THE ROLE OF CONSTRICTOR PROSTANOIDS IN THE DEVELOPMENT OF AORTIC COARCTATION-INDUCED HYPERTENSION IN MALE AND FEMALE RATS

A Dissertation

by

WENDY IRENE BALTZER

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 2003

Major Subject: Veterinary Physiology

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ABSTRACT

The Role of Constrictor Prostanoids in the Development of Aortic Coarctation-Induced Hypertension in Male and Female Rats. (December 2003)

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Vascular reactivity to vasopressin and phenylephrine is potentiated by constrictor prostanoids (CP) in normotensive female (F) but not male (M) rat aorta and CP function is estrogen-dependent. This study investigated the effects of estrogen on CP function and arterial blood pressure (MAP) during development of aortic coarctation-induced hypertension (HT). M and F rats, (15-18 wks.) in four groups: normotensive (NT), hypertensive (HT), ovariectomized (OVX), and OVX estrogen-replaced (OE), underwent abdominal aortic coarctation or sham surgery (NT). At 14 days, SQ 29,548 (SQ, Thromboxane A_2 (TXA_2) receptor antagonist) was given i.v. to the groups. In another experiment, rats received Ridogrel (TXA_2 receptor antagonist+TXA_2 synthase (TXS) inhibitor) or vehicle (methyl cellulose) daily, for 14 days. Thoracic aortae were analyzed for morphology, incubated in Kreb's Henseleit Buffer (KHB) ± angiotensin II (ANG II), or underwent continuous pulsatile flow and pressure experiments (PFP) with KHB ± ANG II. Perfusate was analyzed for thromboxane B_2 (TXB_2) and prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$). RT-PCR and immunohistochemistry were performed for TXS. MAP was higher in F-HT than in M-HT after 14 days. SQ infusion reduced MAP substantially

more in F-HT and OE-HT than in others. Ridogrel prevented increases in MAP in F/OE-HT rats, but not M/OVX-HT. Basal release of TXB_2 and PGF1α increased to a greater extent in F-HT than in M-HT relative to their controls. ANG II-stimulated TXB_2 and PGF1α release increased to a greater extent in F-HT than in M-HT. With or without ANG II, TXB_2 production in HT during PFP increased with estrogen. PGF1α increased during PFP with estrogen, however not with ANG II. Pressurization resulted in less diameter change in F and OE-HT than in OVX-HT. Elastin increased with HT (inhibited by Ridogrel) in all but M. Collagen increased in HT with estrogen (inhibited by Ridogrel). Neither OVX-HT nor Ridogrel had any effect on morphology. Estrogen increased TXS with HT. Estrogen enhanced vascular CP and MAP in F-HT by increased expression of TXS and collagen density in the vasculature indicating that in aortic coarctation-induced HT, CP are upregulated by estrogen. Specific forms of HT in human beings may involve estrogen-induced vascular CP upregulation.

DEDICATION

To my husband, Craig Ruaux, for believing in me even if I don't.

To my brother, Jim, for teaching me what bravery is; to my Dad, Jerry, for teaching me what honor means; and to my Mom, Irene, for teaching me to never give up.

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CHAPTER I

INTRODUCTION

1.1 Hypertension and Vascular Disease Incidence in Women

Coronary artery disease and hypertension are more common in men than in premenopausal women; however, after menopause, the incidence of these diseases in women increases to a level similar to that of men (4; 108; 249). These findings suggest that estrogen exerts a beneficial (protective) effect on cardiovascular health in premenopausal women. In contrast, more recent epidemiological studies have shown that ovarian steroid hormone replacement therapy, particularly with estrogen, does not reduce the incidence of cardiovascular or cerebrovascular disease, and actually increases the risk of myocardial infarction and venous thromboembolic disease in postmenopausal women (108; 176; 233). Despite previous epidemiological data that suggest the protective effects of ovarian steroid hormones on the cardiovascular system, recent findings do not show any significant protection against cardiovascular disease.

For example, recent clinical trials including the Heart and Estrogen/Progestin Replacement Study (HERS) and others, indicate that neither estrogen alone nor estrogen plus medroxyprogesterone acetate affect the risk for cardiovascular events or peripheral arterial events; furthermore, they do not affect the progression of coronary atherosclerosis in women (75; 98; 102).

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

Increased systolic blood pressure and pulse pressure in women aged 45 to 64 years increases the risk of coronary heart disease, stroke, other cardiovascular diseases and all-cause mortality, but in men of the same age, increased systolic pressure increases only the risk of coronary heart disease and not other causes of mortality (6; 214). Most importantly, the incidences of specific vascular diseases such as primary pulmonary hypertension (129; 272), Raynaud's Disease (22; 193; 271), oral contraceptive-induced hypertension and pulmonary hypertension (89; 129) are substantially higher in women than men. Indeed, the incidence of primary pulmonary hypertension is four-fold higher in women than in men (272), and Raynaud's Disease is up to seven-fold higher in women than in men (271). Clearly, the effects of estrogen on the vascular system are not well understood and are more complex than originally thought.

Women with essential hypertension that do not have a nocturnal fall in blood pressure (so called "nondippers") have a significantly higher risk for a future cardiovascular event compared to male nondippers (264; 267). In addition, female nondippers have a significantly larger left ventricular mass than female dippers; but there is no difference in left ventricular mass between male dippers and nondippers (265; 266; 268). Antiestrogens such as tamoxifen appear to protect against the risk of cardiovascular disorders (160). In a clinical trial of tamoxifen use for breast cancer, administration of the drug tended to decrease urinary thromboxane B_2 excretion in hypertensive patients, but not significantly. This was most likely due to a small sample size (n=7) and the fact that the patients were concurrently being treated with antihypertensive drugs such as angiotensin converting enzyme inhibitors (157).

