellet placement, prior to pellet implantation to ensure that circulating levels of E_2 were diminished by OVX. Aortic coarctations were performed in OVX+ER groups 2 weeks after pellet placement. For INT females, vaginal smears were performed on a daily basis and blood samples were taken during the diestrus phase of the cycle to assess basal estrogen levels in the plasma and to avoid the estrogen surge during proestrus.

Protocol 2: Real Time Reverse-Transcriptase-Polymerase Chain Reaction for ERα and ERβ messenger RNA in the Rat Mesenteric Microvasculature

Mesenteric arcades were collected from INT, OVX and OVX+ER normotensive and hypertensive groups and processed to obtain mRNA from each sample, to determine the expression of ERα and ERβ mRNA in NT and during the development of HT in female rats.

Protocol 3: Immunoblots for ERα and ERβ Protein Expression

Proteins were extracted from the same samples from which mRNA was obtained.

3.2.2 Specific Aim II: To determine the mechanisms by which ERα and/or ERβ increase CP pathway function in VSM and endothelium in the rat mesenteric microvasculature.

3.2.2.1 Rationale

With the recent availability of selective estrogen receptor agonists for ERα and ERβ, the effects of each receptor can be studied individually. There are no known studies examining chronic effects of treatment with these agonists on cardiovascular function or during hypertension. This study provides a unique examination of the selective effects of ERα and ERβ during normotension and during the development of hypertension.

Protocol 1: PPT and DPN treatment *in vivo*.

OVX rats were randomly assigned into one of three treatment groups: PPT, DPN or vehicle treatment. INT rats received vehicle treatment only. Rats were then further divided randomly into one of two groups: normotensive or hypertensive. Thus, there were eight treatment groups: INT-NT, OVX-NT, OVX+PPT-NT, OVX+DPN-NT, INT-HT, OVX-HT, OVX+PPT-HT and OVX+DPN-HT. Based upon these treatments, experimental groups include INT females with both ERα and ERβ activity, OVX females with little or no ER activity, PPT-treated OVX females with ERα activity only, and DPN-treated OVX females with ERβ activity only. Rats received subcutaneous injections of PPT, DPN or vehicle once a day for 14-16 days.

Protocol 2: Immunoblots for ERα and ERβ Protein Expression

Mesenteric arcades were collected from INT, OVX, and PPT- or DPN-treated normotensive and hypertensive rats and processed for protein extraction to quantify levels of receptor protein expression for ERα and ERβ.

3.2.3 Specific Aim III: To determine role of ERα and ERβ in mediating the effects of estrogen on rat mesenteric arterial vascular function and on systemic BP.

3.2.3.1 Rationale

In the acute (developmental) phase of ACIH, substantial changes occur both in function and structure (remodeling) of the female vasculature (1). TXA_2 is a potent vasoconstrictor and mitogen that increases vascular tone acutely and induces vascular smooth muscle proliferation chronically (8, 15). Thus, increases in TXA_2 levels are

associated with changes in vascular morphology and function. Inhibition of TXA_2 synthesis or blockade of the TP receptor during ACIH reduces MAP and prevents changes in vascular morphology (8). In addition, OVX of female rats prior to the induction of ACIH reduces the HT during the acute phase (12-14 days), as well as the expression of TxS mRNA, suggesting that TxS and TXA_2 levels are increased in parallel in the presence of estrogen. In the NT female rat aorta, OVX reduces mRNA and expression of TxS and COX-2 and TP receptor. However, the effects of selective activation of either ERα or ERβ on MAP, TXA_2 production and vascular reactivity to VP have not been studied in either NT or ACIH female rats.

It has been proposed that the balance of TXA_2 and PGI_2 is crucial to the regulation of vascular tone and hemostasis (90, 138, 142). There are several interactions between TXA_2 and PGI_2; including the ability of TP receptor agonists to evoke PGI_2 release and IP receptor activation to evoke TP receptor desensitization (24). In light of these interactions, the release of PGI_2 will also be measured in the mesenteric arteries.

Protocol 1: PPT and DPN Treatment

OVX rats were randomly placed into one of three treatment groups: PPT, DPN or vehicle treatment. INT rats received vehicle treatment only. Rats were then further divided randomly into one of two groups: normotensive or hypertensive. Rats received subcutaneous injections once a day for 14-16 days. Uterine weights were recorded to assess ERα stimulation in the PPT treatment group.

Protocol 2: Dose Response Curve to Vasopressin

First and second order mesenteric arterioles from OVX, PPT, DPN and INT normotensive and hypertensive rats were mounted on myographs and a dose response curve to VP (10^{-12} M – 10^{-8} M) was obtained. Four 1.5 mm sections were cut from each artery branch of each animal. Vessels were either incubated in vehicle, SQ 29,548 (1 μM), or Simvastatin (10 μM or 60 μM).

Protocol 3: TXA_2 and PGI_2 Release from Mesenteric Arteries

First and second order mesenteric arterioles from OVX, OVX+PPT, OVX+DPN and INT normotensive and hypertensive rats were incubated in KHB alone or KHB with 10^{-8} M VP to obtain basal and agonist-stimulated TXA_2 and PGI_2 release data.

CHAPTER IV

RESULTS

4.1 Effects of Aortic Coarctation-Induced Hypertension on Plasma Estradiol and Estrone Levels

Body and uterine weights were recorded on the day of sacrifice. OVX increased body weight by an average of 28% and decreased uterine weight by an average of 84%. OVX+PPT treatment increased uterine weights compared to OVX (355%), while OVX+DPN treatment did not alter uterine weight (see Table 2). The uterus expresses ERα protein only, and this measurement served as an index of ERα activity in the PPT group.

Blood samples were taken on the day of coarctation surgery in all groups (day 0), and then on the 4^{th}, 7^{th} and 10^{th} day in OVX-F and OVX+ER. In INT rats, all blood samples were taken during diestrus on the day of surgery and during the development of hypertension. Normotensive INT rats had the expected 4-5 day estrous cycle and low range of 17β-estradiol levels during diestrus (7.96 ± 3.08 pg/ml) and most INT rats continued to cycle during hypertension (21.24 ± 8.57 pg/ml) (Fig. 7). Hypertension did not affect 17β-estradiol levels at matching time points compared to NT (7.77 – 15.64 pg/ml vs. 7.43 – 21.24 pg/ml) groups ($P>0.05$). The day to day measurements showed similar trends between 17β-estradiol and estrone, but due to fluctuations in both 17β-estradiol and estrone levels in cycling rats, there were no significant differences within or between NT and HT groups, except in INT-HT (estrone, sample 2) (Figs. 7, 8).

In OVX-NT rats, there was a small but significant decrease in 17β-estradiol levels from day 0 (6.82 ± 0.53 pg/ml) to days 4 (3.34 ± 0.35 pg/ml) and 7 (2.92 ± 0.69 pg/ml)

($0.0003 \leq P \leq 0.001$). Between OVX-NT and OVX-HT groups, 17β-estradiol levels in OVX-HT were significantly higher at day 0 (11.12 ± 2.08 pg/ml), day 4 (8.76 ± 2.60 pg/ml) and day 7 (13.90 ± 3.82 pg/ml) (Fig. 9). There were no significant differences in estrone levels between OVX-NT and OVX-HT groups (Fig. 10).

Table 2. Body and uterine weights of intact, ovariectomized, ovariectomized with estrogen replacement, ovariectomized with PPT treatment and ovariectomized with DPN treatment Sprague-Dawley rats.

	INT	OVX	OVX+ER	OVX+PPT	OVX+DPN
n	12	15	18	23	26
Body Weight (grams)	230.8 ± 7.5[a]	297.2 ± 9.9[b]	255.0 ± 5.1[c]	277.0 ± 8.0[b]	288.9 ± 8.7[b]
Uterine Weight (mg/100 g body wt.)	172.2 ± 12.6[a]	26.82 ± 1.6[b]	--	95.7 ± 3.3[c]	34.42 ± 3.9[b]

Note: Values are means ± S.E. (*n* = number of rats). [a-c]Within body weights or uterine weights, mean values without common script are significantly different ($0.0001 \leq P \leq 0.0174$).

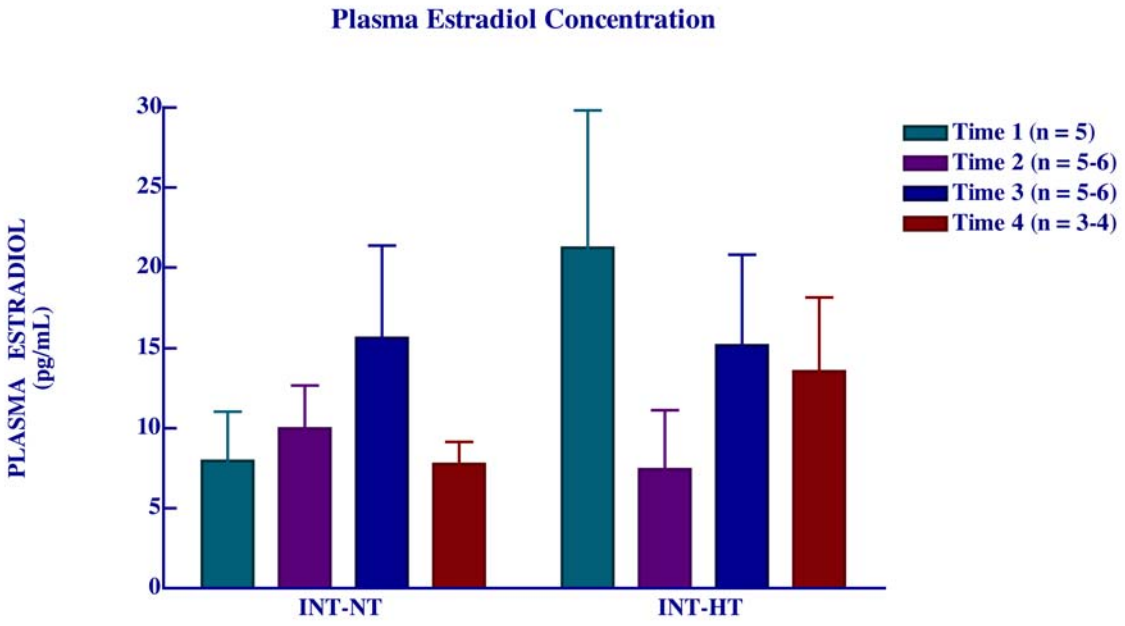

Figure 7: Plasma estradiol concentrations in normotension and during the development of hypertension in intact female rats. Bars are mean ± S.E. (n = number of rats). Samples were taken starting on the day of coarctation and on each day of diestrus thereafter. There were no significant differences within INT-NT, INT-HT or between NT and HT groups ($P > 0.05$).

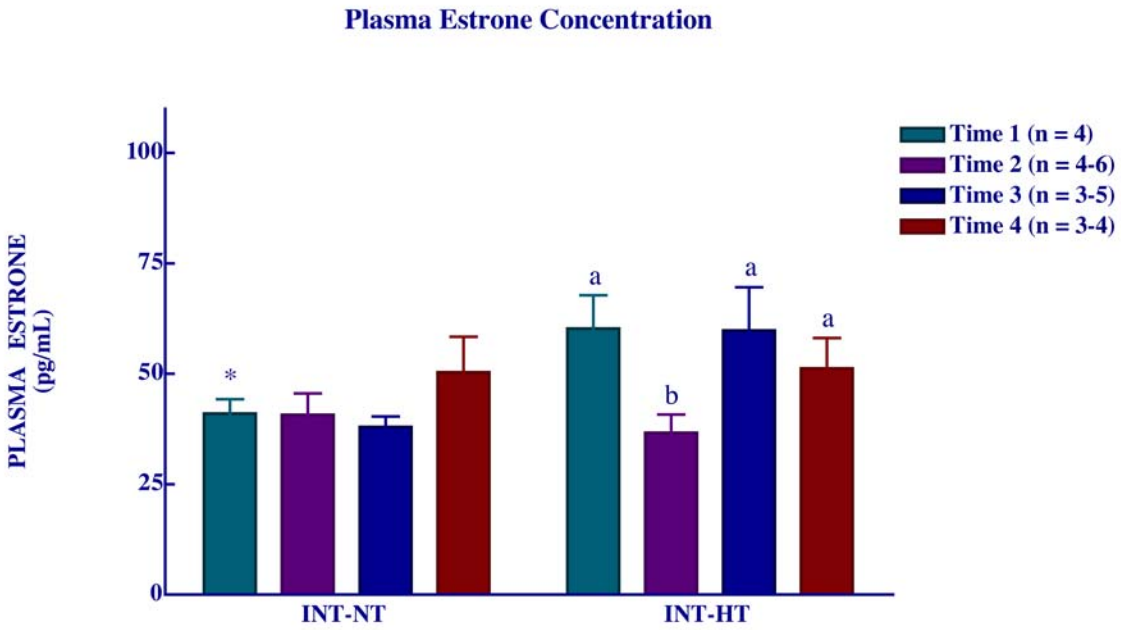

Figure 8: Plasma estrone concentrations in normotension and during the development of hypertension in intact female rats. Bars are means ± S.E. (n = number of rats). Samples were taken starting on the day of coarctation and on each day of diestrus thereafter. [a-b]Within each group (INT-NT or INT-HT), mean values without common script are significantly different ($0.0145 \leq P \leq 0.0329$). [*]Denotes significant differences between NT and HT groups (INT-NT vs. INT-HT) at like time points (Time 1) ($P \leq 0.0445$).

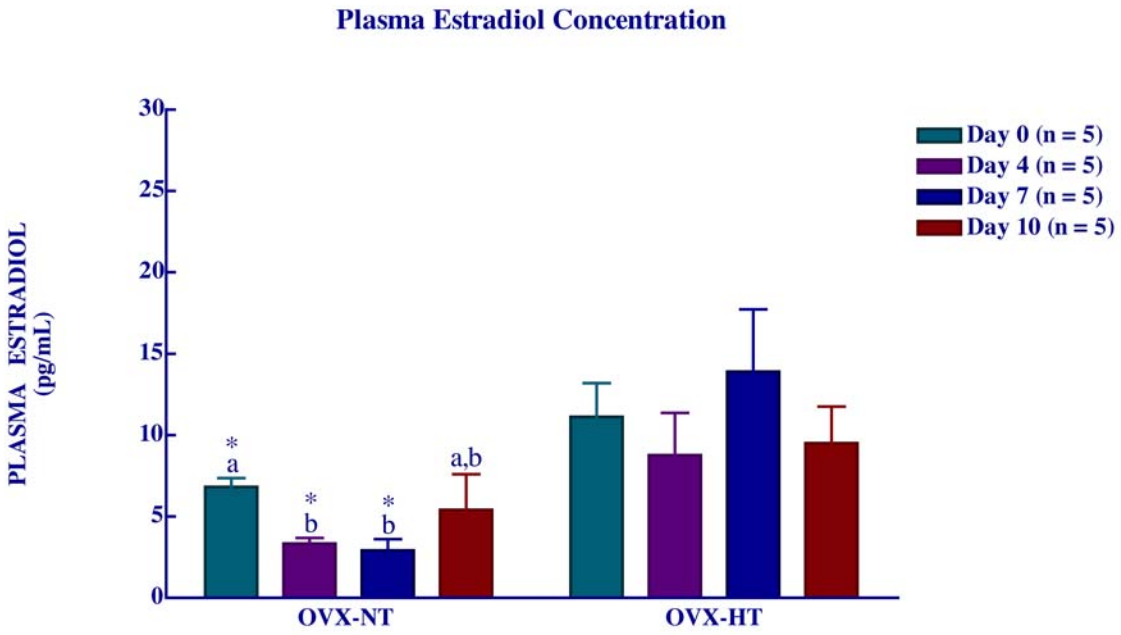

Figure 9: Plasma estradiol concentrations in OVX-NT and OVX-HT female rats at coarctation or sham (day 0) and at days 4, 7 and 10 following coarctation. Bars are means ± S.E. (n = number of rats). [a-b]Within OVX-NT group, mean values without a common script are significantly different ($0.0003 \leq P \leq 0.001$). Mean values in OVX-HT do not differ significantly ($P > 0.05$). * Denotes significant differences between NT and HT groups (OVX-NT vs. OVX-HT), at like time points (days 0, 4 and 7) ($0.0111 \leq P \leq 0.0402$).

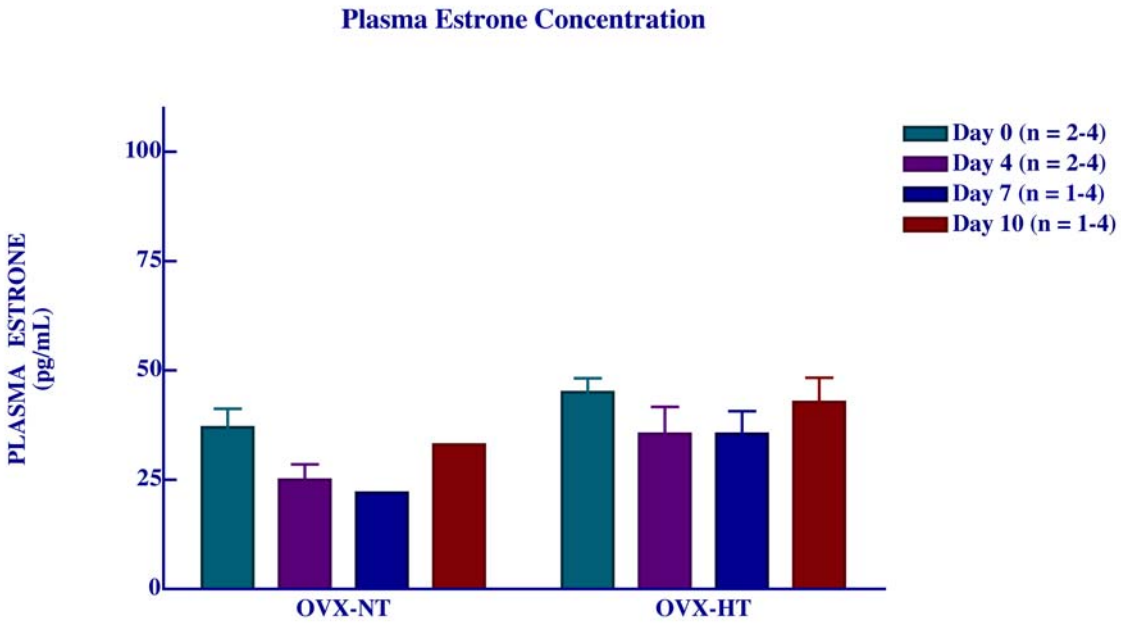

Figure 10: Plasma estrone concentrations in OVX-NT and OVX-HT female rats at coarctation or sham (day 0) and at days 4, 7 and 10 following coarctation. Bars are means ± S.E. (n = number of rats). There were no significant differences within OVX-NT, OVX-HT or between NT and HT groups ($P > 0.05$).

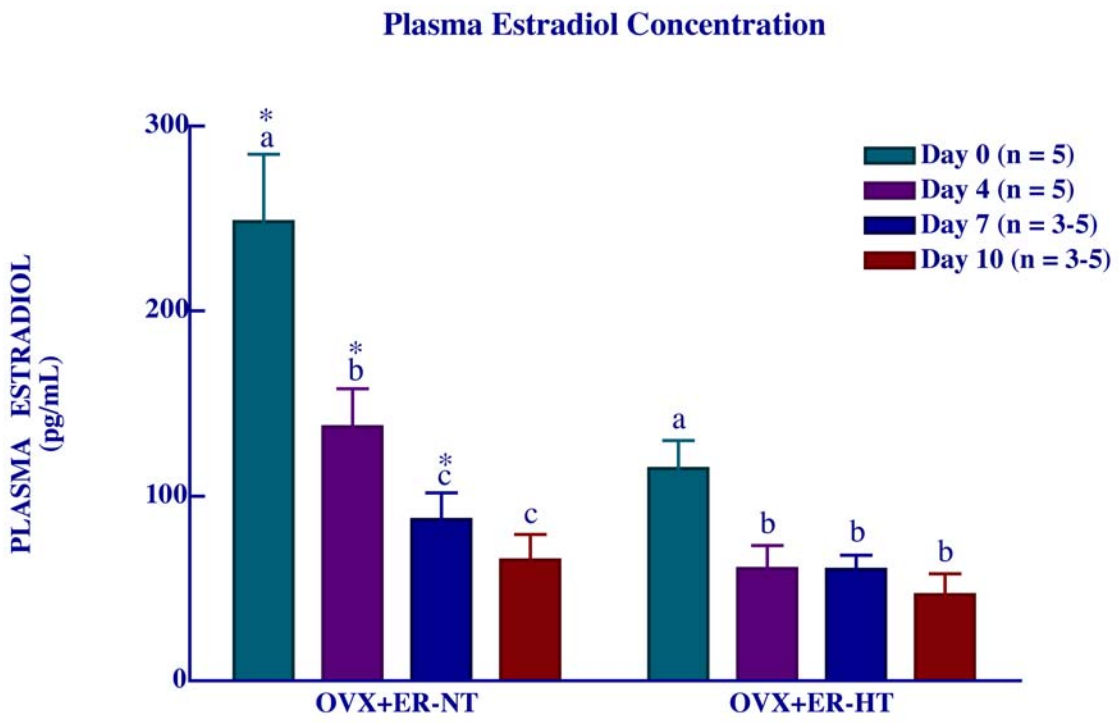

Figure 11: Plasma estradiol concentrations in OVX+ER-NT and OVX+ER-HT female rats at coarctation or sham (day 0) and at days 4, 7 and 10 following coarctation. Bars are means ± S.E. (n = number of rats). [a-c]Within each group (OVX+ER-NT or OVX+ER-HT), mean values without common script are significantly different ($0.0048 \leq P \leq 0.048$). [*]Denotes significant differences between NT and HT groups (OVX+ER-NT vs. OVX+ER-HT) at like time points (Days 0, 4 and 7) ($0.0087 \leq P \leq 0.0116$).

In OVX+ER-NT, plasma concentrations of estradiol exhibited a steady decline from day 0 through day 10. There was a 47% decrease from day 0 to day 4 (248.4 ± 36.42 pg/ml vs. 137.6 ± 20.57 pg/ml) and then subsequent declines of 36% and 25% for the remaining two time points (87.40 ± 14.31 pg/ml and 65.40 ± 13.85 pg/ml).

The plasma concentrations of estradiol in OVX+ER decreased 44% from day 0 to day 4 during the development of hypertension (115.0 ± 15.09 pg/ml vs. 60.75 ± 12.48 pg/ml), but then remained fairly stable throughout the remaining samples, although there was a 23% decline on day 10 (to 46.67 ± 11.32 pg/ml) (Fig. 11).

In OVX+ER, plasma estrone levels remained fairly stable throughout the development of hypertension, unlike plasma estradiol, which declined over time. Plasma estrone remained higher in OVX+ER-NT than in OVX+ER-HT on days 4, 7 and 10 (Fig. 12). There was a slight increase on day 4 in OVX+ER-HT (42.0 ± 7.0 pg/ml vs. 65.5 ± 10.75 pg/ml).

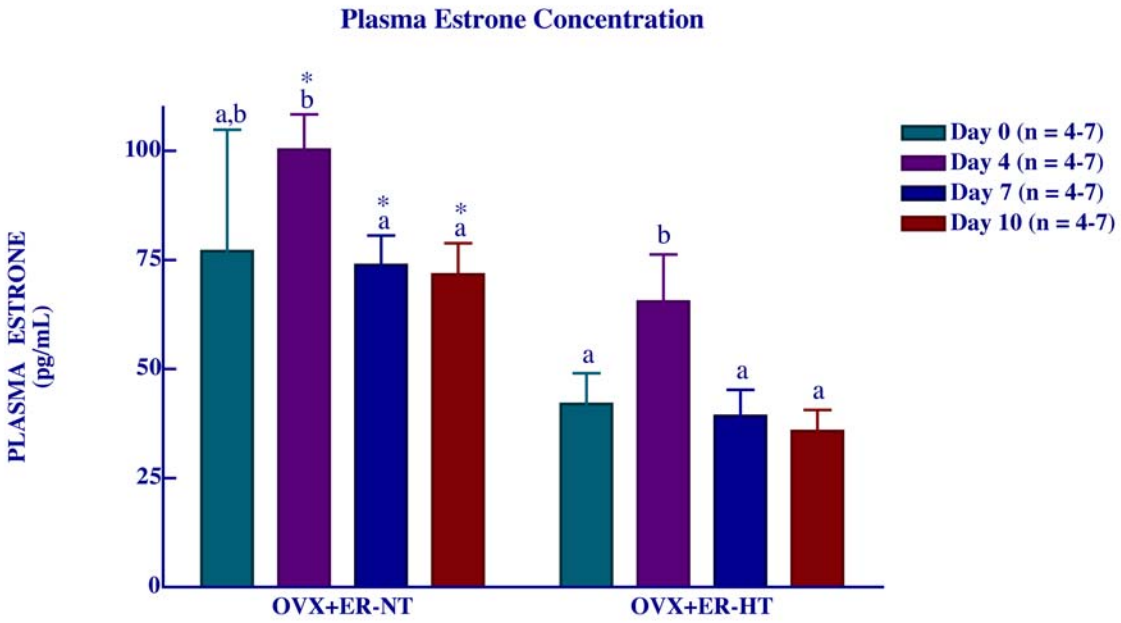

Figure 12: Plasma estrone concentrations in OVX+ER-NT and OVX+ER-HT female rats at coarctation or sham (day 0) and at days 4, 7 and 10 following coarctation. Bars are means ± S.E. (n = number of rats). [a- b]Within each group (OVX+ER-NT or OVX+ER-HT) mean values without common script are significantly different ($0.0106 \leq P \leq 0.05$). [*]Denotes significant differences between NT and HT groups (ER-NT vs. ER-HT) at like time points (Day 4, 7 or 10) ($0.0034 \leq P \leq 0.0147$).