Antihypertensive therapy has proven less successful in women than in men at reducing and controlling elevations in blood pressure (214; 281), but the reasons for these differences are unknown. One explanation for the increase in left ventricular mass in nondipper women may be due to a response to pressure overload by the heart, indicating the presence of a higher systemic pressure in the women. Taken together, these findings suggest the ovarian steroid hormones may exacerbate some cardiovascular diseases in women, particularly those of vascular origin.

Atherosclerosis is characterized by the development of plaques in the walls of arteries. The incidence of atherosclerosis in premenopausal women is lower than in men and is reduced in postmenopausal women by hormone replacement therapy (162). This is in part due to the ability of estrogen to decrease circulating low-density lipoprotein cholesterol levels and increase high density lipoprotein cholesterol (98; 162; 182). The effects of estrogen can be rapid nongenomic in action or genomic (stimulation or inhibition of gene expression). Estrogen rapidly stimulates the release of nitric oxide from endothelial cells by binding to a receptor in the caveolae that then activates the endothelial nitric oxide synthase enzyme. Estrogen can also increase gene expression of prostacyclin synthase and endothelial nitric oxide synthase (93; 162) (21; 125; 166; 217) by acting on the nuclear DNA of endothelial cells. In addition, estrogen acutely modulates vascular smooth muscle tone by opening calcium-activated potassium channels through a nitric oxide-cGMP pathway and by inhibiting migration and proliferation of the cells (21; 125; 162; 166; 182; 280; 283). Estrogen also decreases insulin resistance, serum fibrinogen, clotting factor VII and plasminogen activator

inhibitor-1 (166; 176; 177; 182; 291). These actions combine to reduce systemic blood pressure, prevent plaque deposition in arteries, decrease circulation cholesterol, and increase high-density lipoproteins.

Paradoxically, estrogen only induces small reductions in blood pressure at low doses and causes increases in blood pressure with doses of 1.25 mg or higher in postmenopausal women with hypertension (91). This may be due, at least in part, to the effect of estrogen to elevate gene expression and blood levels of angiotensinogen (92; 214). These previous findings reveal that the actions of estrogen are diverse and multiform and not fully understood in vascular disease.

Several clinical studies support the idea that ovarian steroid hormones play an important role in vascular diseases in women. For example, estradiol levels are higher in both men and women with essential hypertension than in normotensive controls (107) and hypertensive men with increased estradiol levels have a high renin profile and a trend toward an increased risk of myocardial infarction (198). In postmenopausal hypertensive women, hormone replacement therapy increases plasma levels of angiotensin I, angiotensin II, bradykinin and plasma renin activity (258). In the 30 to 50 year old age group with renovascular hypertension, fibromuscular hyperplasia or primary aldosteronism are most often the underlying cause in women, whereas in men the hypertension is commonly due to atherosclerotic stenosis of their proximal renal arteries (154; 207; 300). This would suggest that estrogen protects against atherosclerosis but not other vascular alterations that result in elevated vascular tone and hypertension. Small elevations in plasma angiotensin II (that may not be detected

clinically) may induce significant increases in mean arterial pressure and induce hypertension (212). When postmenopausal women with risk factors for vascular dysfunction (hypertension, hypercholesterolemia, diabetes or coronary artery disease) were infused with 17β-estradiol, their vasodilator responses to acetylcholine and sodium nitroprusside were significantly reduced compared to healthy postmenopausal women (76). Again, these data suggest that the ovarian steroid hormones may exert deleterious effects on the vasculature and underlie male-female differences in vascular disease.

Estrogens and constrictor prostanoids (CP) have been implicated in primary pulmonary and essential systemic hypertension, and have been shown to increase pulmonary vascular tone in normotension and/or pulmonary hypertension (30; 38; 48; 107; 197; 272). Further, in pulmonary hypertension, arachidonic acid induces the release of thromboxane A_2 (TXA_2), a potent vasoconstrictor, which is considered to be an important mediator of this disease (12; 38; 210). In contrast to the pulmonary vasculature, little is known about the role of CP in the systemic vasculature. Although CP production by the systemic vasculature is elevated in some forms of experimental hypertension, little is known about the importance of these substances in the regulation of systemic vascular tone in normotension or during the development of hypertension. In women with gynecologic cancer, production of prostaglandins shifts from the vasodilator prostacyclin (prostaglandin I_2, PGI_2) to the vasoconstrictor TXA_2. In preeclampsia and other chronic placental insufficiency syndromes, there is a deficiency in production of prostacyclin and an overproduction of TXA_2, resulting in the increased vasoconstriction that occurs in hypertensive pregnancies (295). In hypertensive women

with breast cancer, administration of tamoxifen, an antiestrogen, induced a slight decrease in urinary thromboxane B_2 excretion (the stable metabolite of TXA_2, TXB_2). Although this decrease was not significant, the patients were concurrently treated with antihypertensive drugs and no control groups were included (157). Regardless of the shortcomings in these studies, they suggest that in vascular diseases, estrogen may contribute to enhanced CP production.

In addition to their acute effects to potentiate vascular tone, CP also function as mitogens to stimulate vascular smooth muscle cell proliferation (192; 222). This would suggest that the increase in TXA_2 release during the development of hypertension, could be responsible, at least in part, for the vascular remodeling that occurs with hypertension. It would follow that such remodeling may be enhanced in females compared to males if the release of vascular TXA_2 is greater in females, but this has not been investigated. After acute internal vascular injury, estradiol administration reduces arterial wall thickening and neointimal formation, while increasing re-endothelialization of arteries in a dose dependent manner (35; 134; 190; 223). This suggests that estrogen inhibits vascular smooth muscle proliferation, but this is following acute endothelial barotrauma and not induction of hypertension, two very dissimilar disturbances in vascular function. Thus, ovarian steroid hormones may play a very different role in regulation of vascular remodeling in endogenous vascular diseases, compared to acute exogenous, vascular barotrauma; and this remodeling response may occur through a mechanism involving the mitogenic action of CP. Estrogen may affect vascular

remodeling either through direct actions on the endothelium, and/or vascular smooth muscle, or through stimulation of CP production.

1.2 The Effects of Estrogen on the Vascular Wall

The estrogens are a family of steroid hormones in the female, synthesized from the universal steroid precursor molecule, cholesterol. The androgens, the family of testicular steroid hormones in the male, are obligate precursors of estrogen, see Figure 1 for the synthesis of estrogen (17; 17). The key step in all steroid hormone biosynthesis is aromatization of the cholesterol A ring by a cytochrome P-450 enzyme located in the endoplasmic reticulum in the ovary and placenta (where most estrogen synthesis occurs) as well as the adrenal gland and testes. The starting point for the specific synthesis of estrogen is the 17-hydroxylated derivative of Δ5- pregnenolone and progesterone, which yield the androgens, the immediate precursors to estrogens. The second rate-limiting step in estrogen biosynthesis is the conversion of androgens to estrogens via the enzyme aromatase. Once in the circulation, most estrogen is loosely bound to sex-steroid binding globulin albumin but it can also circulate bound to albumin. A small amount of estrogen is also synthesized from testosterone by adipose tissue and the liver (17), which contain variable amounts of aromatase. The aromatase enzyme is also present in the vasculature and can synthesize estrogen locally to have paracrine/autocrine effects on the vasculature without increasing systemic levels of estrogen (234). Human endothelial cells do not contain aromatase, however, vascular smooth muscle cells of the aorta and pulmonary artery do (90).