4.2 Effects of Aortic Coarctation-Induced Hypertension and Estrogen Receptor Agonists on Mean Arterial Pressure

Blood pressures were measured starting on day 2 after coarctation (or sham) surgery and every other day through day 12. In NT (sham) groups (INT, OVX, OVX+PPT and OVX+DPN), BP averaged 125 ± 3 mmHg, 120 ± 1 mmHg, 118 ± 3 mmHg and 124 ± 4 mmHg respectively on day 2, and did not differ over time within groups ($P > 0.05$) or between groups at days 2 or 12 ($P > 0.05$). The only exception was in OVX+DPN rats at day 12 (132 ± 2 mmHg), which was significantly higher than OVX+PPT at day 12 (119 ± 2 mmHg) ($P < 0.01$) (Fig. 13A).

Aortic coarctation increased blood pressure notably in all groups. In HT groups (INT, OVX, OVX+PPT and OVX+DPN), BP averaged 166 ± 3 mmHg, 150 ± 4 mmHg, 151 ± 7 mmHg and 157 ± 5 mmHg, respectively, on day 2 and BP increased over time within groups in INT (183 ± 4 mmHg), OVX+PPT (180 ± 4 mmHg) and OVX+DPN (182 ± 4 mmHg) ($0.0058 \leq P \leq 0.0238$), but not in OVX (153 ± 3 mmHg) ($P = 0.27$). Among groups, BP was higher in INT than in OVX at day 2 ($P = 0.0028$) and higher in INT, OVX+PPT and OVX+DPN than in OVX at day 12 ($0.0005 \leq P \leq 0.0048$) (Fig. 13B).

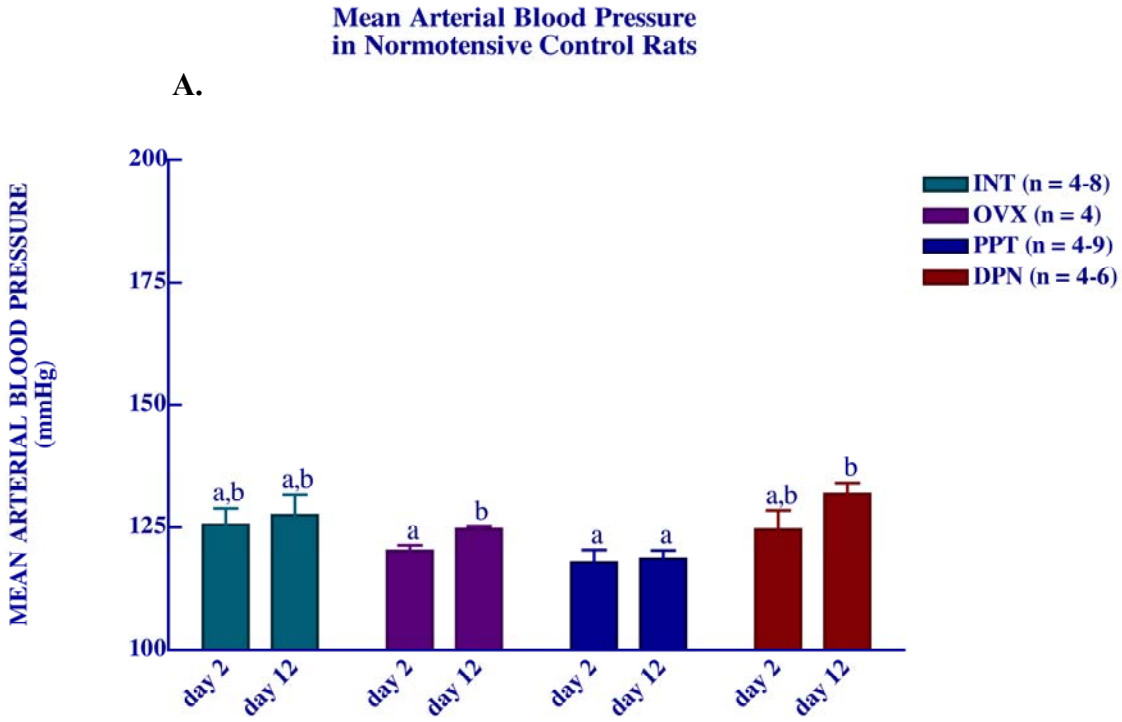

Figure 13: (A) Mean arterial blood pressures (MAP) of rats following sham coarctation (normotension, NT; A) or induction of aortic coarctation-induced hypertension (HT; B) at days 2 and 12.

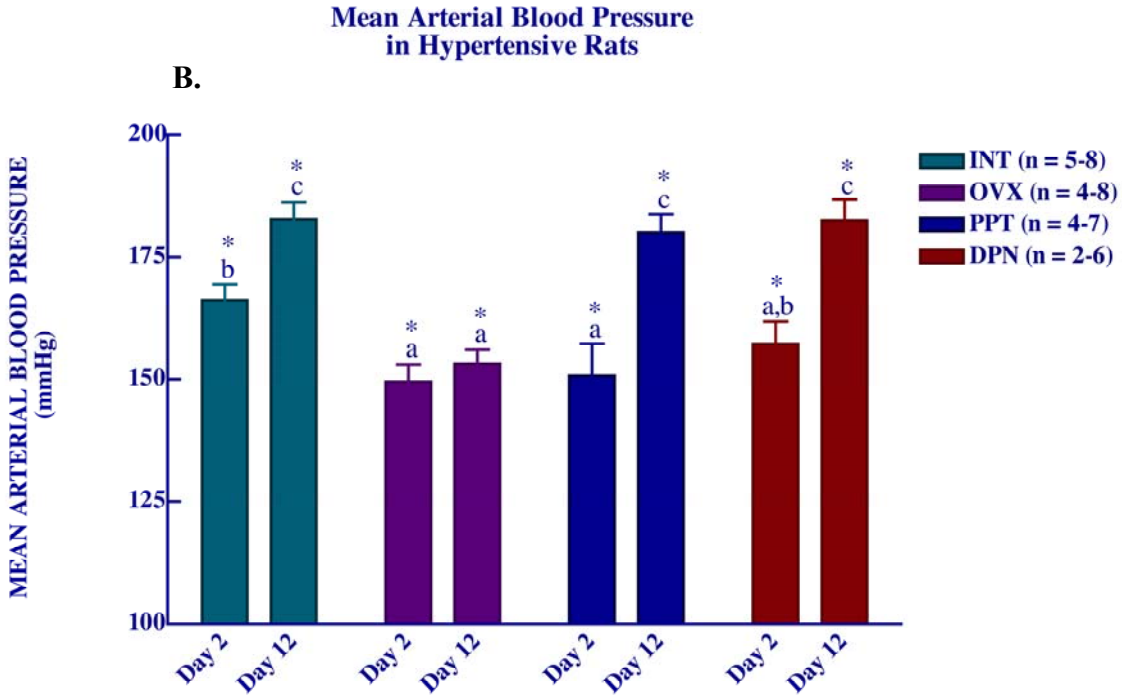

Figure 13 continued: (B) Mean arterial blood pressures (MAP) of rats following sham coarctation (normotension, NT; A) or induction of aortic coarctation-induced hypertension (HT; B) at days 2 and 12. Bars are means ± SE (n = number of rats). [a-c]Mean values within each group (NT or HT) without common script are significantly different ($0.0058 \leq P \leq 0.0238$). [*]Mean values between NT and HT in all groups (INT, OVX, OVX+PPT and OVX+DPN) exhibited significant increases in MAP ($P < 0.001$).

4.3 Effects of Aortic Coarctation and Estrogen Receptors Agonists on TXA_2 and PGI_2 Release from the Mesenteric Arterioles

4.3.1 TXA_2

Basal release of TXA_2 (as the stable metabolite TXB_2) during normotension did not differ among the groups, except for OVX+PPT-NT (82 ± 13 pg/mg tissue/45 minutes), which was about 2-fold higher than OVX+DPN-NT (40 ± 8 pg/mg tissue/45

minutes) ($P < 0.05$) (Fig. 14). The largest increase in response to VP stimulation (10^{-8}M) in NT rats was observed in INT-NT (46 ± 15 vs. 182 ± 55 pg/mg tissue/45 minutes; $P < 0.05$). Stimulation with VP in OVX-NT and OVX+PPT-NT did not produce a significant increase in the release of TXA_2 (51 ± 23 vs. 137 ± 38 and 82 ± 13 vs. 116 ± 24 pg/mg tissue/45 minutes) ($P > 0.05$). Stimulation with VP produced a small but significant increase in the release of TXA_2 in OVX+DPN-NT versus basal release (40 ± 8 vs. 79 ± 14 pg/mg tissue/45 minutes; $P < 0.05$). Aortic coarctation produced a dramatic increase in the basal release of TXA_2 from mesenteric arterioles in all groups (INT, $P < 0.001$; OVX, $P < 0.01$; OVX+PPT, $P < 0.02$; OVX+DPN, $P < 0.01$); however, basal release did not differ among HT groups. INT-HT (58% increase) and OVX+PPT-HT (55% increase) increased TXA_2 release the most in response to VP stimulation during hypertension, while OVX-HT (36% increase) and OVX+DPN-HT (35% increase) groups exhibited smaller increases. Stimulation with VP caused significant increases in all HT groups except OVX-HT ($P > 0.05$) (Fig. 14).

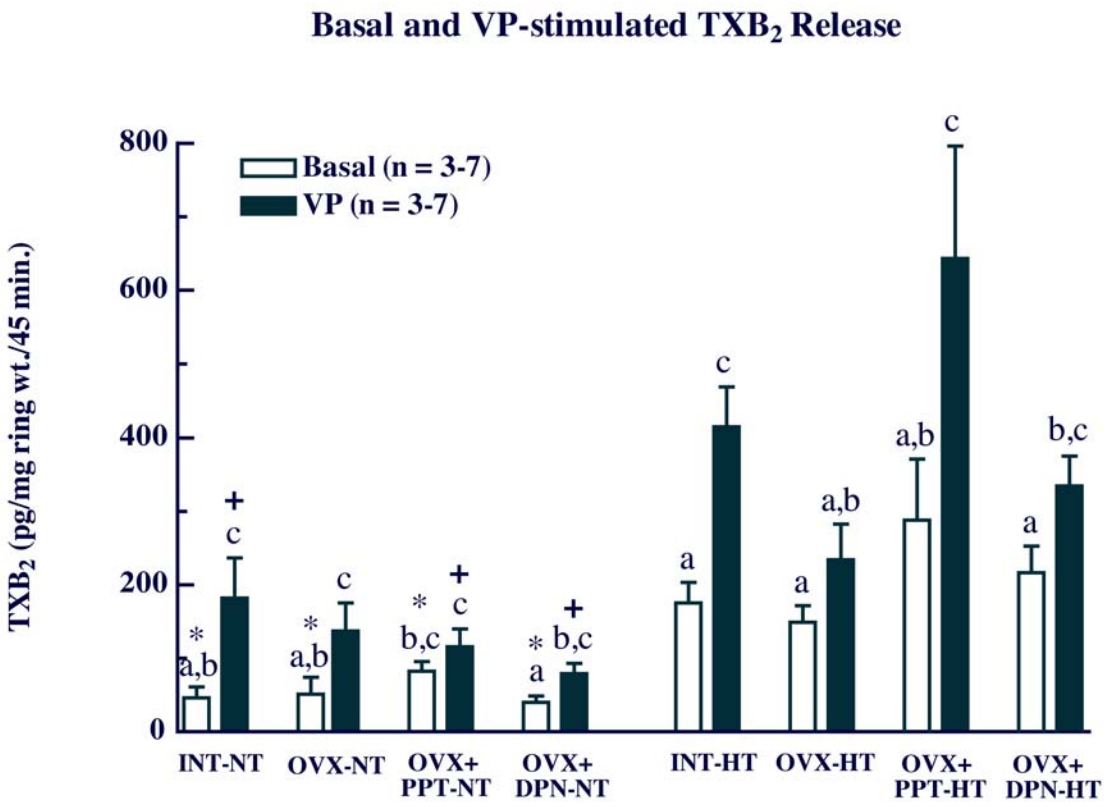

Figure 14: Basal and vasopressin (VP)-stimulated release of TXA_2 (measured as the stable metabolite TXB_2) from mesenteric arterioles in normotensive (NT) and aortic coarctation-induced hypertensive (HT) rats. Bars are means ± S.E. (n = number of animals). INT, intact females; OVX, ovariectomized females; PPT, OVX females treated with PPT; DPN, OVX females treated with DPN. $^{a\text{-}c}$Mean values within each group (NT or HT) without common script are significantly different ($0.0008 \leq P \leq 0.0482$). $^{*+}$Denotes significant differences between groups (NT vs. HT) ($0.0008 \leq P \leq 0.0199$).

4.3.2 PGI_2

Basal release of PGI_2 (as the stable metabolite 6-keto-$PGF_{1\alpha}$) in INT (742 ± 147 pg/mg tissue/45 minutes), OVX (689 ± 75 pg/mg tissue/45 minutes) and OVX+DPN (625 ± 141 pg/mg tissue/45 minutes) did not differ during normotension. Basal release of PGI_2 during normotension was highest in OVX+PPT treated animals (1,577 ± 228 pg/mg tissue/45 minutes) than the other groups ($P < 0.05$) (Fig. 15). VP stimulation produced significant increases in PGI_2 release in INT (2,897 ± 490 pg/mg tissue/45 minutes), OVX (1,451 ± 292 pg/mg tissue/45 minutes) and OVX+DPN (1,418 ± 256 pg/mg tissue/45 minutes), but not in OVX+PPT-NT (2,312 ± 591 pg/mg tissue/45 minutes) ($P > 0.10$) (Fig. 15). Coarctation increased basal release of PGI_2 in all groups, but basal release during HT did not differ among groups. INT-HT (7,969 ± 1,659 pg/mg tissue), OVX+PPT-HT (8,971 ± 1,238 pg/mg tissue) and OVX+DPN-HT (6,030 ± 1,454 pg/mg tissue) produced similar amounts of PGI_2 in response to VP. OVX-HT (4776 ± 865 pg/mg tissue) produced significantly less PGI_2 in response to VP than the other groups (Fig. 15).

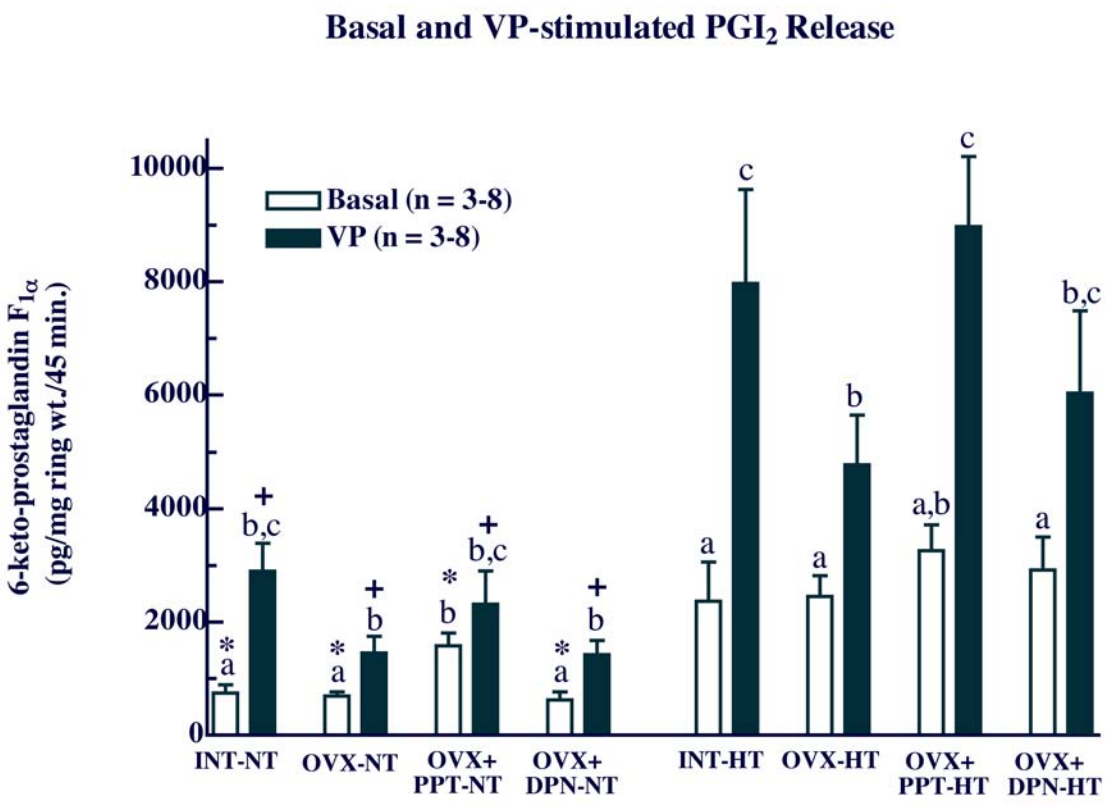

Figure 15: Basal and vasopressin (VP)-stimulated release of PGI_2 (measured as the stable metabolite 6-keto-$PGF_{1\alpha}$) from mesenteric arterioles in normotensive (NT) and aortic coarctation-induced hypertensive (HT) rats. Bars are means ± S.E. (n = number of animals). INT, intact females; OVX, ovariectomized females; OVX+PPT, OVX females treated with PPT; OVX+DPN, OVX females treated with DPN. $^{a\text{-}c}$Mean values within each group (NT or HT) without common script are significantly different ($0.0040 \leq P \leq 0.0498$). $^{*+}$Denotes significant differences between groups (NT vs. HT) ($0.0008 \leq P \leq 0.0338$).

4.4 Effects of Aortic Coarctation and Estrogen Receptor Agonists on the Mesenteric Arteriole Response to Arginine Vasopressin

In NT rats, INT (9.97 ± 0.75 mN/mm) exhibited a maximal response to VP similar to those of OVX (8.63 ± 0.58 mN/mm) and OVX+DPN (9.93 ± 0.53 mN/mm). OVX+PPT treatment in NT rats increased reactivity to VP (12.96 ± 0.64 mN/mm) compared to INT, OVX and OVX+DPN treatments at both middle and maximal concentrations ($0.0002 \leq P \leq 0.02$). INT differed significantly from OVX and OVX+DPN at the middle dose only ($0.0271 \leq P \leq 0.0294$) (Figs. 16A, 17A). However, aortic coarctation abolished the maximal differences in reactivity to VP between INT (11.77 ± 1.40 mN/mm), OVX (10.14 ± 1.01 mN/mm), OVX+PPT (11.39 ± 1.08 mN/mm), and OVX+DPN (11.34 ± 1.39 mN/mm) ($P > 0.05$) (Figs. 16B, 17B). Hypertension did not alter the contractile responses to VP significantly in any group compared to normotensive controls ($P > 0.05$) (Figs. 16B, 17B).

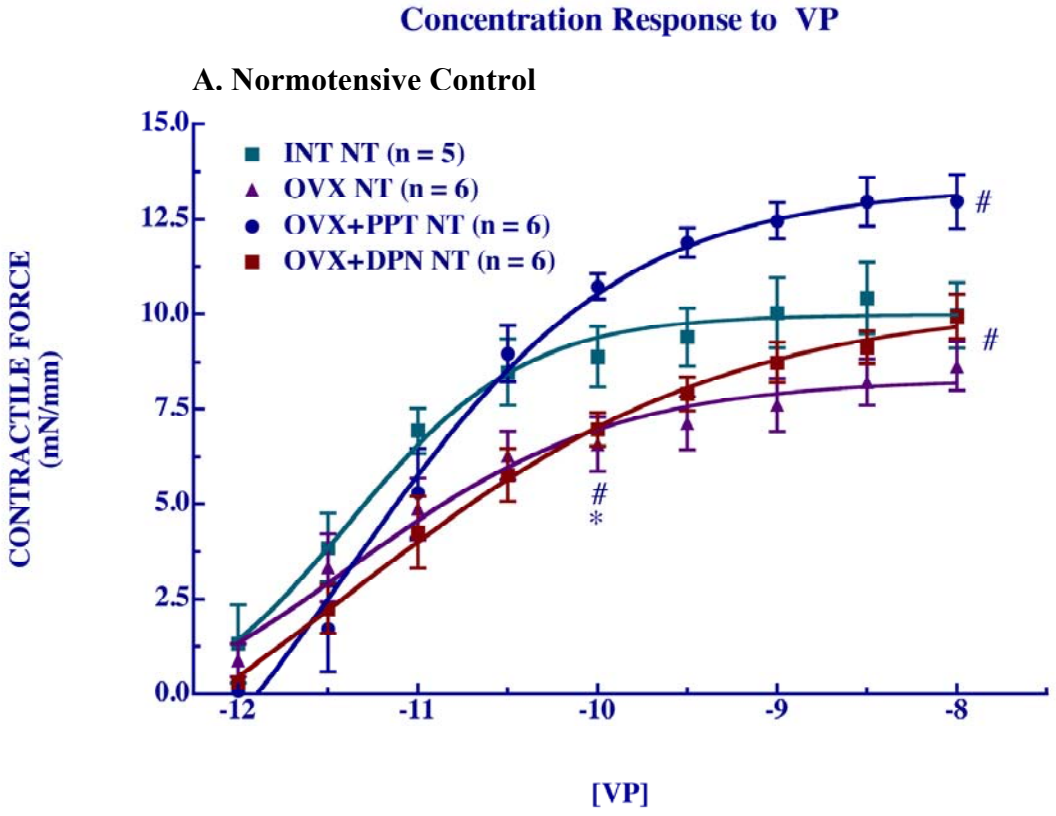

Figure 16: (A) Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from INT, OVX, OVX+PPT- and OVX-+DPN-treated normotensive-control (NT; A) and hypertensive (HT; B) Sprague-Dawley rats. Contractile force was normalized by vessel length. Data points represent means ± S.E. (n = number of animals). [#]Statistically significant differences exist in OVX+PPT-NT vs. OVX+DPN-NT, INT-NT and OVX-NT ($0.0002 \leq P \leq 0.02$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP. [*]Statistically significant differences exist in INT-NT vs. OVX-NT and OVX+DPN-NT at the middle concentrations only ($1x10^{-10}$M) ($0.0271 \leq P \leq 0.0294$). No statistically significant differences exist between OVX+DPN-NT and OVX-NT ($P > 0.05$).

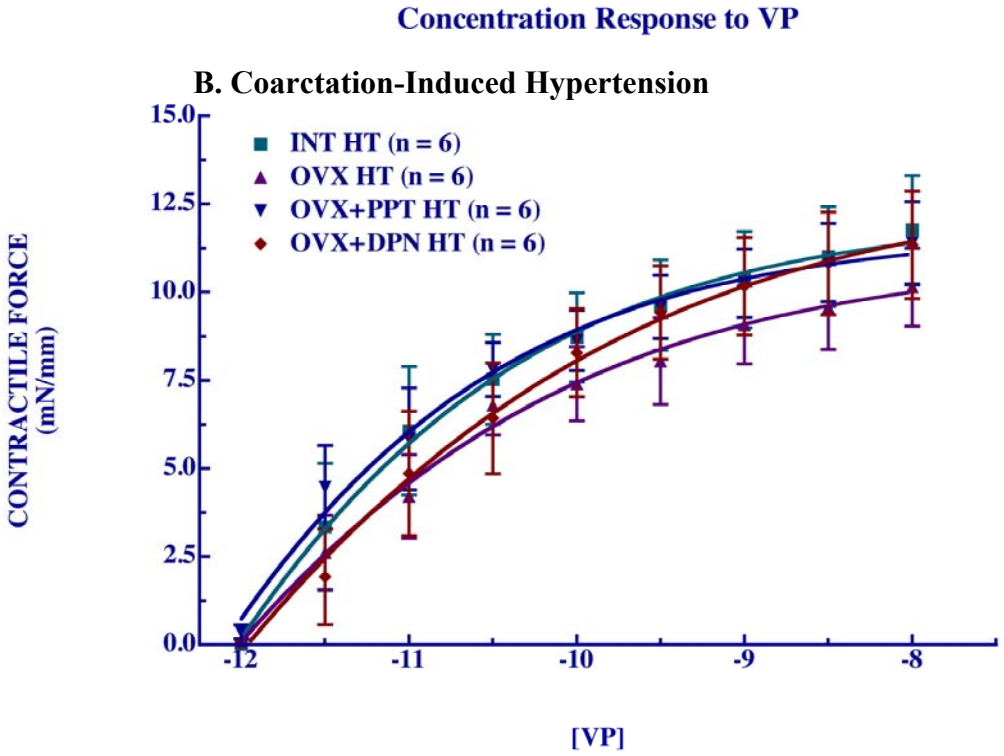

Figure 16 continued: (B) Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from INT, OVX, OVX+PPT- and OVX-+DPN-treated normotensive-control (NT) Sprague-Dawley rats. Contractile force was normalized by vessel length. Data points represent means ± S.E. (n = number of animals). No statistically significant differences exist between the curves ($P > 0.05$).