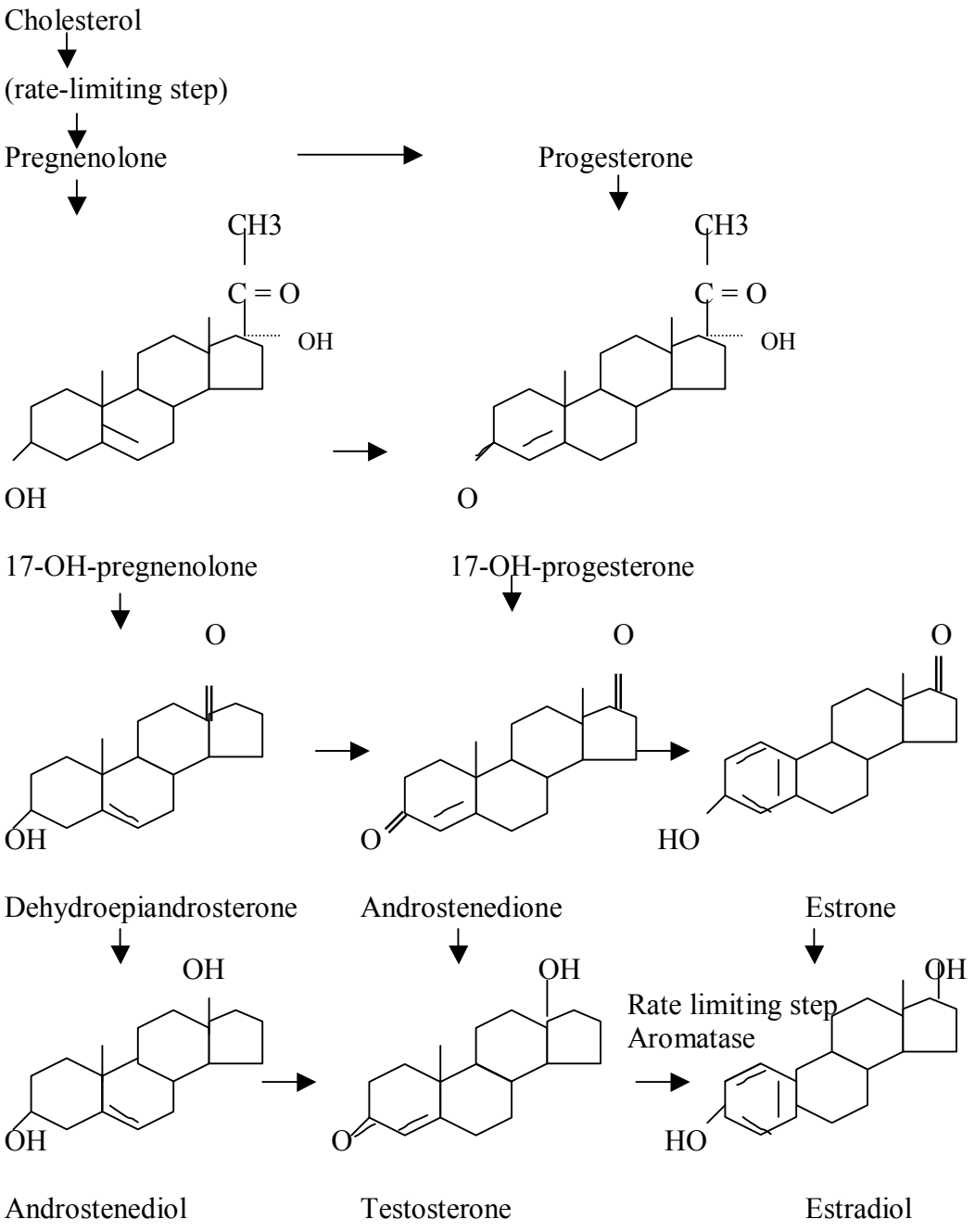


Figure 1: Synthesis of estrogen from cholesterol (adapted from Berne and Levy, eds. *Physiology*, 4th edition)(17).

Estrogen is metabolized to sulfated and glucuronidated derivatives in the liver and excreted in the urine or undergoes 2-hydroxylation to catechol estrogen. Interestingly, catechol estrogens can bind to estrogen receptors but do not activate the receptor and may increase gonadotropin releasing hormone secretion. Further, catechol estrogens bind neoplastic mammary cells and induce proliferation of these cells (225). Catechol estrogens also antagonize uterine artery calcium channels in the pig uterus to decrease arterial tone during pregnancy (66). The metabolites of estrogen may therefore have vascular effects systemically that have yet to be characterized. Table 1 shows the plasma concentration, production and clearance rate of the estrogens (17).

Table 1: Estrogen and progesterone turnover in adult women (adapted from Berne and Levy, eds. *Physiology*, 4th edition)(17).

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
17-hydroxyprogesterone	30-200	600-4000	2000
Progesterone	100-1000	2000-25000	2200

The actions of estrogen include development of female sex organs and female secondary sex characteristics, stimulation of ovulation, inhibition of bone resorption, reabsorption of sodium by the renal tubules and stimulation of hepatic synthesis of

proteins. Some of the proteins stimulated by estrogen include thryroxine binding globulin, cortisol-binding globulin, sex steroid-binding globulin, angiotensinogen, and lipoproteins (very low density and high density) (17).

Estrogen decreases low-density lipoprotein and lipoprotein (a) levels in the blood (17). It also increases high-density lipoprotein-cholesterol (98; 163; 182). The effect is believed to help protect women against the development of atherosclerosis. In addition, estrogen also has other "protective" effects, including decreasing insulin resistance, decreasing serum fibrinogen, clotting factor VII and plasminogen activator inhibitor-1 (163; 182). Estrogen also possesses antioxidant and calcium antagonist activities (163; 182). Monocyte and platelet adhesion to the vascular wall and platelet aggregation are inhibited by estrogen as well (163; 203-205). Therefore, it is not surprising that elevated serum levels of estrogen result in increased fibrinolytic activity and a reduced incidence of atherosclerosis in premenopausal women than in men (163; 289).

1.2.1 The Protective Effects of Estrogen on the Vasculature

Estrogen also exerts direct effects on the vasculature through both genomic and nongenomic mechanisms of action. Via rapid, nongenomic pathways, estrogen can induce vasodilation by acting on the endothelium in two ways. The first involves the release of nitric oxide, a potent vasodilator (202; 288). Estrogen can bind to the estrogen receptor α located in the endothelial cell membrane caveolae (162), which activates the endothelial nitric oxide synthase enzyme (93; 163). These findings were confirmed in a clinical study in which, after ovariectomy, endothelial responses to acetylcholine-

induced vasodilation were impaired, but returned after estrogen replacement therapy (270). In addition, female spontaneously hypertensive rats have a more pronounced vasodilation to acetylcholine than male spontaneously hypertensive rats (116). These two examples could be genomic or nongenomic however, estrogen binds to the cell and activates nitric oxide synthase via a receptor mediated nongenomic system (36; 93).

The second, rapid non-genomic mechanism of vasodilation involves stimulation of calcium-activated potassium channels in vascular smooth muscle cells. Estrogen stimulates cGMP-mediated phosphorylation of calcium-activated potassium ion channels in the cell membrane thus stimulating efflux of potassium from the cell. The efflux of potassium results in relaxation of the cell (162; 283). Supraphysiologic concentrations of estrogen will also acutely inhibit L-type calcium channels (voltage-gated), preventing calcium influx and therefore decreasing vascular smooth muscle cell contraction (162; 163; 227). Whether these effects are clinically relevant is unknown.

Binding of estrogen to DNA can also affect cell function in a genomic manner. Estrogen can affect the vasculature by increasing the expression of genes for prostacyclin synthase and nitric oxide synthase in endothelial cells (63; 163; 288). Estrogen binds to estrogen receptors in the cytosol of the cell and the resulting complex transforms into a dimer, which then moves into the nucleus and binds to DNA at specific promotor sites to increase gene expression (178).

Other effects of estrogen on the production of prostaglandins in the vasculature remain unclear. In addition to the increased expression of genes for prostacyclin synthase, workers have found evidence that estrogen enhances vasodilation induced by

methacholine in rats and cultured bovine coronary endothelial cells; a nitric oxide-dependent mechanism of vasodilation (50; 250). If PGH_2 synthesis or the TXA_2/PGH_2 receptor was blocked in ovariectomized rats, then the vasodilator response to methacholine matched that of intact female rats. However, estradiol in these studies reduced 6-keto-$PGF_{1\alpha}$ and TXB_2 production and did not alter the expression of PGH_2 synthase (50; 250). Therefore in normotension, estrogen may act to enhance vasodilation through nitric oxide synthesis, but potentially through inhibition of vasoconstrictor prostanoids as well. This possibility was also investigated in canine coronary arteries, where estrogen decreased the maximal response to the TXA_2/PGH_2 receptor agonist U-46619 (119). In early, partial aortic coarctation-induced hypertension (up to 10 days post-coarctation), virgin and early-stage pregnant (14-15 days) female rats did not develop an increase in systemic blood pressure (96; 219). There are no published data for blood pressure in chronically aortic coarcted female rats. Nonetheless, many previous studies of the vascular effects of estrogen have indicated that it protects against cardiovascular disease and hypertension; however, there is also evidence that estrogen exerts deleterious effects on the vasculature.

1.2.2 The Detrimental Effects of Estrogen on the Vasculature

Several studies have revealed the potentially deleterious effects of estrogen on the vasculature. The effects of estrogen are no longer believed to influence the progression of coronary atherosclerosis (98). In fact recent evidence suggests that estrogen has the ability to stimulate platelet aggregation and to destabilized

atherosclerotic plaques (94; 108; 187) which may result in potentially life-threatening thromboembolism. Women with thrombosis gene polymorphisms, including Factor V Leiden, prothrombin 20210A, and fibrinogen (-455G/A) variants, receiving estrogen or with elevated serum estrogen levels, have a significantly increased risk of venous thrombosis and myocardial infarction (97). The only independent predictor of reduced primary venous graft patency in women post-surgery is hormone replacement therapy (254). Therefore, one of the major detrimental effects of estrogen on the vasculature is though its promotion of thrombosis.

Table 2: Contracting and relaxing factors derived from the endothelium (Adapted from White et al. 1995)(285).

Vasoconstrictors	**Vasodilators**
Angiotensin II	Endothelium Derived Hyperpolarizing Factor
Arachidonic Acid	Histamine
Endothelin I	Leukotrienes
Endothelium Derived Contracting Factor	Nitric Oxide
Histamine	Prostaglandin E_2
Leukotrienes	Prostaglandin I_2
Prostaglandin H_2	Shear Stress
Stretch	Superoxide Anion
Superoxide Anion	
Thromboxane A_2	

Another mechanism by which estrogen may adversely affect the vasculature is through its influence on vascular tone. Vascular tone is maintained through a balance of

vasoconstrictor and vasodilator mechanisms (Table 2). Most investigators report that estrogen stimulates the nitric oxide pathway and that nitric oxide synthesis is one of the major mechanisms by which estrogen exerts protective effects on the vasculature (228) by decreasing vascular tone, yet others report that estrogen inhibits nitric oxide synthase (298). Estrogen decreases inducible nitric oxide synthase expression and its activity in vascular smooth muscle cells via estrogen receptor α (which is likely the same receptor that stimulates nitric oxide synthase in endothelial cells (298)). Therefore, depending on which nitric oxide synthase enzyme is stimulated or inhibited, and its location will determine if an increase or decrease in vascular tone will result.

Estrogen may act indirectly through other factors. One of these is the renin angiotensin system. In this system, angiotensin II (ANG II) and aldosterone are produced which induce vasoconstriction (ANG II) and sodium retention (Aldosterone and ANG II), resulting in increased systemic blood pressure (17). Hypertensive women receiving estrogen replacement therapy develop elevated plasma renin activity, and plasma angiotensin I and ANG II levels (258). At the molecular level, estrogens may stimulate angiotensinogen synthesis 2-fold (34; 136) over that in males. Estrogen responsive elements (DNA sequences that bind the estrogen-estrogen receptor dimer) have been identified in the 5' flanking region of the rat angiotensinogen gene (7). Functionally, when estradiol is administered to ovariectomized rats, there is a significant increase in the pressor response to angiotensin II (over ovariectomized controls) (219). The actions of estrogen are thus important in the aortic coarctation model of hypertension where the renin-angiotensin system is stimulated. ANG II stimulation in

the presence of estrogen may result in increased blood pressure via a combination of genetic and functional actions.

Another detrimental effect of estrogen is through its action on catecholamines and adrenergic agonists. Male and female rat arteries do not respond similarly to stimulation of contraction by norepinephrine and the α-adrenergic agonist phenylephrine (3; 41; 244; 247; 299). The maximum contractile response to phenylephrine in some studies was 40% greater in males than in females, and this difference was attenuated in the female by administration of a nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) (244; 299). This difference in male and female vasoconstriction in response to phenylephrine was abolished by ovariectomy in the female (245). This again links estrogen to the vasodilator nitric oxide and it also links estrogen to adrenergic influence on vascular tone. In contrast to the Zhang et al investigations where estrogen is thought to suppress the expression of α1-adrenergic receptors in mesenteric arteries (299), studies conducted by Altura (3) and Colucci et al. (41) reported increased vasoconstrictor response to catecholamines and phenylephrine in female rats and male rats treated with estrogen compared to males. Other investigators have found similar results indicating increased vasoconstriction in response to phenylephrine and vasopressin in female rat aortae (68; 244; 245; 247). Mesenteric arteries were used in other experiments and concurred with the studies in rat aortae (3; 243; 246). Given the conflicting results other, as yet unknown, factors may be involved in the vascular response to phenylephrine in the female rat.