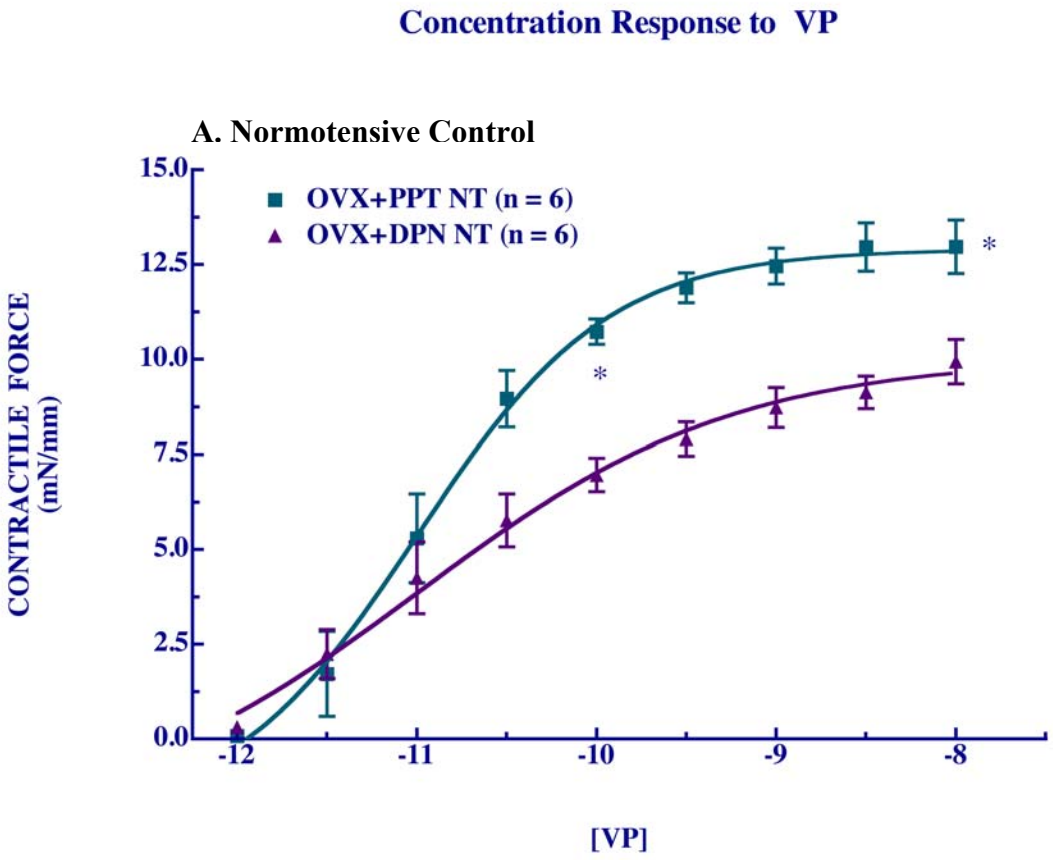

Figure 17: (A) Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX-PPT- and OVX-DPN-treated normotensive-control (NT; A) and aortic coarctation-induced hypertensive (HT; B) Sprague-Dawley rats. Data points represent means ± S.E. (n = number of animals). *Statistically significant differences exist in OVX+PPT-NT vs. OVX+DPN-NT ($0.0001 \leq P \leq 0.004$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

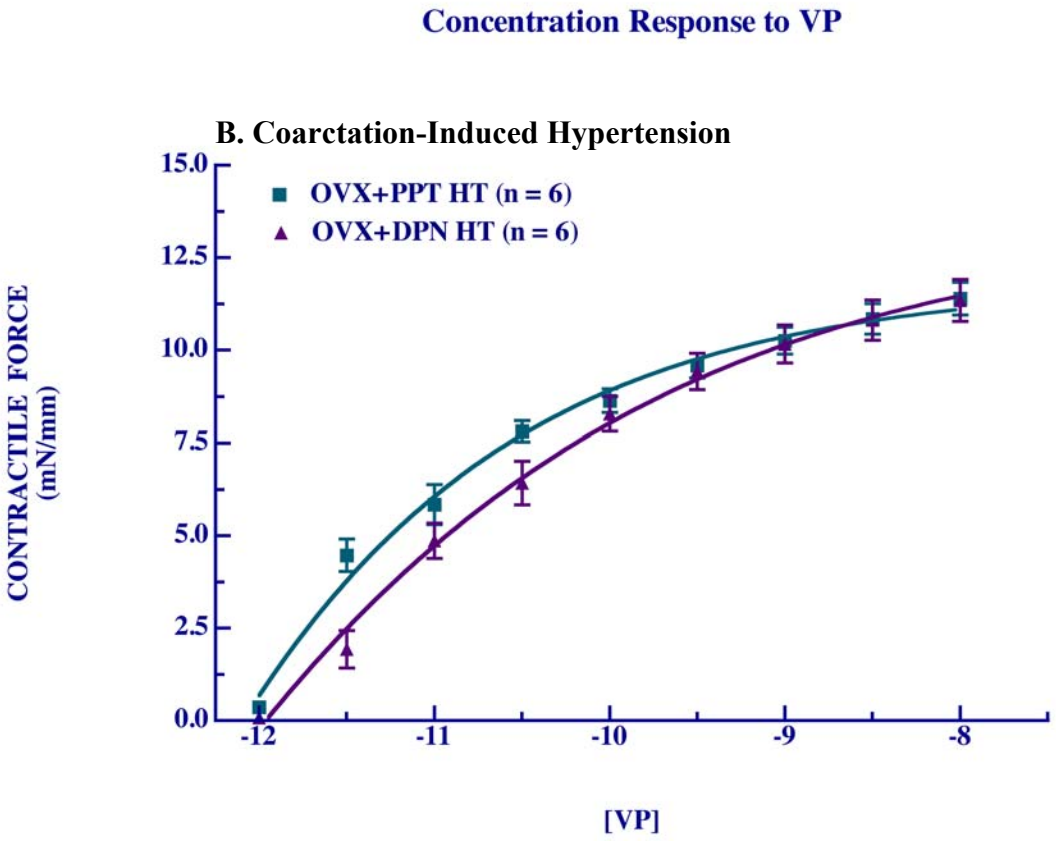

Figure 17 continued: (B) Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+PPT- and OVX+DPN-treated normotensive-control (NT; A) and aortic coarctation-induced hypertensive (HT; B) Sprague-Dawley rats. Contractile force was normalized by vessel length. Data points represent means ± S.E. (n = number of animals). No statistically significant differences exist between OVX+PPT-HT vs. OVX+DPN-HT ($P > 0.05$).

4.4.1 Effects of SQ 29,548 on the Vasopressin Concentration Response

There were no significant differences in any of the groups in the presence of SQ 29,548 (SQ) ($P > 0.05$) (Figs. 18 thru 25).

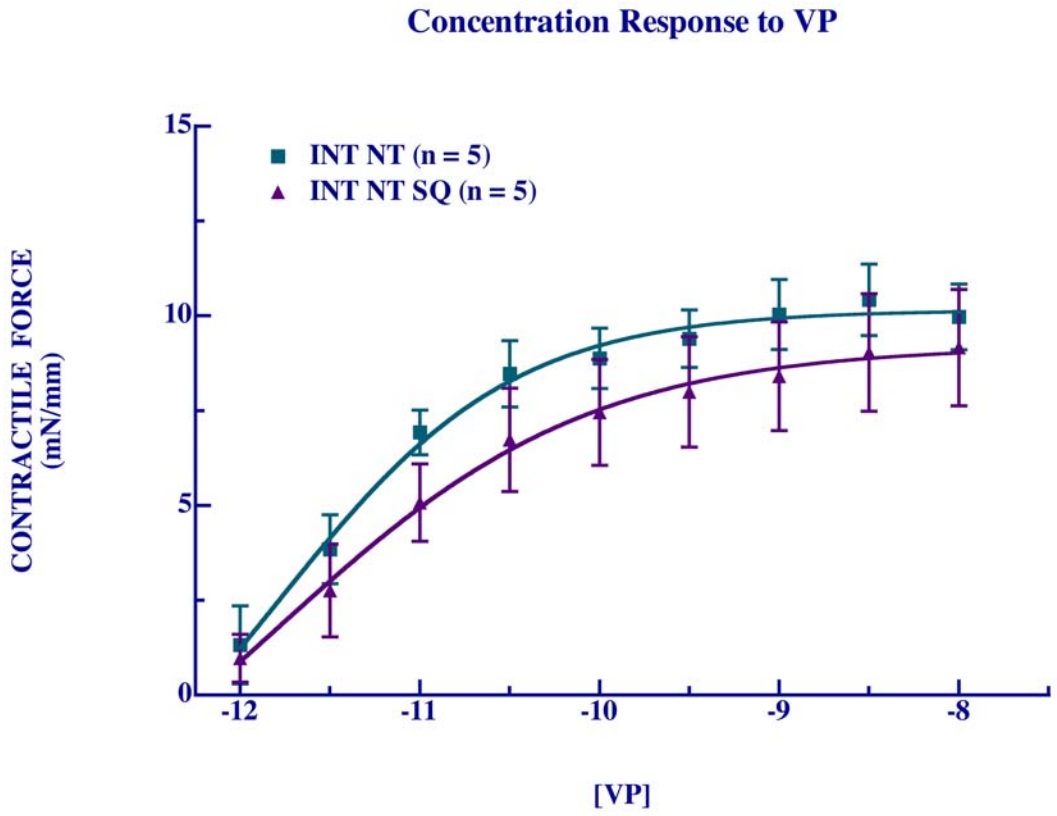

Figure 18: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from INT-NT female Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). There were no statistically significant differences in INT-NT for SQ vs. VEH ($P > 0.05$).

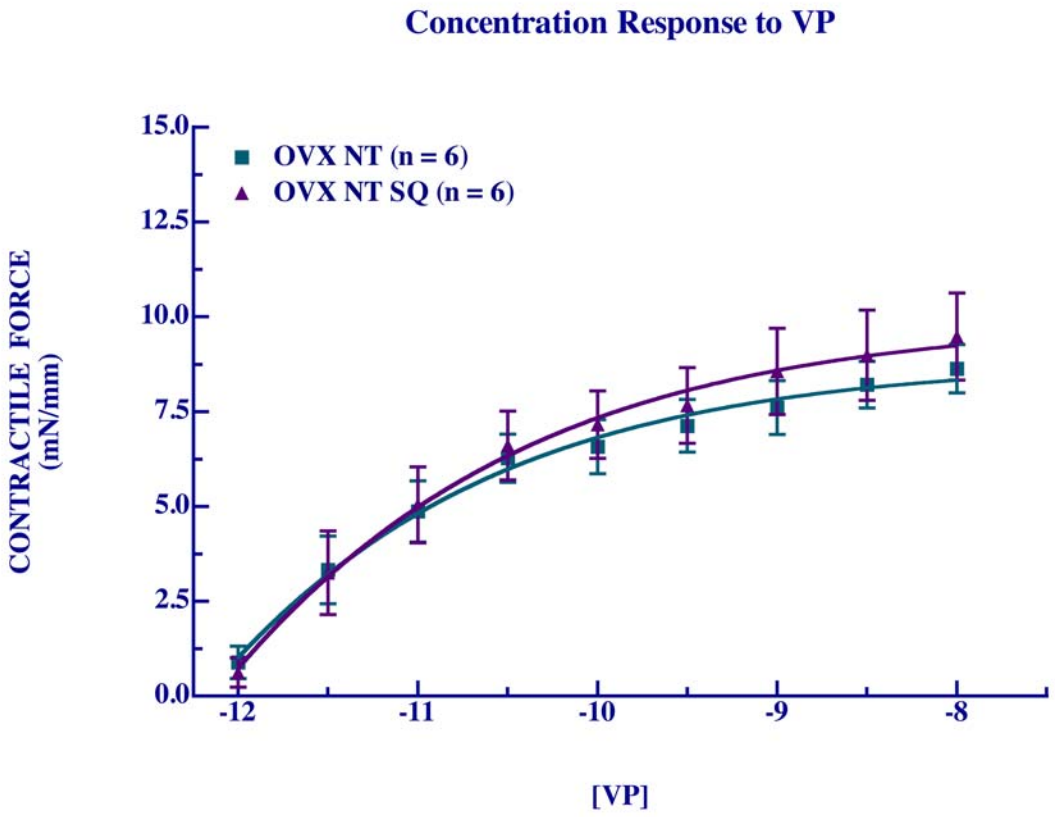

Figure 19: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX-NT female Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E.; (n = number of animals). There were no statistically significant differences in OVX-NT for SQ vs. VEH ($P > 0.05$).

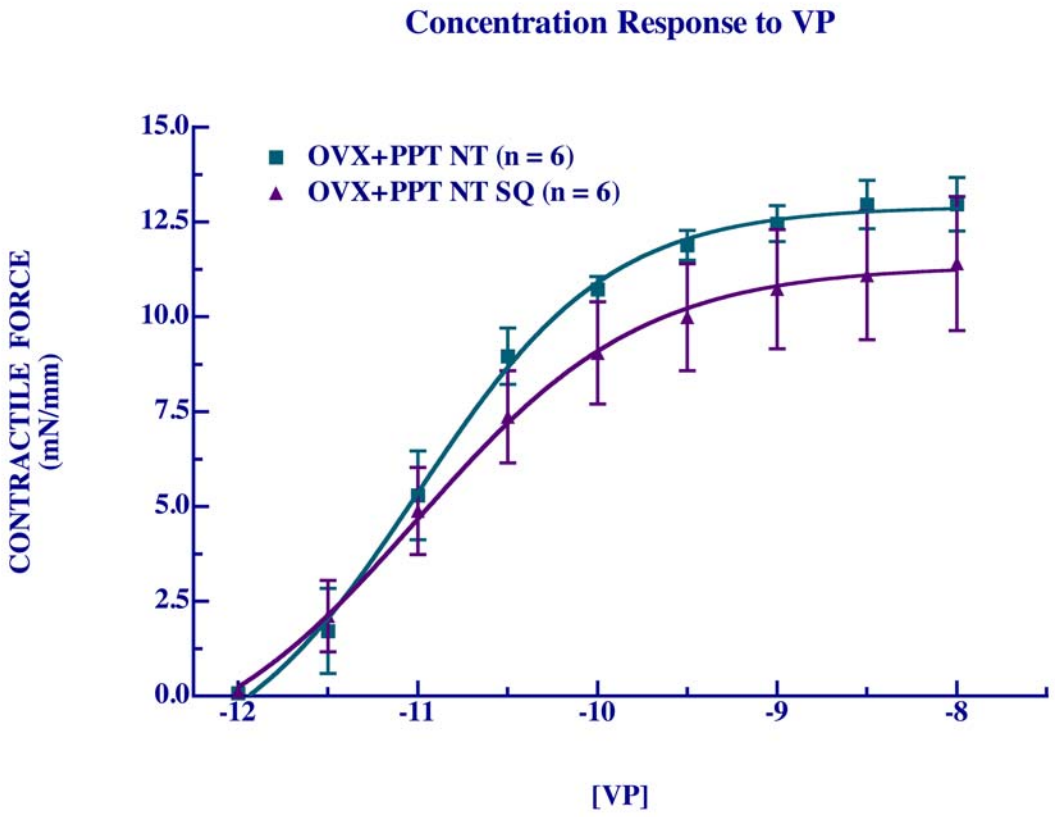

Figure 20: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+PPT-NT Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). There were no statistically significant differences in OVX+PPT-NT for SQ vs. VEH ($P > 0.05$).

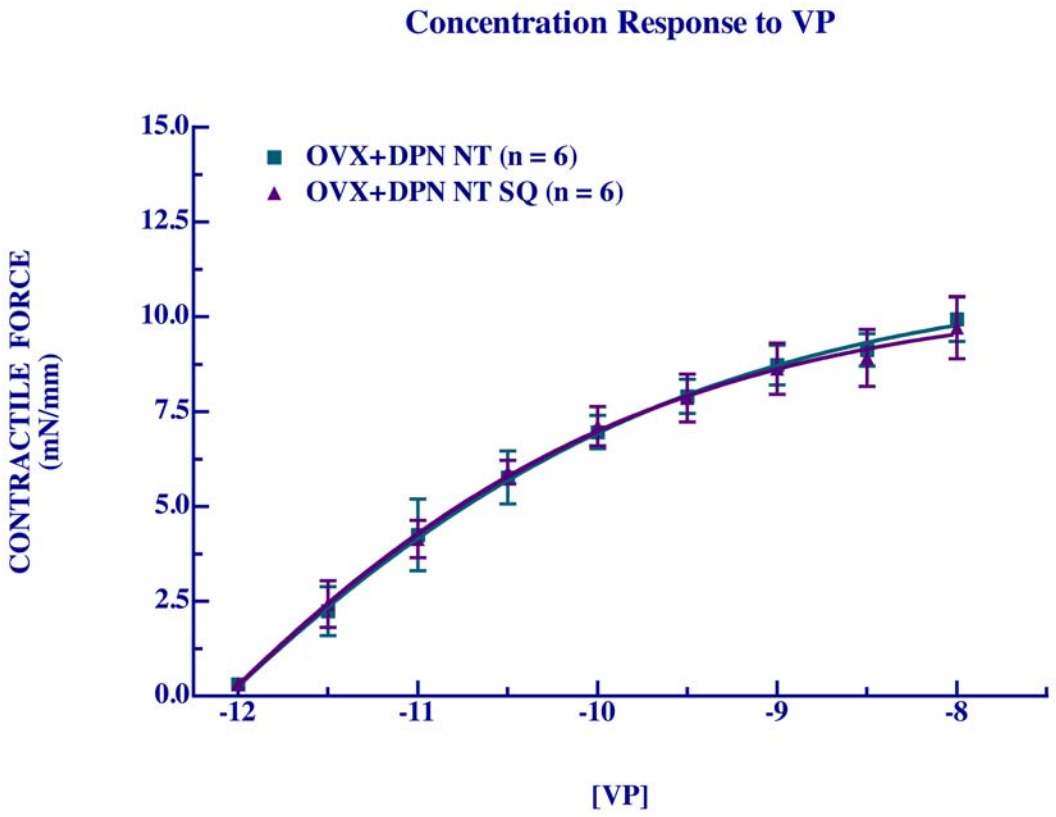

Figure 21: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+DPN-NT Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). There were no statistically significant differences in OVX+DPN-NT for SQ vs. VEH ($P > 0.05$).

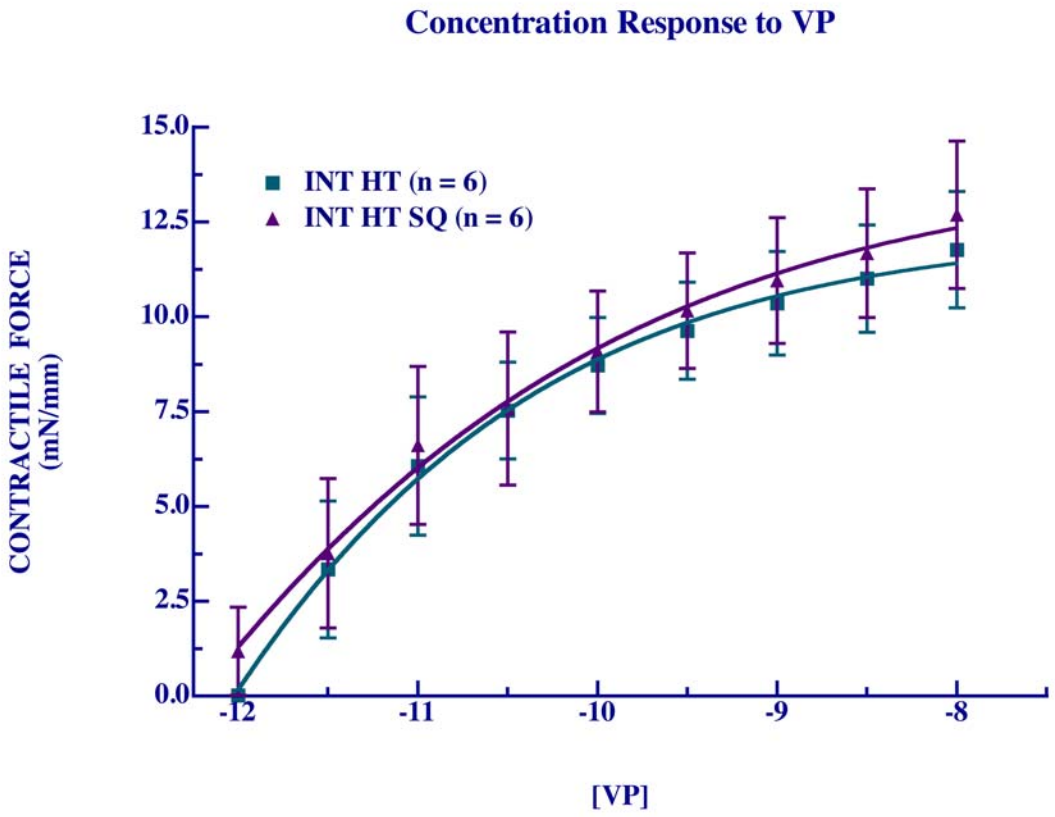

Figure 22: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from INT-HT Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). There were no statistically significant differences in INT-HT for SQ vs. VEH ($P > 0.05$).

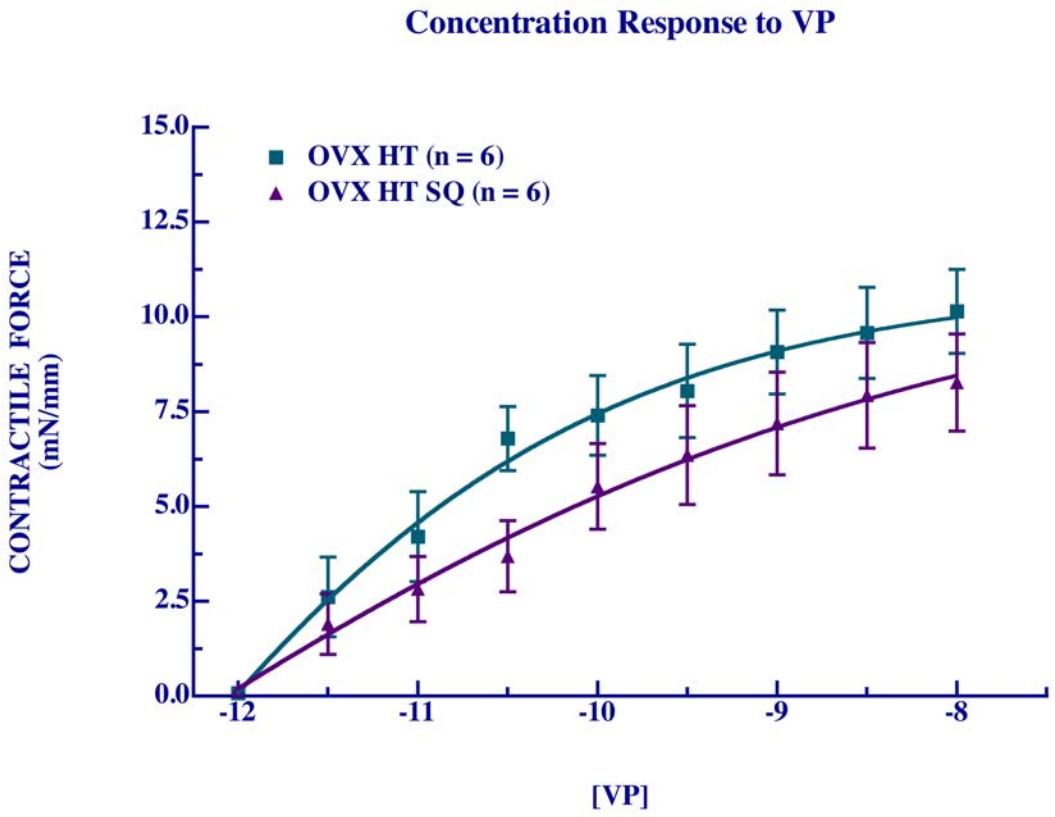

Figure 23: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX-HT Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n, = number of animals). There were no statistically significant differences at either the middle or highest dose in OVX-HT for SQ vs. VEH ($P > 0.05$).