As stated above, arginine vasopressin (AVP) has been found to stimulate larger contractions in females than males (68; 244; 245). AVP is released from the posterior pituitary in response to extracellular fluid hyperosmolality and/or hypovolemia. AVP is the primary regulator of renal water excretion and is also a potent systemic vasoconstrictor. The renal and vascular actions of AVP are important in the maintenance of arterial blood pressure in altered states such as dehydration and hemorrhage (17). The maximal contractile response to AVP is substantially higher in the female rat aorta at any stage of the estrus cycle than in the male (3; 244; 247). The increased response of the female rat aorta to AVP is believed to be due to, at least in part, to CP, since inhibition of cyclooxygenase attenuates vasoconstrictor responses pf the female aorta by 25 to 30% (244). There may be elevated CP synthesis in response to phenylephrine in females where increased vasoconstriction over male aortae occurs as well (68). In chronic deoxycorticosterone acetate (DOCA)-salt induced hypertension, vascular reactivity to AVP was elevated in females compared to males (246). In the acute stage, the female rats had an elevated 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) output and not until the chronic stage did the output increase in males which may account for the differences seen in vascular reactivity to AVP in this particular model of hypertension. Thus, differences in the production of vasodilators (PGI2, nitric oxide) and vasoconstrictors (constrictor prostanoids) may be the defining element to whether increased constriction occurs in the female.

The effects of estrogen on vascular prostaglandin synthesis is not fully understood. Administration of estrogen reduces production of prostacyclin in the uterus

of rats (77) and in the vasculature of rabbits (61). In the rabbit aorta, estrogen enhances endothelium-dependent contractions to arachidonic acid, the precursor to prostaglandins and vasoconstriction to norepinephrine was inhibited by the cyclooxygenase inhibitor indomethacin (168). Similarly, indomethacin also attenuates vasoconstrictor responses to AVP and PE in female but not male rat aortae (68). Ovariectomy abolishes the attenuating effect of indomethacin and SQ 29,548 (thromboxane/endoperoxide receptor antagonist) to the vasoconstrictor response of the female aorta to AVP and PE (67; 67; 68). The vasoconstriction induced by CP does not appear to be mediated by calcium channels in female pulmonary arteries since contraction stimulated by U-46619 (a TXA_2/PGH_2 receptor agonist) was not attenuated by verapamil, a calcium channel blocker. Male rat pulmonary arteries, on the other hand, exhibited attenuated contraction to U-46619 when exposed to verapamil (48). Estrogen stimulates the production of constrictor prostanoids and dilator prostanoids but the balance of these in vascular disease, especially in association with renin-angiotensin system upregulation, may be skewed toward CP production.

In addition, most of estrogen's actions on the vasculature are likely mediated by the endothelium since many vasodilators and vasoconstrictors are derived from endothelial cells(68; 285). The possibility exists that endothelial dysfunction must occur in some vascular diseases to induce alterations in the balance of vasodilator and vasoconstrictor production.

In the spontaneously hypertensive rat, both male and female rats respond similarly to TXA_2/PGH_2 receptor stimulation with U 46619; the vasoconstriction

produced in each sex was not significantly different (123). However, in this model of hypertension, nitric oxide appears to play a larger role in the females. Female aortae have a greater relaxation response to acetylcholine and do not respond to elevated acetylcholine concentrations with contraction as male rats do unless nitric oxide synthesis has first been inhibited by N_G-nitro-L-arginine (123). Therefore, the possibility exists that upregulation of the renin-angiotensin system must occur in conjunction with increased CP production to induce elevated vascular contractility in certain cases in female rats. This would help to explain the conflicting results of previous studies. Regardless, it appears that estrogen has the ability to affect vascular tone negatively by inducing hypertension through imbalances in vasodilators and vasoconstrictors in the female. Since many mediators are likely involved (CP, PGI2, nitric oxide, ANG II, etc) different models and vascular states may result in many seemingly conflicting results.

Estrogen may exert adverse effects on the vasculature depending on the estrogen receptor (ER) it binds to. The endothelium (40) and vascular smooth muscle cells both contain estrogen receptors (101; 120). There are two classical types of estrogen receptors: α and β (115; 178) and both have been identified in vascular endothelium and vascular smooth muscle cells (5). These receptors have been identified in both human and animal vessels with estrogen receptor β predominantly expressed in the vascular smooth muscle (100; 251). Cytosolic estrogen receptors bind to estrogen to form a receptor-dimer complex, move to the nucleus, and act as transcription factors at specific sites on the DNA, called estrogen responsive elements (178). In this way, estrogen can modulate protein synthesis by its target cells. Estrogen receptor α (ERα) and estrogen

receptor β (ERβ) can bind together to act synergistically or act separately so that they may give rise to opposing regulatory signals (178). Endothelial cells also express estrogen receptors located on the cell membrane (in addition to the cytosol and nucleus) that may stimulate mitogen-activated protein kinase and are similar in structure to ERα (216). ERβ has been identified at the plasma membrane as well. Finally, there are estrogen receptors in the cytosol that act solely in the cytosol and do not translocate to the nucleus (178). Estrogen, bound to either ERα or ERβ can activate the Src/Ras/extracellular signal-regulated kinase (MAPK) pathways in the cytosol which then stimulate cellular proliferation (178). The various ways and types of receptors that estrogen may bind to can result in many combinations to have varying effects on a single cell type or organ such as the vasculature. This again not only demonstrates the complexity of estrogen's potential actions but also the variety of results a single model of vascular disease may demonstrate.

There may even be more than just the two classical estrogen receptors α and β. These new receptors appear to be located in the membrane of cells and one receptor has the pharmacologic profile of γ-adrenergic receptors (178; 179). Given that these receptors have not been characterized, their relationship and functions in the vascular wall remain yet to be identified and may help to explain many of the complex actions of estrogen.

The epidemiological and experimental evidence discussed here identify estrogen as a complex hormone with many actions on the vasculature that are not fully

understood, however, estrogen clearly can act on vasodilators and vasoconstrictors to alter vascular tone.

1.2.3 Estrogen as a Mitogen

Estrogen has been said to have atheroprotective effects in that it prevents or slows the progression of atherosclerosis (289). One of the mechanisms for protection is through modulation of vascular endothelial and smooth muscle cell growth and response to injury. Estrogen inhibits smooth muscle cell proliferation (35; 63; 190) and migration (133; 162). In culture, 17β-estradiol inhibits smooth muscle cell proliferation, DNA synthesis, collagen synthesis, platelet-derived growth factor-induced cell migration, and mitogen-activated protein kinase activity (59). Estrogen prevents neointima formation and thickening after vascular injury (35; 63). The inhibition of proliferation may be due to the increased nitric oxide released by estrogen stimulation of nitric oxide synthase, since nitric oxide can directly inhibit vascular smooth muscle cell proliferation (43).

On the other hand, after injury, estrogen enhances re-endothelialization (134). Estrogen induces basic fibroblast growth factor and vascular endothelial growth factor production, and this may be the mechanism by which estrogen stimulates endothelial cell growth and proliferation (176; 223). It also accelerates functional endothelial recovery after injury (134; 284). This is due, at least in part, to increased local expression of vascular endothelial growth factor and the ability of estrogen to inhibit apoptosis in endothelial cells (42; 241; 242). By inhibiting smooth muscle growth and at the same time stimulating endothelial cell proliferation, recovery after injury is enhanced in

females. The effect of estrogen in hypertension on vascular remodeling is unknown. Fischer et al. found in 1985 (65) that inhibition of estrogen receptors with the estrogen antagonist Tamoxifen reduced collagen production after injury, but that progesterone had a more profound stimulatory effect on collagen synthesis than estrogen. Recently however, investigators have found that estrogen and not progesterone increases aortic stiffness, induces left ventricular hypertrophy, increases thoracic aorta pulse pressure, and decreases medial thickness, internal diameter and luminal cross-sectional area in normotensive rats (252). Estrogen has also been found to potentiate endothelial vascular function in the hindquarters but not the carotid vascular bed (252). The changes that occur in hypertension versus injury may result in very different responses of the vessel wall under the influence of estrogen, but regardless, given our current understanding of the mitogenic effects of estrogen, it is likely that the hormone plays a significant role in the response of the vasculature to hypertension. The roles that estrogen may play in vascular responses may vary with the location of the vascular bed.

1.3 Prostaglandins and Their Receptors in the Vascular Wall

Prostaglandins were first identified by von Euler in 1934 and later determined to be acidic lipids responsible for smooth muscle relaxation (224). They were called prostaglandins because they were first isolated from seminal fluid and believed to be released by the prostate. Much later, many different prostanoids were identified and their formation from the "arachidonic acid cascade" was characterized. Prostaglandins, together with leukotrienes and other hydroperoxy and hydroxy fatty acids, are termed the

eicosanoids (224). Eicosanoids function in inter- and intracellular signaling virtually throughout the body, including the cardiovascular system. Their synthesis is regulated by the availability of arachidonic acid, which is formed from polyunsaturated fatty acids by acylhydrolases in the cell membrane (138; 156). The enzyme responsible for prostanoid formation in the cardiovascular system is phospholipase A_2 which forms arachidonic acid. Phospholipase A_2 is present in endothelial cells, smooth muscle cells, macrophages, platelets, neutrophils, eosinophils, alveolar epithelial cells, renal mesangial cells, keratinocytes, fibroblasts, and osteoblasts (138; 156). Regulation of free arachidonic acid levels in the cell is thought to be controlled mainly by the enzymes responsible for incorporation of arachidonic acid into phospholipids in the cell membrane. These enzymes are arachidonyl coenzyme A synthetase and lysophosphatide acyltransferase and their activity is controlled by protein kinase C (156). As the activity of these enzymes that form phospholipids from arachidonic acid increases, less arachidonic acid will be available for prostanoid synthesis.

Many stimuli activate phospholipase A_2 resulting in the formation and release of arachidonic acid. These include ATP, cytokines, growth factors, hormones, interferons, mitogens, monocyte chemotactic protein, neurotransmitters, and vasoactive peptides. Oxygen stress, hyperglycemia, UV light, and shear stress also activate phospholipase A_2 resulting in subsequent increase in free arachidonic acid. These many stimuli increase cytosolic calcium in their target cells, which then causes phospholipase A_2 to move from the cytosol to the endoplasmic reticular and nuclear membranes, from which it can

generate arachidonic acid (138; 156). Arachidonic acid may also be generated from diacylglyerol (DAG) by DAG lipase.