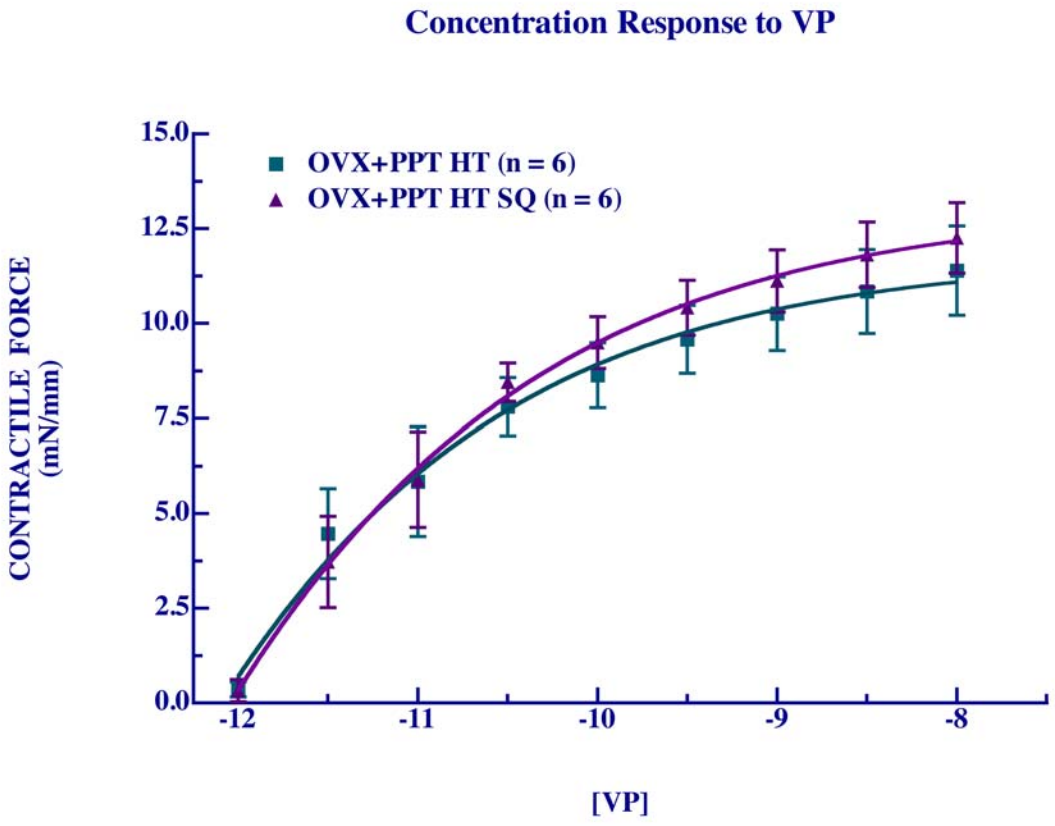

Figure 24: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+PPT-treated HT Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). There were no statistically significant differences in OVX+PPT-HT for SQ vs. VEH ($P > 0.05$).

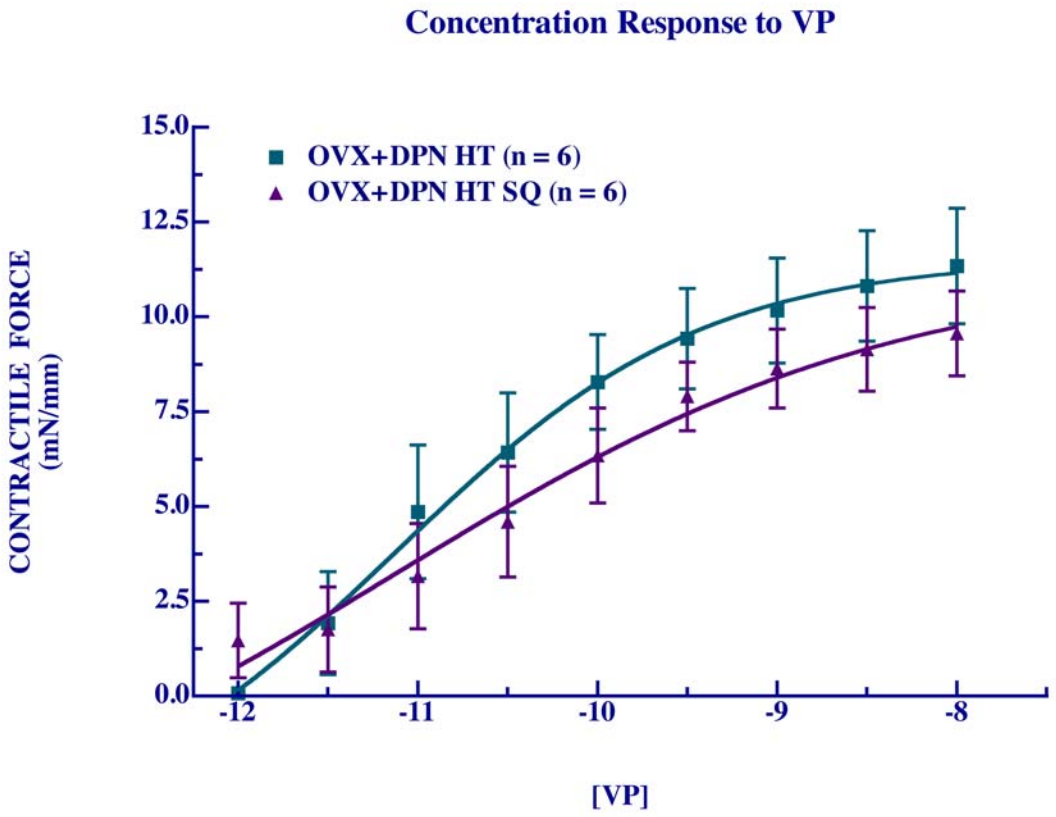

Figure 25: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+DPN-treated HT Sprague-Dawley rats in the presence of SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). There were no statistically significant differences in OVX+DPN-HT for SQ vs. VEH ($P > 0.05$).

Table 3. A. Maximal contractile responses to vasopressin (A) and sensitivity to VP (EC_{50}; B) for vehicle-control and SQ 29,548 treated mesenteric arterioles.

	INT-NT	OVX-NT	OVX+PPT-NT	OVX+DPN-NT	INT-HT	OVX-HT	OVX+PPT-HT	OVX+DPN-HT
n	**5**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Vehicle-Control (mN/mm)	9.97 ± 0.87^{b}	8.63 ± 0.64^{b}	12.96 ± 0.71^{a}	9.93 ± 0.58^{b}	11.77 ± 1.40	10.14 ± 1.01	11.39 ± 1.08	11.34 ± 1.39
SQ 29,548 (mN/mm)	9.16 ± 1.53^{b}	9.48 ± 1.14^{b}	$11.40 \pm 1.76^{a,b}$	9.71 ± 0.82^{b}	12.69 ± 1.77	8.43 ± 1.27	12.25 ± 0.85	9.56 ± 1.02

Table 3. B.

	INT-NT	OVX-NT	OVX+PPT-NT	OVX+DPN-NT	INT-HT	OVX-HT	OVX+PPT-HT	OVX+DPN-HT
n	**5**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Vehicle-Control (pM)	$6.19 \times 10^{-12} \pm 1.49 \times 10^{-12}$	$8.88 \times 10^{-12} \pm 2.87 \times 10^{-12}$	$14.8 \times 10^{-12} \pm 2.39 \times 10^{-12}$	$19.5 \times 10^{-12} \pm 10.9 \times 10^{-12}$	$15.5 \times 10^{-12} \pm 8.04 \times 10^{-12}$	$12.5 \times 10^{-12} \pm 2.8 \times 10^{-12}$	$8.62 \times 10^{-12} \pm 3.23 \times 10^{-12}$	$26.7 \times 10^{-12} \pm 16.3 \times 10^{-12}$
SQ 29,548 (pM)	$10.50 \times 10^{-12} \pm 2.19 \times 10^{-12}$	$15.7 \times 10^{-12} \pm 7.02 \times 10^{-12}$	$16.3 \times 10^{-12} \pm 3.47 \times 10^{-12}$	$17.4 \times 10^{-12} \pm 5.31 \times 10^{-12}$	$22.0 \times 10^{-12} \pm 13.8 \times 10^{-12}$	$20.1 \times 10^{-12} \pm 12.0 \times 10^{-12}$	$9.02 \times 10^{-12} \pm 3.33 \times 10^{-12}$	$54.1 \times 10^{-12} \pm 28.9 \times 10^{-12}$

Note: Values are means ± S.E. (n = number of rats). [a-b]Mean values within normotensive (NT) without common script are significantly different ($0.0002 \leq P \leq 0.02$). There were no significant differences in EC_{50} between vehicle-control and SQ 29,548 treated microvessels.

4.4.2 Effects of Simvastatin on Mesenteric Arteriole Responses to Vasopressin

Arterioles from INT-NT and OVX-NT rats were treated with 60 μM Simvastatin (SIM). At this concentration, SIM nearly eliminated contractile responses to VP throughout the concentration-response curve (maximal responses were 0.12 ± 0.03 mN/mm and 0.11 ± 0.06 mN/mm respectively; 99% reduction from vehicle-control) (Table 4). Subsequently, the remaining six groups were treated with 10 μM SIM. Mesenteric arterioles in all groups exhibited significant attenuation ($0.0001 \leq P \leq 0.05$) of the contractile responses to VP throughout the range of concentrations in the presence of SIM. In OVX+PPT-NT, SIM reduced the contractile response to VP by 73% at the maximal concentration of VP (10^{-8}M) (12.96 ± 0.64 mN/mm vs. 3.52 ± 0.65 mN/mm) (Fig. 26), whereas OVX+DPN-NT exhibited a 92% reduction at the maximal concentration of VP (9.93 ± 0.53 mN/mm vs. 0.78 ± 0.25 mN/mm) (Fig. 27).

In INT-HT, SIM reduced the maximal response to VP by 35% (11.77 ± 1.40 mN/mm vs. 7.62 ± 1.01 mN/mm respectively) (Fig. 28), while in OVX-HT the maximal response to VP was reduced by 61% (10.14 ± 1.01 mN/mm vs. 3.91 ± 0.45 mN/mm respectively) (Fig. 29). In PPT-HT, SIM reduced the maximal response to VP by 28% (11.39 ± 1.08 mN/mm vs. 8.15 ± 1.26 mN/mm respectively) (Fig. 30), while in DPN-HT, SIM reduced the maximal response to VP by 62% (11.34 ± 1.39 mN/mm vs. 4.35 ± 0.76 mN/mm respectively) (Fig. 31). In arterioles from both INT-NT and INT-HT rats, SIM had a profound effect to decrease sensitivity to VP.

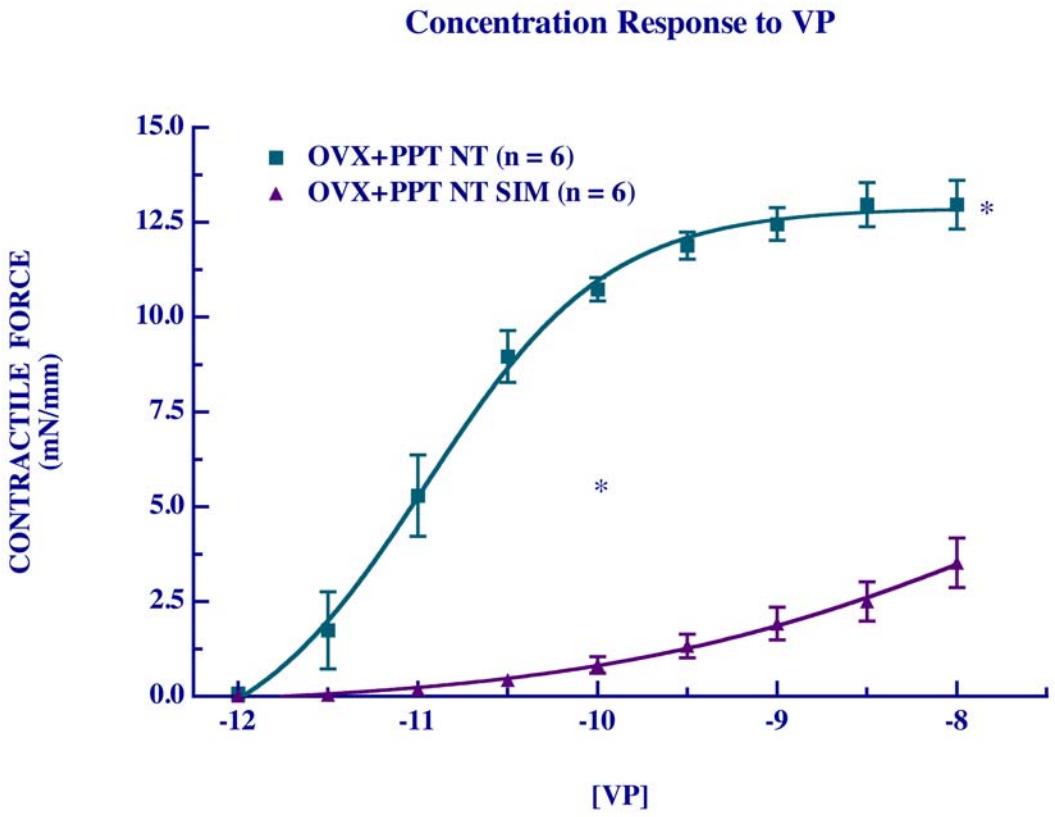

Figure 26: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+PPT-treated NT Sprague-Dawley rats in the presence of Simvastatin (SIM, 10 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). *Statistically significant differences exist in OVX+PPT-NT for SIM vs. VEH (P = 0.0001) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

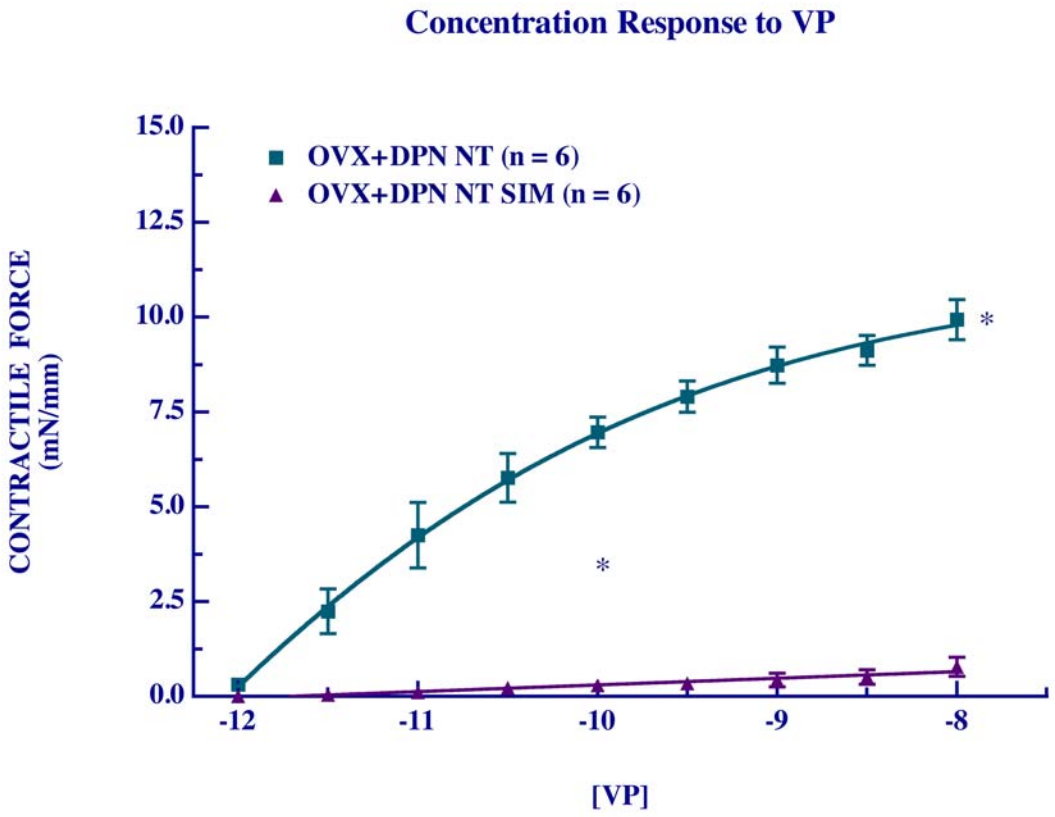

Figure 27: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX+DPN-treated NT Sprague-Dawley rats in the presence of Simvastatin (SIM, 10 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points means ± S.E. (n = number of animals). *Statistically significant differences exist in OVX+DPN-NT for SIM vs. VEH ($0.001 \leq P \leq 0.0084$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

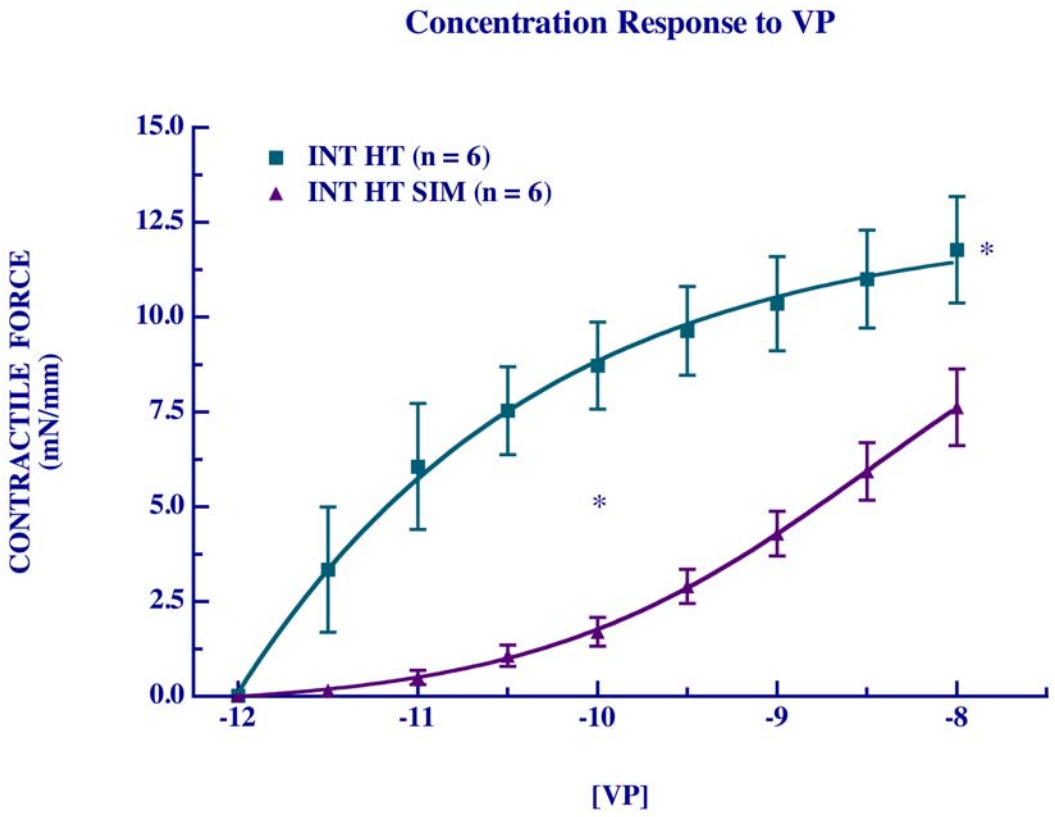

Figure 28: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from INT-HT Sprague-Dawley rats in the presence of Simvastatin (SIM, 10 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). *Statistically significant differences exist in INT-HT for SIM vs. VEH ($0.0012 \leq P \leq 0.0319$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

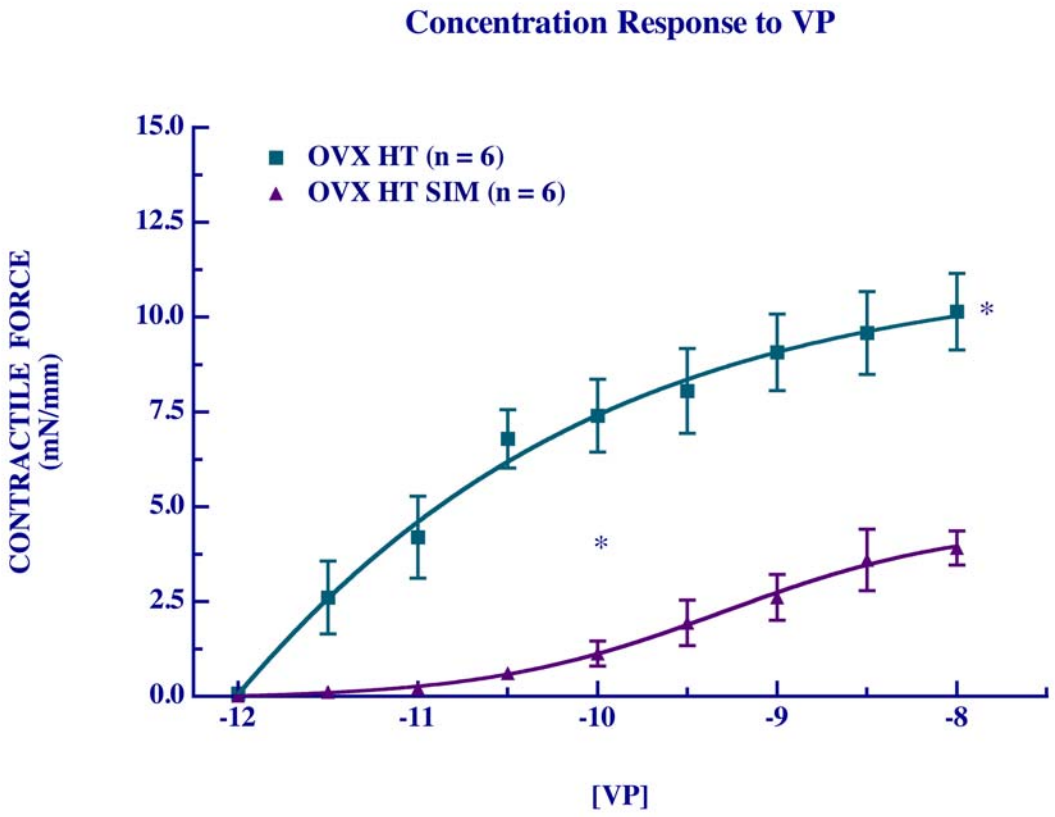

Figure 29: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX-HT Sprague-Dawley rats in the presence of Simvastatin (SIM, 10 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points are means ± S.E. (n = number of animals). *Statistically significant differences exist in INT-HT for SIM vs. VEH ($0.0002 \leq P \leq 0.0005$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

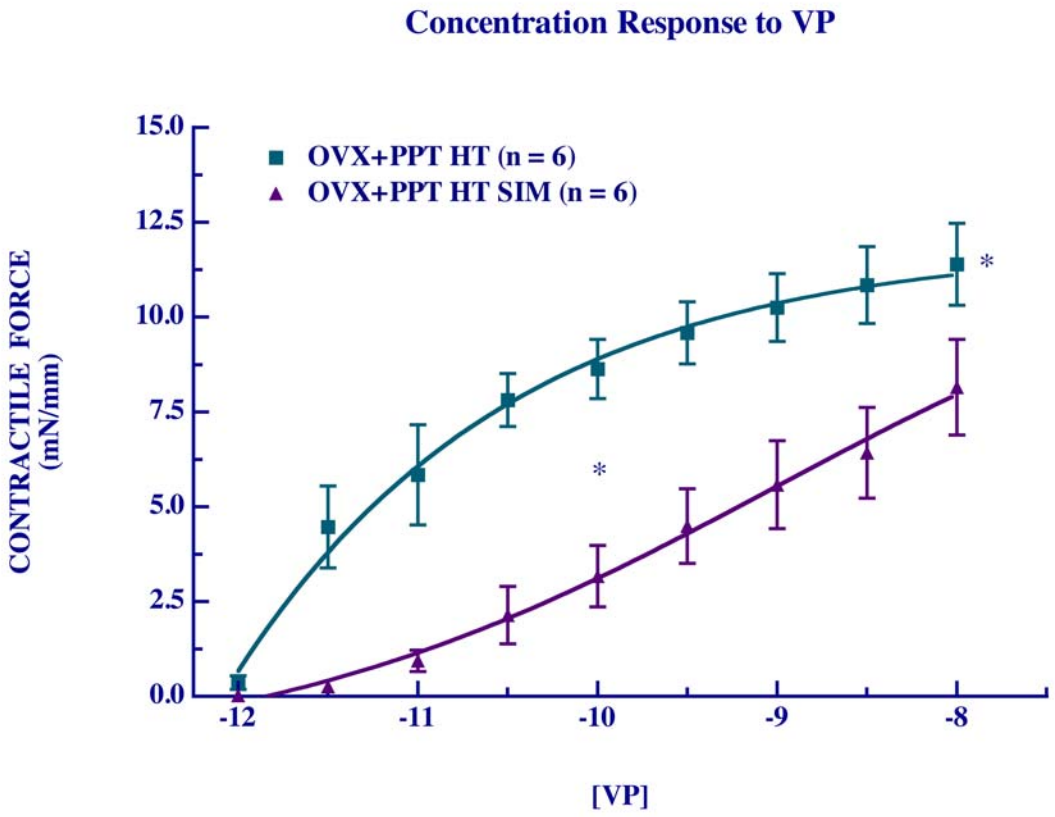

Figure 30: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX-PPT-treated HT female Sprague-Dawley rats in the presence of Simvastatin (SIM, 10 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points represent means ± S.E. (n = number of animals). *Statistically significant differences exist in PPT-OVX-HT for SIM vs. Veh ($0.0044 \leq P \leq 0.05$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

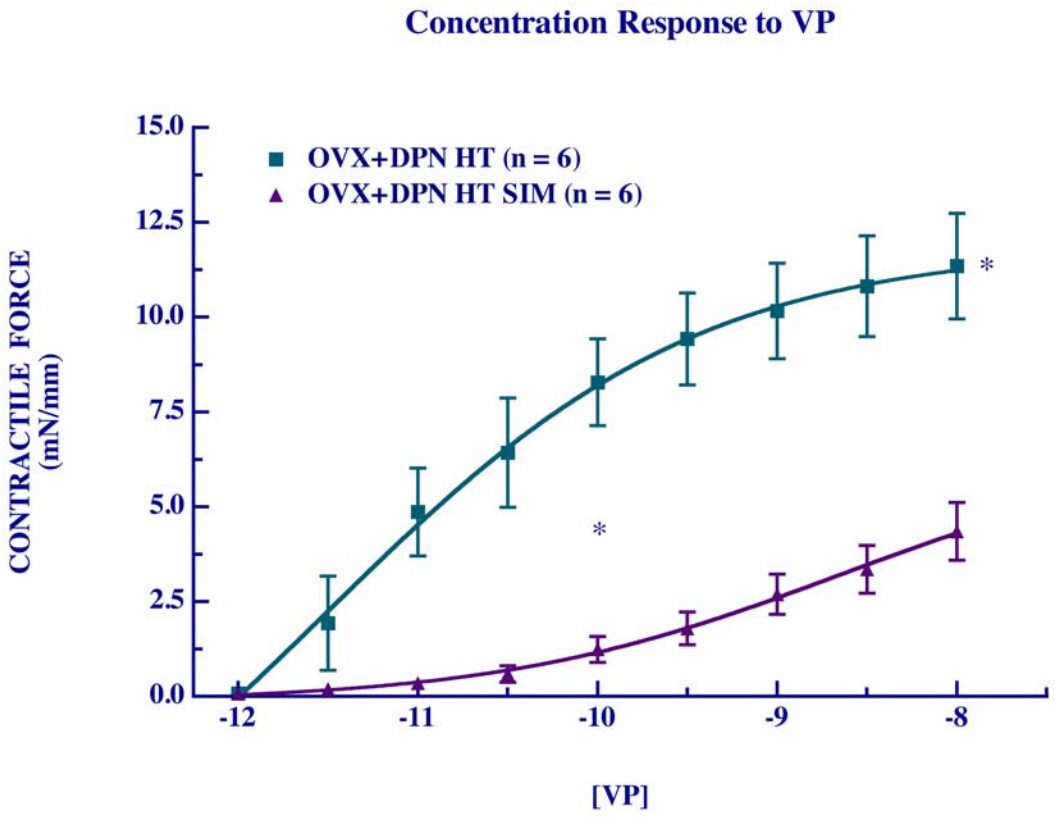

Figure 31: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact mesenteric arterioles from OVX-DPN-treated HT female Sprague-Dawley rats in the presence of Simvastatin (SIM, 10 μM) or vehicle-control. Contractile force was normalized by vessel length. Data points represent means ± S.E.; (n = number of animals). *Statistically significant differences exist in OVX-DPN-HT for SIM vs. VEH ($0.0003 \leq P \leq 0.0019$) at both middle ($1x10^{-10}$M) and maximal ($1x10^{-8}$M) concentrations of VP.

Table 4. A. Maximal contractile responses to vasopressin (A) and sensitivity to VP (EC_{50}; B) for vehicle-control and simvastatin treated mesenteric arterioles.

	INT-NT	OVX-NT	OVX+PPT-NT	OVX+DPN-NT	INT-HT	OVX-HT	OVX+PPT-HT	OVX+DPN-HT
n	**5**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Vehicle-Control (mN/mm)	9.97 ± 0.87^{b}	8.63 ± 0.64^{b}	12.96 ± 0.71^{a}	9.93 ± 0.58^{b}	11.77 ± 1.40^{a}	10.14 ± 1.01^{a}	11.39 ± 1.08^{a}	11.34 ± 1.39^{a}
Simvastatin (mN/mm)	0.12 ± 0.03^{c} (60 μM)	0.11 ± 0.07^{c} (60 μM)	3.52 ± 0.71^{d} (10 μM)	0.78 ± 0.31^{c} (10 μM)	7.62 ± 1.01^{c} (10 μM)	3.91 ± 0.45^{b} (10 μM)	8.15 ± 1.26^{c} (10 μM)	4.35 ± 0.76^{b} (10 μM)

Table 4. B.

	INT-NT	OVX-NT	OVX+PPT-NT	OVX+DPN-NT	INT-HT	OVX-HT	OVX+PPT-HT	OVX+DPN-HT
n	**5**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Vehicle-Control (mN/mm)	$6.19 \times 10^{-12} \pm 1.49 \times 10^{-12b}$	$8.88 \times 10^{-12} \pm 2.87 \times 10^{-12ab}$	$14.8 \times 10^{-12} \pm 2.39 \times 10^{-12*a}$	$19.5 \times 10^{-12} \pm 10.9 \times 10^{-12*ab}$	$15.5 \times 10^{-12} \pm 8.04 \times 10^{-12*}$	$12.5 \times 10^{-12} \pm 2.8 \times 10^{-12*}$	$8.62 \times 10^{-12} \pm 3.23 \times 10^{-12*}$	$26.7 \times 10^{-12} \pm 16.3 \times 10^{-12*}$
Simvastatin (mN/mm)	--	--	$877.0 \times 10^{-12} \pm 213.0 \times 10^{-12*}$	$1040.0 \times 10^{-12} \pm 405.0 \times 10^{-12*}$	$684.0 \times 10^{-12} \pm 81.1 \times 10^{-12*a}$	$902.0 \times 10^{-12} \pm 329.0 \times 10^{-12*ab}$	$325.0 \times 10^{-12} \pm 63.1 \times 10^{-12*b}$	$644.0 \times 10^{-12} \pm 226.0 \times 10^{-12*ab}$

Note: Values are means ± S.E. (n = number of rats). $^{a-d}$Mean values within normotensive (NT) or hypertensive (HT) maximum contraction without common script are significantly different ($0.0002 \leq P \leq 0.05$). *Mean values between vehicle control and simvastatin EC_{50} are significantly different ($0.0001 \leq P \leq 0.0167$). $^{a-b}$Mean values within normotensive (NT) or hypertensive (HT) EC_{50} without common script are significantly different ($0.0048 \leq P \leq 0.0134$).

4.5 Effect of Estrogen Receptor Agonists on Thoracic Aorta Responses to Vasopressin

The contributions of the thromboxane pathway and intracellular calcium to VP-induced contractions of the rat thoracic aorta were quite different than those in the mesenteric arteriole. In OVX+PPT-NT (3,887 ± 247 mg/mg ring wt.), SQ attenuated the contractile response to VP by 34% (2,577 ± 173 mg/mg ring wt.), while in OVX+DPN-NT (3,038 ± 472 mg/mg ring wt.), SQ attenuated the contractile response to VP by 56% (1,328 ± 167 mg/mg ring wt.). In OVX+PPT-NT, SIM (60 μM) attenuated the contractile response to VP by 34%, (2,572 ± 274 mg/mg ring wt.), while in OVX+DPN-NT SIM attenuated the contractile response to VP by only 18% (2,497 ± 164 mg/mg ring wt.) (Figs. 32 and 33).

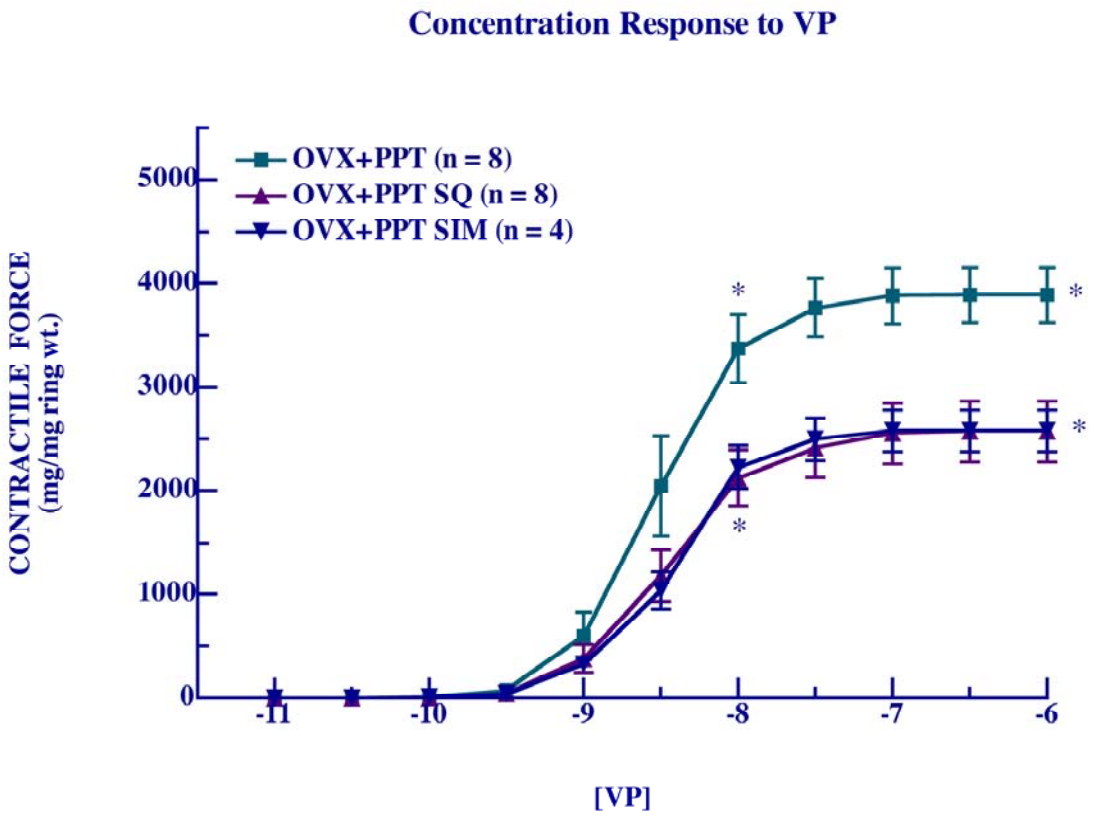

Figure 32: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact aortic ring segments from OVX+PPT-treated NT Sprague-Dawley rats in the presence of Simvastatin (SIM, 60 μM), SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by dry ring weight. Data points are means ± S.E. (n = number of animals). *Statistically significant differences exist in OVX-PPT-NT for SQ and SIM vs. vehicle-control ($0.0025 \leq P \leq 0.0220$) at both middle ($1x10^{-8}$M) and maximal ($1x10^{-6}$ M) concentrations of VP.

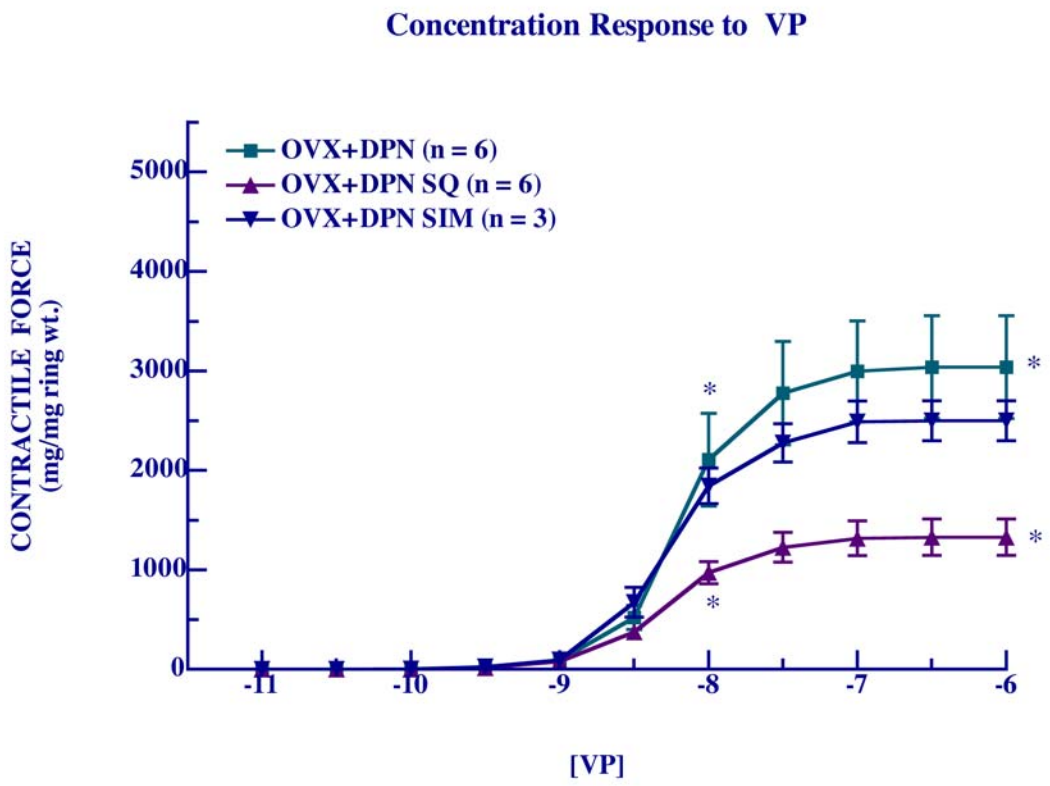

Figure 33: Concentration-response curves for arginine vasopressin (VP) in endothelium-intact aortic ring segments from OVX+DPN-treated NT Sprague-Dawley rats in the presence of Simvastatin (SIM, 60 μM), SQ 29,548 (SQ, 1 μM) or vehicle-control. Contractile force was normalized by dry ring weight. Data points are means ± S.E. (n = number of animals). *Statistically significant differences exist in OVX-DPN-NT for SQ vs. vehicle-control ($0.0055 \leq P \leq 0.0208$) at both middle ($1x10^{-8}$M) and maximal ($1x10^{-6}$ M) concentrations of VP.

4.6 Effect of Aortic Coarctation-Induced Hypertension and Estrogen Treatment on Estrogen Receptor Expression

4.6.1 Real Time RT-PCR

Neither estrogen replacement nor aortic coarctation caused significant changes in the expression of ERα or ERβ ($P > 0.05$) (Figs. 34, 35).

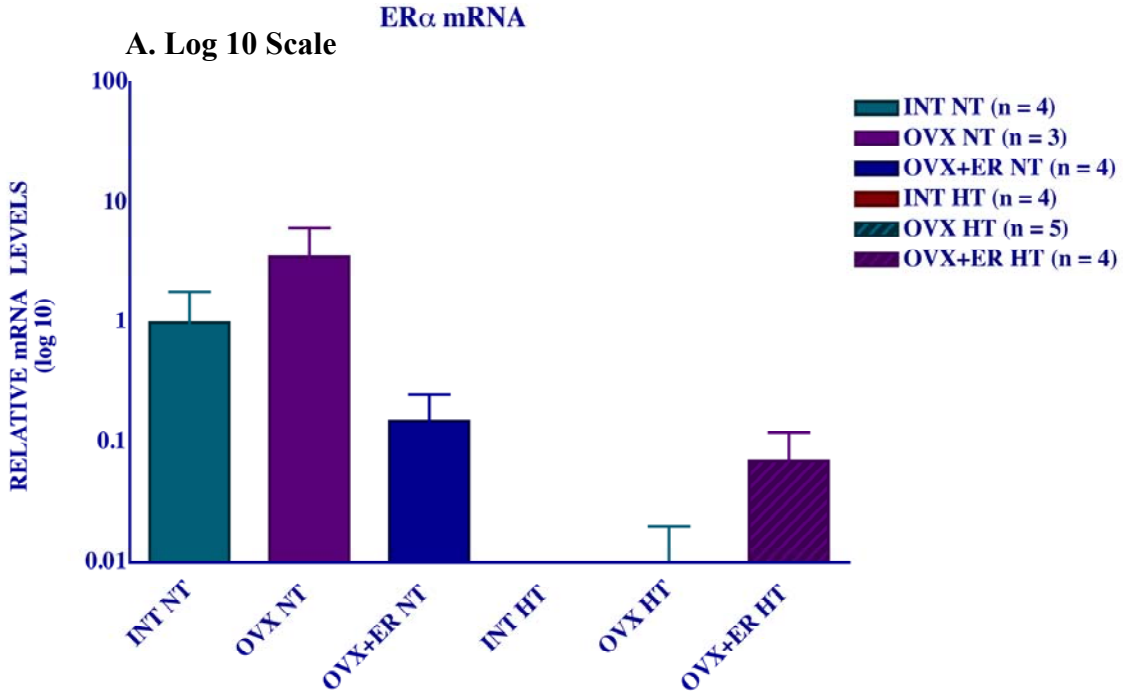

Figure 34: (A) Effect of hypertension and estrogen receptor agonists on mRNA levels of estrogen receptor alpha. ERα was quantified by the ΔC_T method and expressed relative to ERα of INT-NT in both log 10 scale (A) and linear scale (B). Bars are means ± SE. (n = number of animals). There were no significant differences in ERα mRNA levels ($P > 0.05$).

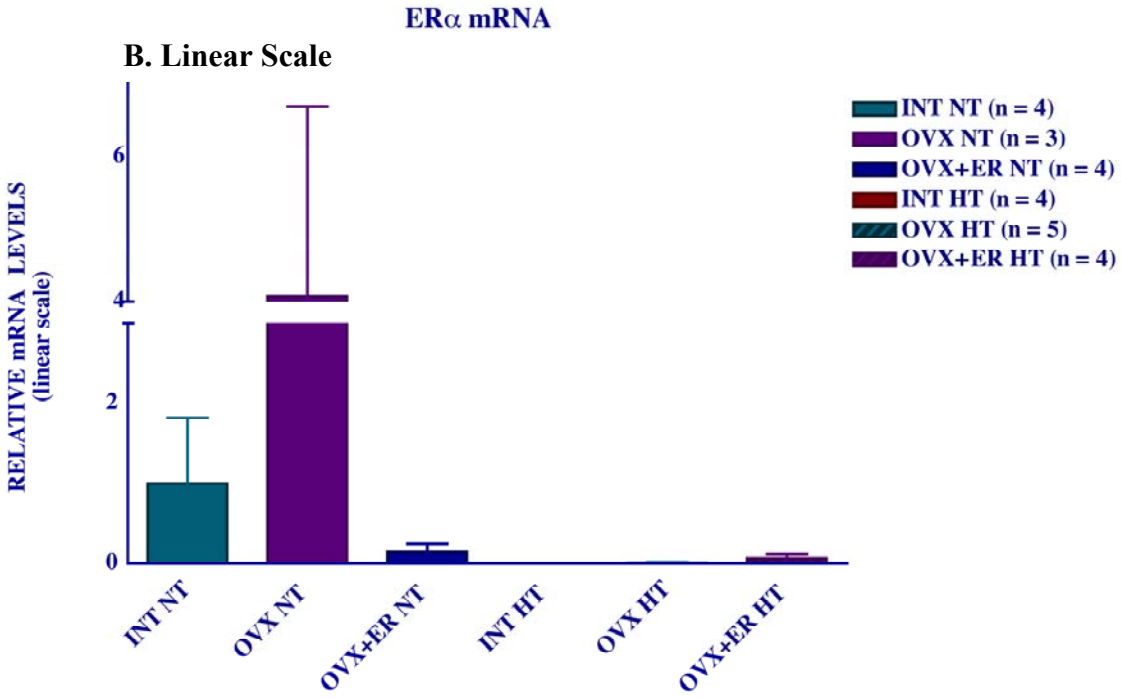

Figure 34 continued: (B) Effect of hypertension and estrogen receptor agonists on mRNA levels of estrogen receptor alpha. ERα was quantified by the ΔC_T method and expressed relative to ERα of INT-NT in both log 10 scale (A) and linear scale (B). Bars are means ± SE. (n = number of animals). There were no significant differences in ERα mRNA levels ($P > 0.05$).

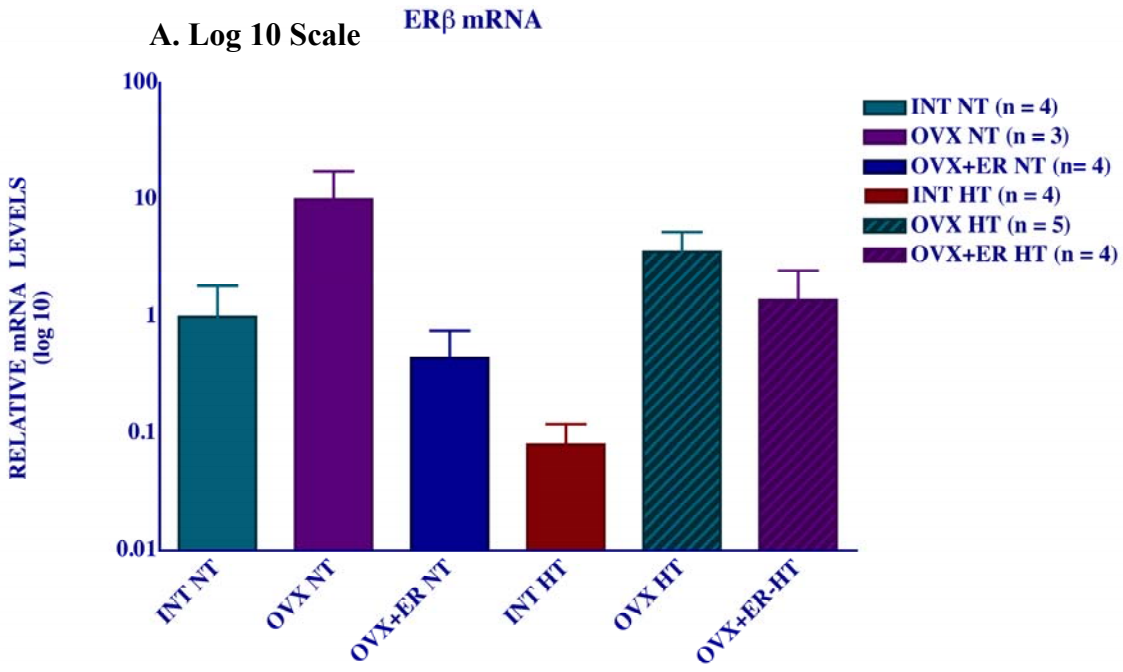

Figure 35: (A) Effect of hypertension and estrogen receptor agonists on mRNA levels of estrogen receptor beta. ERβ was by the ΔC_T method and expressed relative to ERβ of INT-NT in both log 10 scale (A) and linear scale (B). Bars are means ± SE. (n = number of animals). There were no significant differences in ERβ mRNA levels

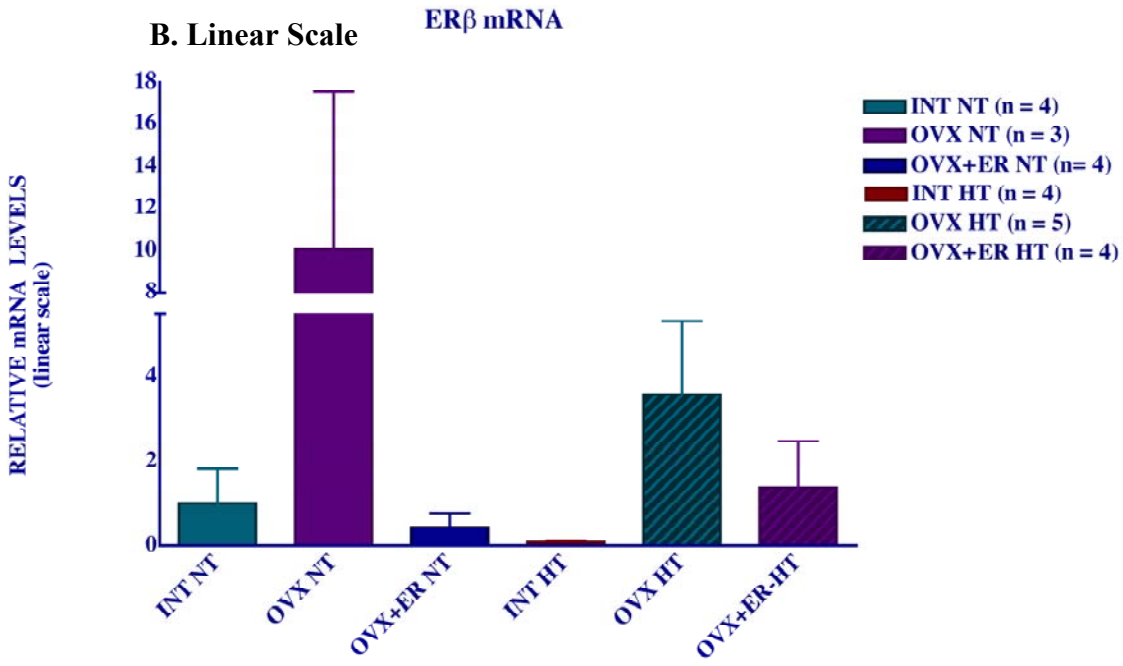

Figure 35 continued: (B) Effect of hypertension and estrogen receptor agonists on mRNA levels of estrogen receptor beta. ERβ was by the ΔC_T method and expressed relative to ERβ of INT-NT in both log 10 scale (A) and linear scale (B). Bars are means ± SE. (n = number of animals). There were no significant differences in ERβ

4.6.2 Immunoblots

Neither estrogen receptor agonist treatment nor aortic coarctation caused significant changes in the protein expression of ERα or ERβ ($P > 0.05$) (Figs. 36, 37).

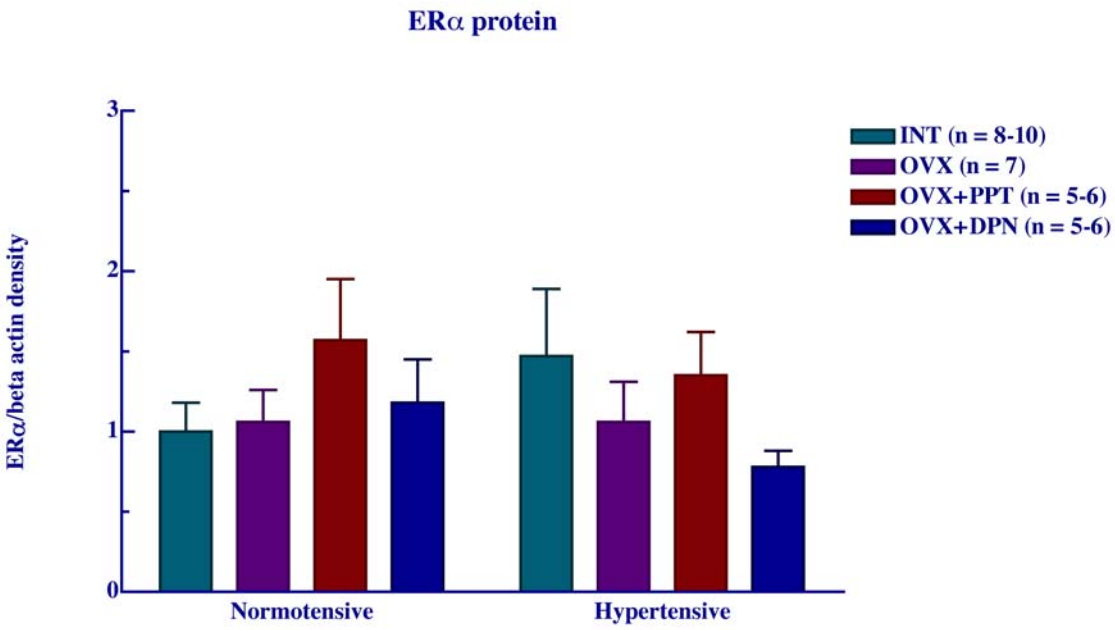

Figure 36: Effect of hypertension and estrogen receptor agonists on the expression of estrogen receptor alpha. ERα was quantified by densitometry analysis, normalized to beta actin and expressed relative to ERα of INT-NT density. Bars are means ± SE. (n = number of animals). There were no significant differences in ERα protein expression ($P > 0.05$).

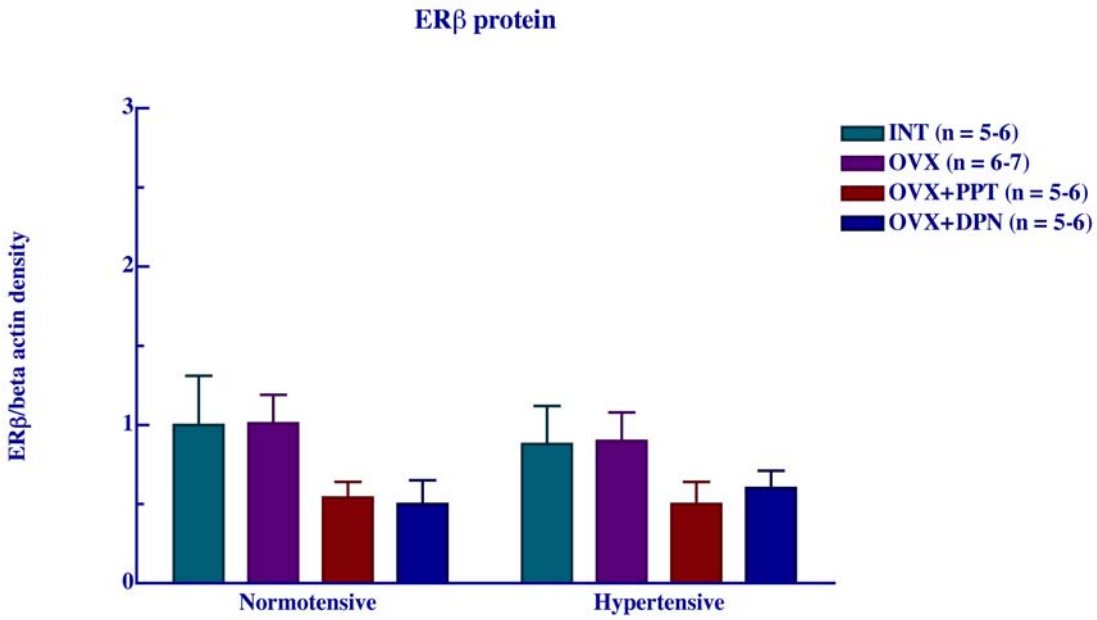

Figure 37: Effect of hypertension and estrogen receptor agonists on the expression of estrogen receptor beta. ERβ was quantified by densitometry analysis, normalized to beta actin and expressed relative to ERβ of INT-NT density. Bars are means ± SE. (n = number of animals). There were no significant differences in ERβ protein expression ($P > 0.05$).

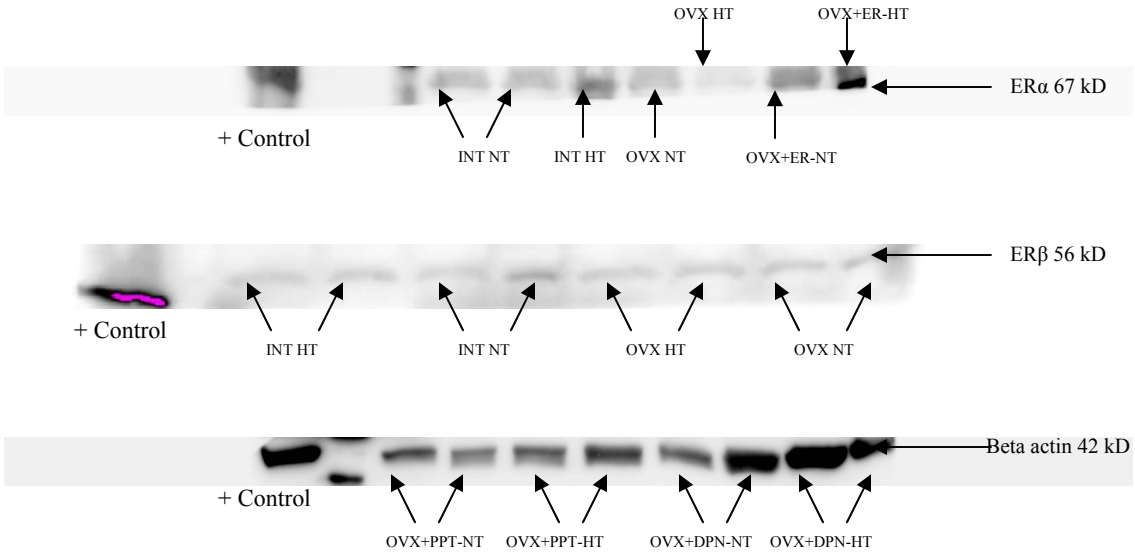

Figure 38: Representative immunoblot analysis of ERα, ERβ and beta actin (loading control) in mesenteric arterioles.

CHAPTER V

SUMMARY AND DISCUSSION

This study investigated the effects of estrogen and selective estrogen receptor agonists on the regulation of mean arterial blood pressure and prostanoid function and on the roles of thromboxane and intracellular calcium in vascular reactivity of mesenteric arterioles to vasopressin in the female Sprague-Dawley rat. The central hypothesis tested was that in aortic coarctation-induced hypertension, estrogen upregulates the constrictor prostanoid pathway in the vascular wall via activation of ERβ, enhancing vascular tone and leading to deleterious effects on blood pressure in the female rat. If so, then rats treated with an ERβ agonist would have higher mean arterial pressures than rats treated with an ERα agonist, as well as increased release of constrictor prostanoids from the vasculature, which would potentiate vascular reactivity to VP. ERβ was suspected of having a main role, based on evidence that it is the more abundant vascular ER. Indeed, rats treated with DPN (ERβ agonist) exhibited mean arterial pressures comparable to those of intact female rats during the development of aortic coarctation-induced hypertension. However, PPT-(ERα agonist) treated rats exhibited similar mean arterial pressures as those seen with DPN treatment. While both treatments resulted in similar increases in blood pressure during aortic coarctation-induced hypertension, which were greater than those in OVX rats, there were significant differences in the release of constrictor prostanoids from the mesenteric vasculature in both normotension and hypertension in ERα vs. ERβ-treated rats. During the development of hypertension, mean arterial pressures in PPT-treated rats can be explained

by the increase in thromboxane release by the mesenteric vascular wall in this group, while the DPN-treated group appears to have another mechanism mediating increases in blood pressure during aortic coarctation-induced hypertension. Prostacyclin release exhibited similar trends as thromboxane, but with a few minor differences.

In mesenteric vascular function studies, blocking the thromboxane receptor with SQ 29,548 did not affect the contractile responses to VP in any groups. However, blocking intracellular calcium release from the sarcoplasmic reticulum with SIM resulted in differential effects in both normotensive and hypertensive groups and among INT, OVX, PPT and DPN groups. SIM had a greater effect in normotensive than in hypertensive rats, and a greater effect in DPN-treated groups than in PPT-treated groups, even during hypertension. The vascular function studies performed in the mesenteric arterioles did not mirror preliminary studies performed in the aorta, where SQ 29,548 treatment attenuated contractile responses to VP to differing extents in normotensive PPT and DPN treated rats, and SIM appeared to have greater effects in the PPT-treated group rather than the DPN-treated group. These studies may reflect regional differences in the roles of ERα and ERβ and/or intracellular Ca^{2+} handling in aorta vs. mesentery.

In most experiments in this study, PPT treatment closely mimicked function in INT rats, while DPN treatment more closely resembled function in OVX rats. This would suggest that most of the actions of estrogen on constrictor prostanoid function and vascular reactivity to VP are mediated by ERα alone. A noticeable instance where DPN treatment had similar results to INT was in the development of ACIH, where MAP was just as severe in the DPN treated group at 12 days as in INT and PPT-treated rats.

5.1 Effects of Aortic Coarctation-Induced Hypertension on Plasma Estradiol and Estrone Levels

In the present studies, plasma levels of 17β-estradiol and estrone were measured in normotensive and ACIH rats in an effort to determine whether the development of ACIH was associated with alterations in the secretion and/or metabolism of estrogens. Studies in humans with essential hypertension revealed that serum levels of estradiol were higher in both men and women (59, 65) compared to their normotensive counterparts. In the present study, estradiol levels in INT-HT females were not altered; however, OVX-HT females had slight but significant increases in plasma estradiol on days 0, 4 and 7 post-coarctation, indicating an increase from an extra-ovarian source of estradiol, most likely the adrenal gland. In OVX+ER-HT, plasma estradiol on days 0, 4 and 7 post-coarctation were significantly lower than in OVX+ER-NT. Although the estradiol level at day 0 in OVX+ER-NT was twice that of OVX+ER-HT (the pellets from the NT group had a higher rate of E_2 release than is normally expected), the rate of estrogen metabolism appeared to differ between the two groups. OVX+ER-NT had a steady rate of decline from day 0 to day 4 to day 7, with no significant decrease between day 7 and day 10. In contrast, OVX+ER-HT had a significant decline from day 0 to day 4, but the levels were steady from day 4 to days 7 and 10. This suggests that during ACIH, exogenous estrogen is metabolized at a faster rate than during NT, while ovarian estrogen secretion does not change during ACIH.

In the present study, plasma estrone levels in INT and OVX females were very similar, both in normotension and during the development of ACIH. This suggests that the

production of estrone is independent of ovarian function, and likely arises from peripheral conversion of adrenal derived E_2. The levels of estrone in the present study in INT rats were similar to those in a previous study (121). In the present study, the presence of the exogenous estrogen source in OVX+ER groups resulted in higher levels of estrone, due to the conversion of 17β-estradiol to estrone by 17β-hydroxysteroid dehydrogenase in the periphery.

One interesting observation of the present study was the trend of estrone to closely follow 17β-estradiol, indicating that, for the most part, where 17β-estradiol increased or decreased, estrone also exhibited parallel increases or decreases. This serves as an internal control and verifies that the changes in estrogen levels measured by RIA are authentic.

5.2 Effects of Aortic Coarctation-Induced Hypertension and Estrogen Receptor Agonists on Mean Arterial Pressure

The present study revealed that INT-HT rats exhibited a higher MAP at day 2 than OVX-HT or OVX+PPT-HT, while MAP of OVX+DPN-HT did not differ from INT-HT on day 2. However by 12 days post-coarctation, the differences among INT, OVX+ER-agonist and OVX treated groups had progressively widened to reveal marked statistically significant differences in MAP. At 12 days post-coarctation, MAP during treatment with either ERα or ERβ agonists closely resembled that of INT rats while OVX rats exhibited a significantly lower MAP.

These results support previous work by Baltzer *et al.* (6, 7) which reported that the presence of estrogen in INT or OVX+ER rats results in a higher MAP than in OVX or male rats at days 12-14 of ACIH (6, 7, 8). One difference between this and the present

study is the significantly higher MAP at day 2 in INT-HT rats when compared to OVX-HT rats in this study. Prior to the studies by Baltzer *et al.* (6, 7, 8), there were only two short-term investigations that examined MAP in female rats with ACIH. One study revealed that chronic estradiol treatment increased the sensitivity of the carotid arteries to ANG II induced-vasoconstriction (116), while another study compared the effects of partial aortic coarctation in pregnant vs. virgin female rats and found that in pregnant females there was an increase in MAP (54). While the Henzel study did not find any change in the MAP of virgin female rats, this is most likely due to the use of partial aortic coarctation, rather than the complete aortic coarctation that was used in the present study as well as the Baltzer study (6, 7, 8).

While there were subtle differences in MAP between INT-HT, OVX+PPT-HT and OVX+DPN-HT at day 2, these differences were abolished at day 12. The higher MAP of OVX+DPN-HT rats at day 12 is likely due to a different mechanism (perhaps neural mechanisms involved in ACIH) than that of INT-HT and OVX+PPT-HT rats, as mesenteric arteriolar TXA_2 production in response to VP-stimulation was nearly half that of OVX+PPT-HT, but not significantly different than arterioles from INT-HT rats. Previous studies by Baltzer *et al.* (6, 7, 8) revealed that acute infusion of SQ 29,548 (TP receptor antagonist) at day 14 or chronic treatment with Ridogrel (TP receptor antagonist/TXS inhibitor) during the development of ACIH during days 1-14 significantly attenuated the MAP of INT and OVX+ER females at 12-14 days to a much greater extent than in OVX or male rats, indicating that TXA_2 and TP receptor activation are central to the effects of estrogen to exacerbate MAP in ACIH. In the present study, increased

arteriolar TXA_2 production in OVX+PPT-HT and INT-HT rats can be associated with the high MAP observed at day 12, based on the findings in the present studies and those of Baltzer *et al.* (6, 7, 8) involving systemic administration of SQ, which lowered MAP markedly in INT-HT rats. However, the high MAP observed in OVX+DPN-HT cannot be explained by a massive increase in TXA_2, since the arterioles from this group did not have similar increases in TXA_2 production when stimulated with VP.

A previous study examined blood pressure and vascular function in male ERβ knock-out mice and found that the absence of ERβ resulted in systolic and diastolic hypertension with aging, suggesting that ERα, when unopposed by ERβ, possesses the ability to exert detrimental effects on blood pressure (152). In the present study, both selective and non-selective ER agonist activity clearly exacerbate the development of ACIH. Based on the data in the present study, there is no difference at day 12 in MAP whether PPT (ERα), DPN (ERβ) or 17β-estradiol (ERα and ERβ) are present. It is apparent that the activity of both of the ERs is detrimental and exacerbates MAP in ACIH.

Based on previous studies of the effects of 17β-estradiol to upregulate TXA_2 production in the vascular wall, as well as TxS and TP receptors (73), and studies demonstrating that females rely more on intracellular calcium release in the contractile response to VP (36), the mechanisms by which ERα and ERβ modulate blood pressure may lie in differences in these two pathways (see sections 5.3.1 and 5.3.2).

5.3 Effects of Aortic Coarctation and Estrogen Receptor Agonists on TXA_2 and PGI_2 Release from the Mesenteric Vasculature

In the present study, basal release of PGI_2 and TXA_2 was similar across all NT groups; however, OVX+PPT treatment resulted in a higher basal release of TXA_2 than OVX+DPN treatment. Similarly, basal release of PGI_2 also was higher in OVX+PPT than in all other groups. The effect of OVX+PPT to increase both TXA_2 and PGI_2 release in arterioles from normotensive rats suggests that a common precursor is upregulated by PPT, possibly COX-2. Recent studies by Li *et al.* (73) support this idea, since 17β-estradiol replacement therapy upregulated expression of both COX-2 and TxS, enhancing the release of both TXA_2 and PGI_2 in the NT female rat aorta (73). Other studies have demonstrated that estrogen treatment increases COX-2 protein in cultured endothelial cells (2, 131).

Likewise, the dramatic increase in basal release of both TXA_2 and PGI_2 across all the groups during the development of ACIH in the present study demonstrates that a precursor molecule common to both TXA_2 and PGI_2 is upregulated during hypertension, likely COX-1 or COX-2, and possibly both. Previous studies have shown that prostaglandin release is increased during hypertension (7, 74, 75) and that 17β-estradiol upregulates the prostaglandin pathway, from COX-2 to TxS to the TP receptor (73). Therefore, there is a synergistic effect of hypertension and the presence of estrogen to increase prostaglandin production and activity. However, the effects of specific ER agonists demonstrated that significant differences exist in the extent to which ERs can affect TXA_2 and PGI_2 production during the development of ACIH.

5.3.1 TXA_2

The present study quantified basal and VP-stimulated TXA_2 release from mesenteric arterioles in normotension and during the development of ACIH hypertension. In normotensive rats, basal release did not differ among the groups with the exception of OVX+PPT arterioles, which exhibited higher basal release of TXA_2 than OVX+DPN treatment. For INT and OVX groups, this consistency in basal release is in agreement with previous studies of the female rat aorta, where basal release did not differ between INT, OVX and OVX+ER females (73).

In the present study, arterioles from INT-NT rats demonstrated the largest increase in TXA_2 release of all NT groups in response to VP-stimulation. While arterioles from OVX and OVX+DPN rats also exhibited significant increases in TXA_2 release in response to VP, agonist-induced increases were much smaller than in INT rats. In OVX+PPT rats, VP-induced release of TXA_2 did not differ significantly from basal release.

During the development of hypertension, basal release among groups did not differ from one another, although ACIH increased basal release of TXA_2 in all of the groups compared to NT rats. Arterioles from INT, OVX+PPT and OVX+DPN groups all exhibited higher agonist-induced release of TXA_2 compared to OVX. While the statistical analysis did not detect any significant differences in VP-stimulated TXA_2 release between OVX+DPN-HT and OVX+PPT-HT, this is likely due to the small sample size of the OVX+DPN-HT group ($n = 3$), because the mean difference in TXA_2 production by OVX+PPT-HT arterioles was nearly two-fold higher than that of OVX+DPN-HT vessels,

suggesting that this difference is likely to be biologically significant, based upon previous functional and prostanoid release studies in the rat aorta (72, 73).

During the development of ACIH, TXA_2 release appears to be reduced about half in the combined presence of both ERα and ERβ activity in the INT group, when compared to ERα activity alone in the PPT-treated group, where TXA_2 production is quite high. This suggests that ERβ may have some inhibitory effects on ERα activities, which has been suggested by several previous studies on the interactions of ERs (28, 80, 81, 95, 130). It is possible that homodimer formation of ERα leads to the detrimental effects seen in the PPT-treated group, since it appears that ERβ exerts any inhibitory effects on ERα through the preferential formation of heterodimers (80, 96). This formation of the homodimers alone in the PPT-treated group combined with ACIH may result in the significantly increased production of TXA_2 and PGI_2 seen in this study.

5.3.2 PGI_2

In NT rats, basal release of PGI_2 by arterioles from OVX+PPT rats was 2-2.5 fold higher than that of arterioles from INT, OVX+DPN or OVX rats. INT, OVX+DPN and OVX groups all exhibited significant increases in PGI_2 release in response to VP, while arterioles from OVX+PPT rats did not. In HT rats, differences in basal release among the groups were abolished, although ACIH caused significant increases (2-3 fold) in the basal release of PGI_2 in all the groups compared to NT rats. Arterioles from OVX+PPT-HT and INT-HT rats exhibited the highest VP-stimulated release of PGI_2 overall, and significantly higher release than OVX-HT rats, while OVX+DPN-HT treatment did not differ from

OVX-HT. The differences observed between INT-HT and OVX-HT groups were expected, as 17β-estradiol is known to increase PGIS and PGI_2 production (37, 98, 99, 114) as well as COX-2 and the production of PGI_2 (73). While the evidence that estrogen upregulates COX-1, COX-2 and PGIS expression and PGI_2 production is seemingly abundant in the literature, more often than not, these studies have failed to measure the effects of estrogen on TxS expression and TXA_2 production. Based on the findings of the present study, it is clear that both pathways are upregulated not only by estrogen, but by any activity of the ERs, especially ERα.

The findings of the present study suggest that ERα possesses the ability to upregulate PGIS as well as TxS, and likely the COX enzymes, while ERβ appears to have very little effect on TxS and the COX enzymes, but may act more on PGIS, at least during ACIH. Further, the present study suggests that the differential changes in basal and VP-stimulated release of TXA_2 and PGI_2 in the presence of OVX+PPT vs. OVX+DPN vs. INT could result from the formation of hetero- or homodimers by the ERs, which could play a role in determining the physiological response or pathways that are influenced by estrogen.

While TXA_2 production is greatly increased during ACIH, PGI_2 is increased to a much greater extent, raising the question of why PGI_2 does not appear to provide a protective effect on blood pressure or vascular reactivity. The answer may reside in the chronic effects of TXA_2 on the vasculature. While TXA_2 acutely promotes platelet aggregation and powerful vasoconstriction (greater than that of angiotensin II), its long-term effects include VSM hypertrophy and abnormal remodeling of the vascular wall (15, 52). On the other hand, PGI_2 works to combat these effects, by exerting anti-aggregatory

effects on platelets and inhibiting VSM hypertrophy (22, 24). These findings reveal that a delicate balance must be maintained between these two molecules in order for vascular homeostasis to prevail. Therefore, in the case of ACIH, this balance is severely disrupted, and the increased biological activity of TXA_2 is unable to be overridden by the possible protective effects of PGI_2, resulting in enhanced vascular reactivity to VP, thrombosis and MAP, especially in female rats.

5.4 Effects of Aortic Coarctation and Estrogen Receptor Agonists on the Mesenteric Vascular Responses to Arginine Vasopressin

The present study demonstrated clear differential effects of chronic treatment with selective ER agonists on mesenteric vascular function. In normotension, OVX+PPT treatment resulted in markedly higher contractile responses to VP compared to OVX+DPN, INT or OVX. These differences were abolished during ACIH, but the contractile force developed in mesenteric arterioles from ACIH groups were not significantly higher than that from NT rats, and in fact, OVX+PPT-HT developed slightly less contractile force than OVX+PPT-NT. At the middle concentrations only, VP produced significantly higher contractions in INT than in OVX or OVX+DPN. This result was expected in the INT and OVX groups, based on previous findings by Stallone *et al.* (72, 126, 127, 128), which established that intact and estrogen-replaced females exhibit higher responsiveness to VP compared to males or OVX females, both in the aorta and the isolated perfused mesenteric vasculature.

A previous study of the effects of estrogen replacement on mesenteric arteriolar reactivity to VP found no differences between INT, OVX and OVX+ER rats (150). One possible reason for the differences between that study and the present study is the use of pressurized mesenteric vessels in the previous study, which were normalized to a maximal constriction to 120 mM KCl. While this is an acceptable format for reporting this type of data, normalizing the vessels in this manner can mask differences in maximal force developed by the different groups, and so it is difficult to determine whether there were any true differences in the maximal contractile responses to the vasoconstrictors. Further, the rats in this previous study were likely fed standard laboratory rat chow (it was not discussed in the paper), which is known to contain phytoestrogens (119, 120), which confound the effects of ovariectomy on vascular reactivity to VP (43, 72). The present study used an alfalfa- and soy-free diet, which is free of phytoestrogens.

The increased responsiveness to VP in OVX+PPT-NT rats compared to INT-NT and OVX+DPN-NT in the present study was unexpected, since VP-stimulated release of TXA_2 is not different between INT-NT, OVX+PPT-NT and OVX-DPN-NT groups, and therefore it is reasonable to assume that TXA_2 production is not the only pathway differentially regulated by ERα and ERβ in the response to VP. In fact, this idea is supported by two previous studies, one involving sexual dimorphism and vascular reactivity to phenylephrine (PE) in rat aorta, which demonstrated that the female rat aorta was more reactive to PE than male aorta (43), and another demonstrating that estrogen augmented vasoconstriction to PE in male ERβ knock-out mice (152).

The present study also examined the role of ERα and ERβ in the mesenteric arterial response to VP during ACIH. While there were differences in the maximal response to VP in normotensive rats, these differences were abolished in hypertensive rats. While INT-HT, OVX+PPT-HT and OVX+DPN-HT developed slightly higher contractile force than OVX-HT, due to the inherent animal to animal variability of the mesenteric arteriolar response to VP, they were not significantly different. However, the significantly lower MAP in OVX-HT compared to the other ACIH groups would suggest that responses to constrictors should be blunted when compared to those of the ER treated groups. That they were not different may be due to the possible interactions between TXA_2 and PGI_2, as suggested by the effects of TP receptor blockade with SQ 29,548 (see next section).

The fact that OVX+DPN-HT developed nearly identical contractile force as OVX+PPT-HT was unexpected. Although the TXA_2 release in these two groups was not statistically different, the mean TXA_2 production was 2-fold higher in OVX+PPT-HT, and this difference likely has biological relevance. The 2-fold difference in TXA_2 production of these two groups would suggest that OVX+PPT-HT would possess greater reactivity to VP, as in the NT groups. However, the mechanism for the contractile responses of OVX+DPN-HT to VP remains unknown at this time. Further investigation into this mechanism is warranted.

5.4.1 Effects of SQ 29,548 on Concentration-Responses to Vasopressin

To determine the role of ERs on TXA_2 production and TP receptor function in the mesenteric vascular reactivity to VP in the present studies, the TP receptor antagonist SQ 29,548 was employed in vascular function studies. INT-NT, OVX-NT, OVX+DPN-NT, OVX+DPN-HT, INT-HT and OVX+PPT-HT groups all produced significant amounts of thromboxane in response to VP, and yet SQ 29,548 did not have a significant effect on the contractile responses to VP (10^{-12} M – 10^{-8} M) (Figs. 18-25).

In contrast, studies by Fulton *et al.* (43) demonstrated that contractile responses of the female rat aorta to VP were significantly higher in females than in males and that non-selective inhibition of COX enzymes with Indo reduced contractile responses of female aorta to the same extent as SQ 29,548, suggesting that TXA_2 and/or TP receptor activation were responsible for about 30% of VP-mediated contractions of the female rat aorta (43). Studies by Li *et al.* (72) further supported this idea, showing that the maximal response to TXA_2 receptor analog U-46619 was significantly higher in female than in male rats, and that the maximal response in OVX rats was attenuated when compared to INT rats and that OVX+ER restored the contractile responses to U-46619 (72). Studies by Baltzer *et al.* (7) provided further *in vivo* support of this idea, since in ACIH, intravenous infusion of SQ during the acute phase of the hypertension (on day 12-14) in conscious rats resulted in a significantly greater decrease in MAP in INT female and OVX+ER female rats, compared to OVX or male rats. These findings consistently indicate that the TXA_2 pathway is important in the regulation of arterial tone, which is responsible for controlling blood pressure. One earlier study found that OVX increased the sensitivity and maximal

contractility of mesenteric arterioles exposed to U-46619 when compared to OVX+ER (150); however, the vessels used in that study were endothelium-denuded, whereas those in the Fulton and Li studies (43, 72) and the present study were all endothelium intact vessels. Therefore, based upon the previous findings from the studies of Fulton, Li and Baltzer (6-8, 43, 72, 73), a significant effect of SQ 29,548 to attenuate the contractile responses to VP was expected in the vascular function portion of the present study. The failure of TP receptor blockade to attenuate contractile responses to VP suggests that either the estrogen-TXA_2 mechanism is absent from the mesenteric vasculature, or that other local vasoactive factors may be masking the actions of TXA_2.

Indeed, more recent studies have suggested the possibility that other prostaglandins, especially PGI_2, which is normally believed to be vasodilatory, may also contribute to vasoconstrictor responses of VSM to VP. In the Spontaneously Hypertensive Rat (SHR) aorta, the vasodilator agonist acetylcholine (ACh) induces contractile responses via agonist-induced release of a local vasoconstrictor factor (49, 107, 133). The Rapoport study (107) found that ACh-induced contractions of SHR and Wistar-Kyoto (normotensive) rat aortas are composed of two components: activation of the TP receptor, and activation of an additional PG receptor or receptors. That study determined that the amount of PGI_2 produced by the aorta in response to ACh stimulation likely resulted in local concentrations of PGI_2 in the micromolar range, and based on functional studies with carbacyclin (a PGI_2 analogue), this concentration of PGI_2 would be high enough to elicit the contractile responses observed in the aorta. Rapoport *et al.* (107) proposed that PGI_2

was causing the constrictions through activation of the prostaglandin E (EP) receptor, since the constriction with carbacyclin occurred in the presence of SQ 29,548.

Similarly, in the present study, the levels of PGI_2 produced by the mesenteric arterioles in response to VP were 1.6 to 1.8 times higher than those in the Rapoport study. In fact, the groups with the highest output of PGI_2, INT-HT and OVX+PPT-HT, actually demonstrated slightly *higher* contraction to VP in the presence of SQ (although not statistically significant). The abundance of PGI_2 released by the mesenteric arterioles may mask the inhibitory effect of SQ 29,548 on TXA_2-enhanced contraction via the TP receptor. If the PGI_2 produced in response to VP stimulation is adequate to stimulate an EP receptor (likely EP_3, as this receptor subtype is associated with contraction of vascular smooth muscle) (105), then this may mask the effects of SQ 29,548 to block effects of TXA_2 in the contractile response to VP. The PGI_2 contractile theory seems a likely candidate, given the amounts of TXA_2 produced by the mesenteric arterioles in response to VP stimulation (especially during ACIH), as well as the substantially higher amounts of PGI_2 being produced simultaneously by these arterioles. That production of these two prostanoids is upregulated in parallel suggests that several points along the AA metabolism pathway are upregulated, mainly COX-2, as well as PGIS and TxS.

5.4.2 Effects of Simvastatin on the Vasopressin Concentration Dose Response

To determine the role of ERs on intracellular calcium release by the sarcoplasmic reticulum in the mesenteric vascular response to VP, simvastatin (blocker of IP_3 mediated IC release) (38, 94) was employed in the functional studies.

In the present studies, SIM significantly reduced the response to VP in all groups. The response was greater in NT than in HT, and greater in OVX+DPN and OVX than in INT and OVX+PPT. The greater dependence on IC release in the normotensive groups suggests that during hypertension there are other mechanisms contributing to the contractile response to VP, or that extracellular Ca^{2+} entry contributes more to contraction during ACIH. Another possibility is that blockade by SIM during ACIH is not as effective, due to greater contribution by intracellular calcium in contractile responses. Indeed, a previous study demonstrated that hypertensive rats exhibited a calcium dependent basal tone during ACIH, and that this tone was dependent upon TP receptor and COX (33).

A previous study by Eatman *et al.* (36) demonstrated that a clear sexual dimorphism exists in the role of intracellular vs. extracellular calcium in the vascular reactivity to VP in the rat aorta, revealing that female rats were more dependent on IC release for VP-induced contraction, whereas male rats were more dependent upon extracellular calcium influx. In the present study, the observed differences between INT and OVX rats were not expected, as OVX resulted in greater dependence on IC release for VP-induced contraction. The change in calcium contribution with OVX demonstrates that in the absence of estrogens, the mechanisms of vascular function are altered and this might be central to understanding why post-menopausal women are more susceptible to cardiovascular disease. Although OVX did not produce the expected response, it is another example of how function of PPT-treated rats closely resembles function of INT rats while the function of DPN-treated rats resembles function of OVX rats, suggesting that ERα alone is capable of mediating the effects of estrogen on IC release. Previous studies have

shown the effects of 17β-estradiol to relax VSM by reducing IC concentration, either by increasing efflux or reducing influx via inhibition of L-type Ca^{2+} channels (50, 93, 104, 113, 124). While it appears evident that estrogen receptors exert effects on Ca^{2+} in the vasculature, it remains unclear at this stage exactly what those effects are in differing tissues, as well as the differences in acute and chronic treatments.

5.5 Effect of Estrogen Receptor Agonists on the Concentration-Response to Vasopressin in Rat Aorta

In the present study, preliminary aortic function studies demonstrated clear differences in the effects of OVX+PPT vs. OVX+DPN treatment on the functional responses to VP. Similar to the mesenteric arterioles, OVX+PPT treatment resulted in a greater contractile force in the control response to VP. The responses to SQ and SIM in the OVX+PPT group were almost identical (34% reduction in contraction), unlike the mesenteric arterioles, where SQ did not have an effect and SIM reduced the response to VP by 73%. However, OVX+DPN treatment exhibited differential responses to SQ and SIM treatment. In the aorta, SIM reduced the response to VP by approximately 12%, whereas in the mesenteric arterioles, the reduction was much higher, at 92%. SQ also had a more prominent effect in aorta, where it reduced the maximum response to VP by 56%, while it had no significant effects on mesenteric arterioles.

These data suggest that while TXA_2 and TP appear to play a more significant role in the response to VP in aorta, the mesenteric response is either independent of TXA_2 and TP, or the TXA_2 contribution is masked by the effects of another PG, possibly PGI_2. It is

possible that the mesenteric vasculature is more sensitive to the effects of other PGs, while the aorta is unresponsive to any interference of other PGs that are released, especially PGI_2. Indeed, neither PGI_2 nor PGI_2 analogues produce relaxation in rat aorta (49, 107). In addition, aortic responses to VP appear less dependent on intracellular Ca^{2+} release than the mesenteric arterioles.

5.6 Effect of Aortic Coarctation-Induced Hypertension and Estrogen Treatment on Estrogen Receptor Expression

5.6.1 Real Time RT-PCR

In the present study, there was a trend for OVX to increase the levels of ERα and ERβ mRNA, but there were no statistically significant differences due to animal to animal variability in the data.

Other studies have shown that ERα mRNA is upregulated during OVX in the uterus (110) as well as in the kidney and cerebral cortex (88). However, Mohamed *et al.* (88) found that in the liver, heart, brainstem, cerebellum and thoracic and abdominal aorta there were no significant changes in mRNA of ERα. ERβ was not measured in these studies; in the Rosser study, ERβ had not yet been identified. It would appear that the regulation of the ERs by estrogen is tissue specific.

While there were not any statistically different levels of mRNA in the present study, this may not have made much impact, since the protein expression levels for ERα and ERβ did not change with either estrogen treatment or hypertension, and thus, are in agreement with the mRNA expression data.

5.6.2 Immunoblots

In the present study, there were no significant differences in the protein expression of the estrogen receptors; however, there were some general trends. OVX+PPT treatment during normotension and hypertension tended to slightly increase the expression of ERα when compared to INT-NT. In contrast, ERβ expression in OVX+PPT-NT, OVX+DPN-NT, OVX+PPT-HT and OVX+PPT-NT tended to be lower than INT-NT. This might indicate that ERα activation increases its own receptor protein, but has little effect on ERβ protein levels, while activation of ERβ does not appear to affect either its own receptor protein or ERα protein levels.

Previous investigations have stated that ERβ was the more dominant ER in the vascular system in both humans and rats (3, 57). Using the method of immunoblotting to detect protein levels makes comparisons between ERα and ERβ unreliable, due to differences in antibody binding affinity. Therefore, in the present study, we were unable to make such comparisons and each group is normalized to the reference group, in this case INT-NT.

In the present study, levels of both ERs were strikingly similar in both INT and OVX groups, suggesting that the regulation of ERs is independent of ovarian function. Another recent study found that protein levels of ERα and ERβ in the VSM of the sheep uterine artery did not differ between OVX and OVX+ER sheep and that there were no differences in either ERα or ERβ protein in omental, renal and coronary endothelium from ewes in luteal, follicular and pregnant stages. An exception was the higher levels of ERβ protein in the coronary endothelium of pregnant ewes, compared to non-pregnant ewes

during the luteal phase ewes (17). An earlier study found that treatment of endothelial cells with 17β-estradiol decreased ERα after short-term exposure, but increased it after longer exposure (60). Clearly, the effect of estrogen to regulate its receptors is both tissue and treatment dependent.

The lack of change in protein expression during HT likely indicates that upregulation of ERs in the vasculature is not the mechanism by which HT affects the PG pathway during the development of HT, and that changes in expression of ERs are not important in ACIH. However, the present study provides the unique opportunity to examine ER cross-regulation and protein levels in the presence of selective receptor activation. Specifically, whether the expression of each ER is modulated by its own activity, or the activity of the opposing receptor, or whether the interaction of the two ERs together changes their expression differentially.

5.7 Conclusions

Historically, TXA_2 and other constrictor prostanoids were considered important only in hypertension or other vascular disease states, and only in males (22, 30, 90). More recent studies have revealed that not only is TXA_2 important in maintaining vascular tone during normotension, but that the prostanoid pathway appears to contribute to vascular tone more in females than in males (6, 7, 8, 43, 72, 73). The findings of the present study confirm that ERα and ERβ contribute selectively to the actions of estrogen on the prostanoid pathway and are important in the regulation of constrictor prostanoid mediated mechanisms and blood pressure during the development of aortic coarctation-induced

hypertension. Further, the increased release of constrictor prostanoids by the vasculature is likely responsible for the increased reactivity to vasopressin seen in both the aorta and mesenteric vasculature (43, 73, 126, 127). Although aortic coarctation was used as the model of hypertension in the present study, essential human hypertension may also involve the effects of estrogens and constrictor prostanoids. Levels of estradiol in men and women with essential hypertension were higher than their normotensive counterparts (59, 65, 103), suggesting that estradiol may contribute to essential hypertension. In addition, aortic coarctation-induced hypertension is renin-angiotensin-dependent, a major factor in human essential hypertension as well (65, 70, 103); therefore, this model provides the unique opportunity to study the mechanisms of and changes to the vasculature involved in the development of hypertension.

Although women appear to be protected from cardiovascular disease in the pre-menopausal years, there is increasing evidence that the protective effects of estrogen on the vasculature have an "expiration date", because post-menopausal women receiving hormone replacement therapy have higher incidences of cardiovascular events (51, 58, 111) and are no longer protected. Clearly, there are many details of current hormone replacement therapy which mandate investigation, including the type of hormone used, timing of replacement and risk factors at the time of therapy. If it is possible to separate the beneficial effects of estrogen from the detrimental effects, then new possibilities will evolve in the treatment of cardiovascular disease for women, involving selective estrogen receptor modulation.

The present study is the first to study the effects of chronic selective estrogen receptor stimulation during normotension and the development of aortic coarctation-induced hypertension, and to examine the effects of the estrogen receptors on the production of PGI_2 and TXA_2, as well as the contribution of the estrogen receptors to vascular reactivity to vasopressin in normotension and during the development of hypertension.

The present study demonstrates that ERα mediates the majority of the effects of estrogen on the constrictor prostanoid pathway and on the vascular reactivity to vasopressin in the rat mesenteric arterioles. Although ERβ replacement therapy did not increase levels of PGI_2 and TXA_2 production to the same extent as did ERα therapy, nor did it increase vascular reactivity to vasopressin to the same extent, stimulation of this receptor caused increases in mean arterial pressure that rivaled those of 17β-estradiol and ERα stimulation, suggesting that ERβ affects other pathway(s) involved in this type of hypertension. Further investigation into the vasoconstrictor and vasodilator mechanisms associated with the estrogen receptors are needed to clarify the true effects of estrogen on vascular homeostasis and blood pressure.

REFERENCES

1. **Aavik E, du Toit D, Myburgh E, Frösen J and Hayry P.** Estrogen receptor beta dominates in baboon carotid after endothelial denudation injury. *Mol Cell Endocrinol* 182: 91-98, 2001.

2. **Akarasereenont P, Techatraisak K, Thaworn A and Chotewuttakorn S.** The induction of cyclooxygenase-2 by 17β-estradiol in endothelial cells is mediated through protein kinase C. *Inflamm Res* 49: 460-465, 2000.

3. **Andersson C, Lydrup ML, Ferno M, Idvall I, Gustafsson J and Nilsson BO.** Immunocytochemical demonstration of oestrogen receptor β in blood vessels of the female rat. *J Endocrinol* 169: 241-247, 2001.

4. **Armstrong RA and Wilson NH.** Aspects of the thromboxane receptor system. *Gen. Pharmacol* 26: 463-472, 1995.

5. **Bailie MD, Donoso VS and Gonzalez NC.** Role of the renin-angiotensin system in hypertension after coarctation of the aorta. *J Lab Clin Med* 104: 553-562, 1984.

6. **Baltzer WI, Kuo L and Stallone JN.** Estrogen enhances constrictor prostanoids and blood pressure in aortic coarctation-induced hypertension in female rats. *FASEB J* 17: A1234, 2003.

7. **Baltzer W and Stallone JN.** Aortic coarctation-induced hypertension is greater in females vs. males and is more constrictor prostanoid dependent. *FASEB J* 16: A81, 2002.

8. **Baltzer WI, Sellers MM and Stallone JN.** Estrogen enhances vascular wall structure and blood pressure in aortic coarctation-induced hypertension. *FASEB J* 18: A1211, 2004.

9. **Barrett-Connor E and Bush TL.** Estrogen and coronary heart disease in women. *JAMA* 265: 1861-1867, 1991.

10. **Bayard F, Clamens S, Meggetto F, Blaes N, Delsol G and Faye J.** Estrogen synthesis, estrogen metabolism, and functional estrogen receptors in rat arterial smooth muscle cells in culture. *Endocrinology* 136: 1523-1529, 1995.

11. **Berne RM and Levy MN, (eds).** *Physiology.* St. Louis, Mo: Mosby, 1998.

12. **Bolla M, Matrougui K, Loufrani L, Maclouf J, Levy BI, Levy-Toledano S, Habib A and Henrion D.** P38 mitogen-activated protein kinase activation is required for thromboxane-induced contraction in perfused and pressurized rat mesenteric resistance arteries. *J Vasc Res* 39: 353-360, 2002.

13. **Bolla M, You D, Loufrani L, Levy BI, Levy-Toledano S, Habib A and Henrion D.** Cyclooxygenase involvement in thromboxane-dependent contraction in rat mesenteric resistance arteries. *Hypertension* 43: 1264-1269, 2004.

14. **Bonnelye E and Aubin JE.** Review: estrogen receptor-related receptor α: a mediator of estrogen response in bone. *J Clin Endocrinol Metab* 90: 3115-3121, 2005.

15. **Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP and Versteeg HH.** Prostanoids and prostanoid receptors in signal transduction. *Int J Biochem Cell Biol* 36: 1187-1205, 2004.

16. **Bunting S, Moncada S, and Vane JR.** The prostacyclin-thromboxane A_2 balance: pathophysiological and therapeutic implications. *British Medical Bulletin* 39: 271-276, 1983.

17. **Byers MJ, Zangl A, Pernetton TM, Lopez G, Chen DB and Magness RR.** Endothelial vasodilator production by ovine uterine and systemic arteries: ovarian and steroid pregnancy control of ERalpha and ERbeta levels. *J Physiol* 565: 85-99, 2005.

18. **Carvalho MHC, Fortes ZB, Nigro D, Oliveira MA and Scivoletto R.** The role of thromboxane A_2 in the altered microvascular reactivity in two-kidney, one-clip hypertension. *Endothelium* 5: 167-178, 1997.

19. **Caughey GE, Cleland LG, Penglis PS, Gamble JR and James MJ.** Roles of cyclooxygenase (COX)-1 and (COX)-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. *J Immunol* 167: 2831-2838, 2001.

20. **Chambliss KL and Shaul PW.** Estrogen modulation of endothelial nitric oxide synthase. *Endocrine reviews* 23: 665-686, 2002.

21. **Chambliss KL, Yuhanna IS, Anderson RGW, Mendelsohn ME and Shaul PW.** ERβ has nongenomic action in caveolae. *Molecular Endocrinol* 16(5): 938-946, 2002.

22. **Chan PS and Cervoni P.** Prostaglandins, prostacyclin and thromboxane in cardiovascular diseases. *Drug Dev Res* 7: 341-359, 1986.

23. **Chang J, Musser JH and McGregor H.** Phospholipase A_2: function and pharmacological regulation. *Biochem Pharmacol* 36: 2429-2436, 1987.

24. **Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA and FitzGerald GA.** Role of prostacyclin in the cardiovascular response to thromboxane A_2. *Science 2*96(5567): 539-541, 2002.

25. **Chi OZ, Barsoum S, Wen Y, Liu X and Weiss HR.** 17β-Estradiol prevents blood-brain barrier disruption induced by VEGF. *Horm Metab Res* 36: 272-276, 2004.

26. **Clapham DE.** Calcium signaling. *Cell* 131(6): 1047-1058, 2007.

27. **Cooper CL and Malik KU.** Mechanism of action of vasopressin on prostaglandin synthesis and vascular function in the isolated rat kidney: effect of calcium antagonists and calmodulin inhibitors. *J Phamacol Exp Ther* 229: 139-147, 1984.

28. **Cruz MN, Douglas G, Gustafsson J, Poston L and Kublickiene K.** Dilatory response to estrogenic compounds in small femoral arteries of male and female estrogen receptor-β knockout mice. *Am J Physiol Heart Circ Physiol* 290: H823-H829, 2006.

29. **Dantas APV, Franco MCP, Tostes RCA, Fortes ZB, Costa SG, Nigro D and Carvalho MHC.** Relative contribution of estrogen withdrawal and gonadotropins increase secondary to ovariectomy on prostaglandin generation in mesenteric microvessels. *J Cardiovasc Pharmacol* 43: 48-55, 2004.

30. **Dantas APV, Scivoletto R, Fortes ZB, Nigro D and Carvalho MHC.** Influence of female sex hormones on endothelium-derived vasoconstrictor prostanoid generation in microvessels of spontaneously hypertensive rats. *Hypertension* 34(2): 914-919, 1999.

31. **Davidge ST and Zhang Y.** Estrogen replacement suppresses a prostaglandin H synthase-dependent vasoconstrictor in rat mesenteric arteries. *Circ Res* 83: 388-395, 1998.

32. **DelliPizzi A and Nasjletti A.** Involvement of nitric oxide and potassium channels in the reduction of basal tone produced by blockade of thromboxane A_2/ prostaglandin H_2 receptors in aortic rings of hypertensive rats. *Clin and Exper Hypertension* 20(8): 903-916, 1998.

33. **DelliPizzi A, Pucci ML, Mosny AY, Deseyn K and Nasjletti A.** Contribution of constrictor prostanoids to the calcium-dependent basal tone in the aorta from rats with aortic coarctation-induced hypertension: relationship to nitric oxide. *J Pharmacol Exp Ther* 283: 75-81, 1997.

34. **Dorn GW, Becker MW and Davis MG.** Dissociation of the contractile and hypertrophic effects of vasoconstrictor prostanoids in vascular smooth muscle. *J Biol Chem* 267(34): 24897-24905, 1992.

35. **Dubey RK, Jackson EK, Gillespie DG, Zacharia LC, Wunder D, Imthurn B and Rosselli M.** Medroxyprogesterone abrogates the inhibitory effects of estradiol on vascular smooth muscle cells by preventing estradiol metabolism. *Hypertension* 51: 1197-1202, 2008.

36. **Eatman D, Stallone JN, Rutecki GW and Whittier FC.** Sex differences in extracellular and intracellular calcium-mediated vascular reactivity to vasopressin in rat aorta. *European J Pharmacol* 361: 207-216, 1998.

37. **Egan KM, Lawson JA, Fries S, Koller B, Rader DJ, Smyth EM, and Fitzgerald GA.** COX-2-derived prostacyclin confers atheroprotection on female mice. *Science* 306: 1954-1957, 2004.

38. **Escobales N, Castro M, Altieri PI, and Sanabria P.** Simvastatin releases Ca^{2+} from a thapsigargin-sensitive pool and inhibits $InsP_3$-dependent Ca^{2+} mobilization in vascular smooth muscle cells. *J Cardiovasc Pharacol* 27: 383-391, 1996.

39. **Fetalvero KM, Martin KA and Hwa J.** Cardioprotective prostacyclin signaling in vascular smooth muscle. *Prostaglandins Other Lipid Mediators* 82: 109-118, 2007.

40. **Fitzgerald DJ, Rocki W, Murray R, Mayo G and Fitzgerald GA.** Thromboxane A_2 synthesis in pregnancy-induced hypertension. *Lancet* 335: 751-754, 1990.

41. **Flavahan NA.** Balancing prostanoid activity in the human vascular system. *TRENDS in Pharmacol Sci* 28: 106-110, 2007.

42. **Florian M, Lu Y, Angle M, and Magder S.** Estrogen induced changes in Akt-dependent activation of endothelial nitric oxide synthase and vasodilation. *Steroids* 69: 637-645, 2004.

43. **Fulton CT and Stallone JN.** Sexual dimorphism in prostanoid-potentiated vascular contractions: roles of endothelium and ovarian steroids. *Am J Physiol Heart Circ Physiol* 283: H2062-H2073, 2002.

44. **Furchgott RF and Vanhoutte PM.** Endothelium-derived relaxing and contracting factors. *FASEB J* 3: 2007-2018, 1989.

45. **Ganong WF.** *Review of Medical Physiology.* New York: McGraw-Hill, 2005.

46. **Geary GG, Krause DN and Duckles SP.** Estrogen reduces mouse cerebral artery tone through endothelial NOS- and cyclooxygenase-dependent mechanisms. *Am J Physiol Heart Circ Physiol* 279: H511-H519, 2000.

47. **Geary GG, McNeill AM, Ospina JA, Krause DN, Korach KS and Duckles SP.** Cerebrovascular NOS and cyclooxygenase are unaffected by estrogen in mice lacking estrogen receptor-α. *J Appl Physiol* 91: 2391-2399, 2001.

48. **Gilligan DM, Badar DM, Panza JA, Quyyumi AA, and Cannon RO, 3rd.** Acute vascular effects of estrogen in postmenopausal women. *Circulation* 90: 786-791, 1994.

49. **Gluais P, Lonchampt M, Morrow JD, Vanhoutte PM, and Feletou M.** Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin. *Br J Pharmacol* 146: 834-845, 2005.

50. **Gonzales RJ and Kanagy NL.** Endothelium-independent relaxation of vascular smooth muscle by 17β-estradiol. *J Cardiovasc Pharmacol Therapeut* 4(4): 227-234, 1999.

51. **Grady D, Wenger NK, Herrington D, Khan S, Furberg C, Hunninghake D, Vittinghoff E and Hulley S.** Postmenopausal hormone therapy increases risk for venous thromboembolic disease. The heart and estrogen/progestin replacement study. *Ann Intern Med* 132: 689-696, 2000.

52. **Halushka PV, Allan CJ and Davis-Bruno KL.** Thromboxane A2 receptors. *J Lipid Mediat Cell Signal* 12: 361-378, 1995.

53. **Heard DJ, Norby PL, Holloway J and Vissing H.** Human ERRγ, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue-specific isoforms are expressed during development and in the adult. *Mol Endocrinol* 14: 382-392, 2000.

54. **Henzel MK and Alsip NL.** Systemic vascular reactivity in an aortic coarctation model of preeclampsia in the rat. *Pathophysiology* 8: 133-140, 2001.

55. **Herrington DM and Klein KP.** Pharmacogenetics of estrogen replacement therapy. *J Appl Physiol* 91: 2776-2784, 2001.

56. **Hewitt SC, Deroo BJ, and Korach KS.** Signal trandsduction. A new mediator for an old hormone? *Science* 307: 1572-1573, 2005.

57. **Hodges YK, Tung L, Yan XD, Graham JD, Horwitz KB and Horwitz LD.** Estrogen receptors α and β: Prevalence of estrogen receptor β mRNA in human vascular smooth muscle and transcriptional effects. *Circulation* 101: 1792-1798, 2000.

58. **Hodis HN, Mack WJ, Azen SP, Lobo RA, Shoupe D, Mahrer PR, Faxon DP, Cashin-Hemphill L, Sanmarco ME, French WJ, Shook TL, Gaarder TD, Mehra AO, Rabbani R, Sevanian A, Shil AB, Torres M, Vogelbach KH and Selzer RH.** Hormone therapy and the progression of coronary-artery atherosclerosis in postmenopausal women. *N Engl J Med* 349: 535-545, 2003.

59. **Hughes GS, Mathur RS and Margolius HS.** Sex steroid hormones are altered in essential hypertension. *J Hypertens* 7: 181-187, 1989.

60. **Ihionkhan CE, Chambliss KL, Gibson LL, Hahner LD, Mendelsohn ME and Shaul PW.** Estrogen causes dynamic alterations in endothelial estrogen receptor expression. *Circ Res* 91: 814-820, 2002.

61. **Jesmin S, Hattori Y, Sakuma I, Liu MY, Mowa CN and Kitabatake A.** Estrogen deprivation and replacement modulate cerebral capillary density with vascular expression of angiogenic molecules in middle-aged female rats. *J Cerebral Blood Flow and Metab* 23: 181-189, 2003.

62. **Jun SS, Chen Z, Pace MC, and Shaul PW.** Estrogen upregulates cyclooxygenase-1 gene expression in fetal pulmonary artery endothelium. *J Clin Invest* 102: 176-183, 1998.

63. **Katušić ZS and Shepherd JT.** Endothelium-derived vasoactive factors: II Endothelium-dependent contraction. *Hypertension* 18 [suppl III]: III-86-III-92, 1991.

64. **Kawka DW, Ouellet M, Hétu PO, Singer II and Riendeau D.** Double-label expression studies of prostacyclin synthase, Thromboxane synthase and COX isoforms in normal aortic endothelium. *Biochimica et Biophysics Acta* 1771: 45-54, 2007.

65. **Khaw K-T and Barrett-Connor E.** Blood pressure and endogenous testosterone in men: an inverse relationship. *J Hyper* 6: 328-332, 1988.

66. **Khazaei M, Moien-afshari F and Laher I.** Vascular endothelial function in health and diseases. *Pathophysiology* 15: 49-67, 2008.

67. **Klabunde RE.** *Cardiovascular Physiology Concepts.* Philadelphia, PA: Lippincott Williams & Wilkins, 2005.

68. **Kleiger RE, Boxer M, Ingham RE and Harrison DC.** Pulmonary hypertension in patients using oral contraceptives. *Chest* 69: 143-147, 1976.

69. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson and Gustafsson J.** Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci* 93: 5925-5930, 1996.

70. **Lappe RW and Brody MJ.** Hemodynamic, neural, and humoral mechanisms of aortic coarctation hypertension in the rat. *J Cardio Pharmacol* 8: 656-662, 1986.

71. **Levin ER.** Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol* 91: 1860-1867, 2001.

72. **Li M and Stallone JN.** Estrogen potentiates vasopressin-induced contraction of female rat aorta by enhancing cyclooxygenase-2 and thromboxane function. *Am J Physiol Heart Circ Physiol* 289: H1542-H1550, 2005.

73. **Li M, Kuo L, and Stallone JN.** Estrogen potentiates constrictor prostanoid function in female rat aorta by upregulation of cyclooxygenase-2 and thromboxane pathway expression. *Am J Physiol Heart Circ Physiol* 294: H2444-H2455, 2008.

74. **Lin L, Mistry M, Stier CT and Nasjletti A.** Role of prostanoids in renin-dependent and renin-independent hypertension. *Hypertension* 17: 517-525, 1991.

75. **Lin L and Nasjletti A.** Role of endothelium-derived prostanoid in angiotensin-induced vasoconstriction. *Hypertension* 18: 158-164, 1991.

76. **Lindner V, Kim SK, Karas RH, Kuiper GGJM, Gustafsson JA and Mendelsohn MM.** Increased expression of estrogen receptor-ß mRNA in male blood vessels after vascular injury. *Circ Res* 83: 224-229, 1998.

77. **Losordo DW and Isner JM.** Estrogen and Angiogenesis: a review. *Arterioscler Thromb Vasc Biol* 21: 6-12, 2001.

78. **Lüscher TF.** The endothelium in hypertension: bystander, target or mediator? *J Hypertension* 12(Suppl 10): S105-S116, 1994.

79. **Martin F, Fletcher D, Chauvin M and Bouhassira D.** Constitutive cyclooxygenase-2 is involved in central nociceptive processes in humans. *Anesthesiol* 106: 1013-1018, 2007.

80. **Matthews J and Gustafsson J-A.** Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Intervent* 3: 281-292, 2003.

81. **Matthews J, Wihlén B, Tujague M, Wan J, Strom A and Gustafsson J-A.** Estrogen receptor (ER) β modulates ERα-mediated transcriptional activiation by altering the recruitment of c-Fos and c-Jun to estrogen-responsive promoters. *Mol Endocrinol* 20: 534-543, 2006.

82. **McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA and FitzGerald GA.** Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: The human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci* 96: 272-277, 1999.

83. **McEwen BS.** Estrogens effects on the brain: multiple sites and molecular mechanisms. *J Appl Physiol* 91: 2785-2801, 2001.

84. **McEwen BS.** Non-genomic and genomic effects of steroids on neural activity. *TIPS* 12: 141-147, 1991.

85. **Mendelsohn ME and Karas RH.** Molecular and cellular basis of cardiovascular gender differences. *Science* 308: 1583-1587, 2005.

86. **Meyer MR, Haas E and Barton M.** Gender differences of cardiovascular disease. *Hypertension* 47: 1019-1026, 2006.

86a. **Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS and Katzenellenbogen JA.** Estrogen receptor-β potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem* 44: 4230-4251, 2001.

87. **Michel F, Silvestre JS, Waeckel L, Corda S, Verbeuren T, Vilaine JP, Clergue M, Duriez M and Levy BI.** Thromboxane A_2/prostaglandin H_2 receptor activation mediates angiotensin II-induced postischemic neovascularization. *Arterioscler Thromb Vasc Biol* 26: 488-493, 2006.

88. **Mohamed MK and Abdel-Rahman AA.** Effect of long-term ovariectomy and estrogen replacement on the expression on estrogen receptor gene in female rats. *Eur J Endocrinol* 142: 307-314, 2000.

89. **Moini H, Bilsel S, Bekdemir T and Emerk K.** 17β-estradiol increases intracellular free calcium concentrations of human vascular endothelial cells and modulates its response to acetylcholine. *Endothelium* 5: 11-19, 1997.

90. **Moncada S and Higgs E.** Arachidonate metabolism in blood cells and the vessel wall. *Clinics in Haematology* 15: 273-287, 1986.

91. **Mügge A, Riedel M, Barton M, Kuhn M, and Lichtlen PR.** Endothelium independent relaxation of human coronary arteries by 17 beta-oestradiol in vitro. *Cardiovasc Res* 27: 1939-1942, 1993.

92. **Mulvany MJ and Halpern W.** Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* 41(1): 19-26, 1977.

93. **Nakajima T, Kitazawa T, Hamada E, Hazama H, Omata M and Kurachi Y.** 17β-estradiol inhibits the voltage-dependent L-type Ca2+ currents in aortic smooth muscle cells. *Eur J Pharmacol* 294: 625-635, 1995.

94. **Ng LL, Davies JE and Wojcikiewicz RJH.** 3-Hydroxy-3-methyl glutaryl coenzyme A reductase inhibition modulates vasopressin-stimulated Ca2+ responses in rat A10 vascular smooth muscle cells. *Circ Res* 74: 173-181, 1994.

95. **Nilsson B-O, Ekblad E, Heine T and Gustafsson J-A.** Increased magnitude of relaxation to oestrogen receptor β knock-out mice. *J. Endocrinol* 166: R5-R9, 2000.

96. **Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y and Muramatsu M.** Molecular cloning and characterization of human estrogen receptor βcx: a potential inhibitor of estrogen action in human. *Nucleic Acids Res* 26: 3505-3512, 1998.

97. **Okamoto T and Hino O.** Expression of cyclooxygenase-1 and -2 mRNA in rat tissues: tissue specific difference in the expression of the basal level of mRNA. *Int J Mol Med* 6: 455-457, 2000.

98. **Ospina JA, Krause DN and Duckles SP.** 17β-estradiol increases cerebrovascular prostacyclin synthesis by elevating cyclooxygenase-1 and prostacyclin synthase. *Stroke* 33: 600-605, 2002.

99. **O'Sullivan MG, Goodrich JA and Adams MR.** Increased prostacyclin synthesis by atherosclerotic arteries from estrogen-treated monkeys. *Life Sci* 69: 395-401, 2001.

100. **Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson J, Kushner PJ and Scanlan TS.** Differential ligand activation of estrogen receptors ERα and ERβ at API sites. *Science* 277: 1508-1510, 1997.

101. **Palmer KT, Griffin MJ, Syddall H, Pannett B, Cooper C and Coggon D.** Prevalence of Raynaud's phenomenon in Great Britain and its relation to hand transmitted vibration: a national post survey. *Occup Environ Med* 57: 448-452, 2000.

102. **Parente L and Perretti M.** Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochem Pharmacol* 65: 153-159, 2003.

103. **Phillips GB, Jing TY, Laragh JH and Sealey JE.** Serum sex hormone levels and renin-sodium profile in men with hypertension. *Am J Hypertens* 8: 626-629, 1995.

104. **Prakash YS, Togaibayeva AA, Kannan MS, Miller VM, Fitzpatrick LA and Sieck GC.** Estrogen increases Ca2+ efflux from female porcine coronary arterial smooth muscle. *Am J Physiol Heart Circ Physiol* 45: H926-H934, 1999.

105. **Qian Y, Jones RL, Chan K, Stock AI and Ho JKS.** Potent contractile actions of prostanoid EP_3-receptor agonists on human isolated pulmonary artery. *Br J Pharmacol* 113: 369-374, 1994.

106. **Quest DW and Wilson TW.** Effects of ridogrel, a thromboxane synthase inhibitor and receptor antagonist, on blood pressure in the spontaneously hypertensive rat. *Jpn J Pharmacol* 78: 479-486, 1998.

107. **Rapoport RM and Williams SP.** Role of prostaglandins in acetylcholine-induced contraction of aorta from spontaneously hypertensive and Wistar-Kyoto rats. *Hypertension* 28: 64-75, 1996.

108. **Rauschemberger MB, Sellés J and Massheimer V.** The direct action of estrone on vascular tissue involves genomic and non-genomic actions. *Life Sci* 82: 115-123, 2008.

109. **Rich S, Hart K, Kieras K and Brundage BH.** Thromboxane synthetase inhibition in primary pulmonary hypertension. *Chest* 91(2): 356-360, 1987.

110. **Rosser M, Chorich L, Howard E, Zamorano P and Mahesh VB.** Changes in rat uterine estrogen receptor messenger ribonucleic acid levels during estrogen- and progesterone- induced estrogen receptor depletion and subsequent replenishment. *Biol of Repro* 48: 89-98, 1993.

111. **Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, and Ockene J.** Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. *JAMA* 288: 321-333, 2002.

112. **Rubanyi GM, Kauser K and Johns A.** Role of estrogen receptors in the vascular system. *Vascular Pharmacol* 38: 81-88, 2002.

113. **Rubio-Gayosso I, Sierra-Ramirez A, García-Vasquez A, Martinez-Martinez A, Munoz-García O, Morato T and Ceballos-Reyes G.** 17β-estradiol increases intracellular calcium concentration through a short-term and nongenomic mechanism in rat vascular endothelium in culture. *J Cardiovasc Pharmacol* 36: 196-202, 2000.

114. **Rupnow HL, Phernetton TM, Modrick ML, Wiltbank MC, Bird IM and Magness RR.** Endothelial vasodilator production by uterine and systemic arteries. VIII. Estrogen and progesterone effects on $cPLA_2$, COX-1, and PGIS protein expression. *Biol of Repro* 66: 468-474, 2002.

115. **Salgado HC and Salgado MCO.** Acute aortic coarctation hypertension: role of vasopressin and angiotensin II. *Heart Circ Physiol* 26: H1480-H1484, 1989.

116. **Salgado MCO, Castania JA, Ballejo G and Salgado HC.** Effect of chronic estradiol administration on the acute pressor response to aortic coarctation in conscious rats. *Braz J Med Biol Res* 28: 881-888, 1995.

117. **Sellers MM and Stallone JN.** Sympathy for the devil: the role of thromboxane in the regulation of vascular tone and pressure. *Am J Physiol Heart Circ Physiol* 294: H1978-H1986, 2008.

118. **Selles J, Polini N, Alvarez C and Massheimer V.** Novel action of estrone on vascular tissue: regulation of NOS and COX activity. *Steroids* 70: 251-256, 2005.

119. **Setchell KDR.** Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin Nutr* 68 *Suppl:* 1333S-1334S, 1998.

120. **Setchell KDR and Cassidy A.** Dietary isoflavones: biological effects and relevance to human health. *Am J Nutr* 129 *Suppl:* 758S-767S, 1999.

121. **Shaikh AA.** Estrone and estradiol levels in the ovarian venous blood from rats during the estrous cycle and pregnancy. *Biol Reprod* 5: 297-307, 1971.

122. **Shepherd JT and Katušić ZS.** Endothelium-derived vasoactive factors: I endothelium-dependent relaxation. *Hypertension* 18[III]: III-76-III-85, 1991.

123. **Sherman TS, Chambliss KL, Gibson LL, Pace MC, Mendelsohn ME, Pfister SL and Shaul PW.** Estrogen acutely activates prostacyclin synthesis in ovine fetal pulmonary artery endothelium. *Am J Respir Cell Mol Biol* 26: 610-616, 2002.

124. **Sierra-Ramirez A, Morato T, Campos R, Rubio I, Calzada C, Mendez E and Ceballos G.** Acute effects of testosterone on intracellular Ca^{2+} kinetics in rat coronary endothelial cells are exerted via aromatization to estrogens. *Am J Physiol Heart Circ Physiol* 286: H63-H71, 2004.

125. **Sohrabji F.** Guarding the blood-brain barrier: a role for estrogen in the etiology of neurodegenerative disease. *Gene Expression* 13: 311-319, 2006.

126. **Stallone JN.** Mesenteric vascular responses to vasopressin during development of DOCA-salt hypertension in male and female rats. *Am J Physiol Regul Integr Comp Physiol* 37: R40-R49, 1995.

127. **Stallone JN.** Role of endothelium in sexual dimorphism in vasopressin-contraction of rat aorta. *Heart Circ Physiol* 34: H2073-H2080, 1993.

128. **Stallone JN.** Sex differences in nitric oxide-mediated attenuation of vascular reactivity to vasopressin are abolished by gonadectomy. *Eur J Pharmacol* 259: 273-283, 1994.

129. **Stallone JN, Crofton JT and Share L.** Sexual dimorphism in vasopressin-induced contraction of rat aorta. *Am J Physiol Heart Circ Physiol* 260: H453-H458, 1991.

129a. **Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen, BS and Katzenellenbogen JA.** Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-α-selective agonists. *J Med Chem* 43: 4934-4947, 2000.

130. **Ström A, Hartman J, Foster JS, Kietz S, Wimalasena J and Gustafsson J-A.** Estrogen receptor β inhibits 17β-estradiol-stimulated proliferation of the breast cancer cell line T47D. *PNAS* 101: 1566-1571, 2004.

131. **Tamura K, Deb S, Sebastian S, Okamura K and Bulun SE.** Estrogen up-regulates cyclooxygenase-2 via estrogen receptor in human uterine microvascular endothelial cells. *Fertil Steril* 81: 1351-1356, 2004.

132. **Tamura K, Yamaguchi K and Kogo H.** 17β-estradiol inhibits ovariectomy-induced expression of inducible nitric oxide synthase in rat aorta in vivo. *Life Sci* 66: PL259-PL264, 2000.

133. **Tang EHC and Vanhoutte PM.** Gene expression changes of prostanoid synthases in endothelial cells and prostanoid receptors in vascular smooth muscle cells caused by aging and hypertension. *Physiol Genomics* 32: 409-418, 2008.

134. **Tatchum-Talom R, Martel C and Marette A.** Influence of estrogen on aortic stiffness and endothelial function in female rats. *Am J Physiol Heart Circ Physiol* 282: H491-H498, 2002.

135. **Thomas W, Coen N, Faherty S, Flatharta CO, and Harvey BJ.** Estrogen induces phospholipase A2 activation through ERK 1/2 to mobilize intracellular calcium in MCF-7 cells. *Steroids* 71: 256-265, 2006.

136. **Tsang SY, Yao X, Wong CM, Chan FL, Chen ZY and Huang Y.** Differential regulation of K^+ and Ca^{2+} channel gene expression by chronic treatment with estrogen and tamoxifen in rat aorta. *Euro J Pharmacol* 483: 155-162, 2004.

137. **Ueno N, Murakami M, Tanioka T, Fujimori K, Tanabe T, Urade Y and Kudo I.** Coupling between cyclooxygenase, terminal prostanoid synthase, and phospholipase A_2. *J Biological Chem* 276: 34918-34927, 2001.

138. **Vane JR.** Back to an aspirin a day? *Science* 296: 474-475, 2002.

139. **Voulgari PV, Alamanos Y, Papazisi D, Christou K, Papanikolaou C and Drosos AA.** Prevalence of Raynaud's phenomenon in a healthy Greek population. *Ann Rheum Dis* 59: 206-210, 2000.

140. **Wagenvoort CA and Wagenvoort N.** Primary pulmonary hypertension. A pathologic study of the lung vessels in 156 clinically diagnosed cases. *Circ* 42: 1163-1184, 1970.

141. **Wang L and Kulmacz RJ.** Thromboxane synthase: structure and function of protein and gene. *Prostaglandins Other Lipid Mediators* 68-69: 409-422, 2002.

142. **Wang D, Wang M, Cheng Y, and Fitzgerald GA.** Cardiovascular hazard and non-steroidal anti-inflammatory drugs. *Curr Opin Pharmacol* 5: 204-210, 2005.

143. **Wenger NK, Speroff L and Packard B.** Cardiovascular health and disease in women. *N Engl J Med* 329: 247-256, 1993.

144. **Wilcox CS and Welch WJ.** Thromboxane mediation of the pressor response to infused angiotensin II. *Am J Hypertens* 3: 242-249, 1990.

145. **Williams JK, Adams MR, Herrington DM, and Clarkson TB.** Short-term administration of estrogen and vascular responses of atherosclerotic coronary arteries. *J Am Coll Cardiol* 20: 452-457, 1992.

146. **Wilson JD, Foster DW, Kronenberg HM, and Larsen PR (eds.)**. *Williams Textbook of Endocrinology.* Philadelphia: W.B. Saunders Company, 1998.

147. **Wilson SJ, Dowling JK, Zhao L, Carnish E and Smyth EM.** Regulation of thromboxane receptor trafficking through the prostacyclin receptor in vascular smooth muscle cells: Role of heterodimerization. *Arterioscler Thromb Vasc Biol* 27: 290-296, 2007.

148. **Ylikorkala O and Makila UM.** Prostacyclin and thromboxane in gynecology and obstetrics. *Am J Obstet Gynecol* 152: 318-329, 1985.

149. **Zancan V, Santagati S, Bolego C, Vegeto E, Maggi A and Puglisi L.** 17β-estradiol decreases nitric oxide synthase II: Synthesis in vascular smooth muscle cells. *Endocrinology* 140: 2004-2009, 1999.

150. **Zhang Y and Davidge ST.** Effect of estrogen replacement on vasoconstrictor responses in rat mesenteric arteries. *Hypertension* 34: 1117-1122, 1999.

151. **Zhao L and Brinton RD.** Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. *Brain Res* 1172: 48-59, 2007.

152. **Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thorén P, Smithies O, Gustafsson J and Mendelsohn ME.** Abnormal vascular function and hypertension in mice deficient in estrogen receptor β. *Science* 295: 505-508, 2002.

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