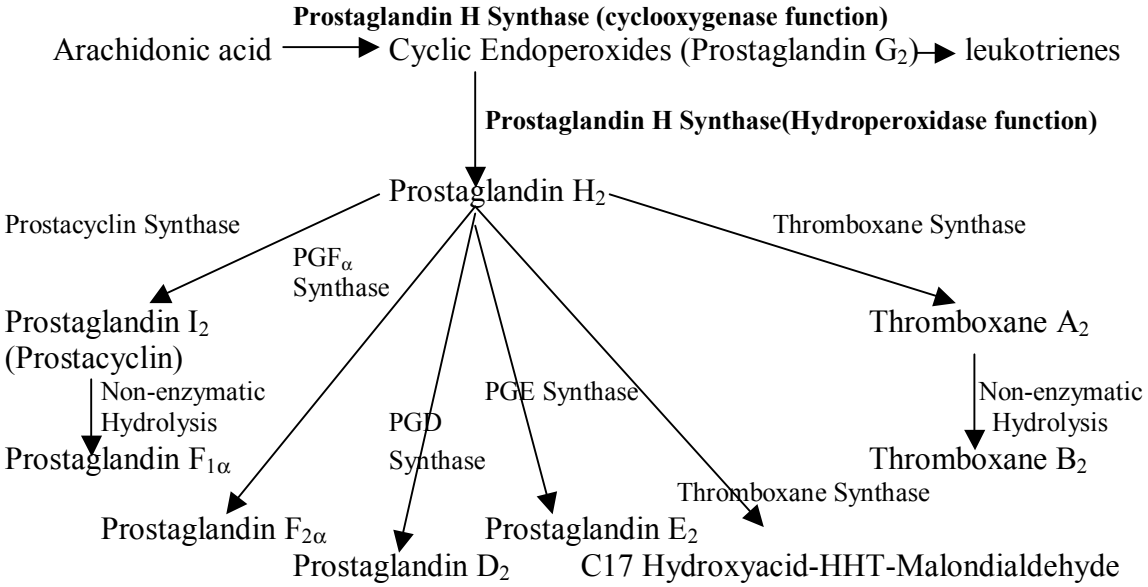


Figure 2: Metabolism of arachidonic acid (adapted from Moncada and Higgs, 1986; and Marks and Furstenberger, eds. 1999)(156; 175).

Thus, changes in arachidonic acid production in the cell, which is the rate-limiting step in eicosanoid synthesis, are mediated in 3 ways: 1) generation by phospholipase A_2, 2) rate of removal by incorporation into phospholipids by arachidonyl coenzyme A synthetase and lysophosphatide acyltransferase, and 3) generation via the DAG pathway (156).

Once arachidonic acid is free in the cytosol, it can be processed by prostaglandin endoperoxide synthase-cyclooxygenase into prostaglandin G_2 endoperoxide (See Figure 2), located in the endoplasmic reticulum and nuclear membranes (183). Cyclooxygenase or COX is a membrane-bound protein that has two isoforms: constitutive COX-1 and inducible COX-2. COX-2 is produced after cell stimulation by inflammatory cytokines, growth factors, and bacterial endotoxin (156; 183). It is interesting to note that some of the same stimuli that increase free arachidonic acid also stimulate the COX-2, enzyme most likely to quickly increase prostaglandin production. COX-2 then forms prostaglandin H_2 from G2. As shown in Figure 2, PGH_2 is processed into many different prostaglandins including prostacyclin (prostaglandin I_2 or PGI_2) and thromboxane A_2 (TXA_2).

1.3.1 Prostaglandin I_2 or Prostacyclin

Prostacyclin is produced in most endothelial cells and in all vascular and nonvascular smooth muscle cells. The PGI_2 synthase enzyme is located in the plasma and nuclear membranes of smooth muscle cells (183). Thromboxane A_2 and PGH_2 analogues as well as thrombin, histamine, bradykinin, shear stress, trypsin, platelet derived growth factor, epidermal derived growth factor, choline esters, interleukin-1 and adenosine nucleotides all stimulate PGI_2 synthesis in endothelial cells (19; 183). Inhibitors of PGI_2 synthesis include glucocorticosteroids, lipocortin, low density lipoproteins loaded with lipid hydroperoxides and vitamin K_1(19). PGI_2 has a half-life of only 2-3 minutes because it spontaneously hydrolyzes to 6-keto-prostaglandin $F_{1\alpha}$

($PGF_{1\alpha}$) which is then excreted in the urine (175). PGI_2 may stimulate proliferation and differentiation of some cells such as preadipocytes (112; 183). PGI_2 mainly functions as a vasodilator produced by the endothelium, and has an antiaggretory influence on platelets, as well as causing disaggregation of platelets *in vitro* (81; 183; 224). The antiaggretory effect on platelets is mediated by increasing cAMP levels within the cell or platelet, and this is accomplished within a very short half-life of the prostaglandin (2-3 minutes) (224; 229). PGI_2 also acts on platelets to prevent their aggregation by inhibiting platelet phospholipase and cyclooxygenase (224). Endothelial cells can make PGI_2 from endoperoxides released by platelets as well as from endoperoxides generated by the endothelial cells themselves (224). PGI_2 synthesis decreases from the endothelium outward toward the vessel wall adventitia, and the reason for this has been hypothesized to be that PGI_2 protects the vasculature from platelet adhesion and aggregation (174; 183; 224). Obviously, if a blood vessel was damaged or torn out to the adventitia contraction rather than dilation would serve to decrease bleeding and help to form a clot. Therefore, it makes sense that little or no PGI_2 is produced in the outer layers of the vessel wall. As mentioned above, PGI_2 inhibits platelet aggregation by increasing cAMP levels (by stimulating the adenyl cyclase enzyme) thereby, increasing sequestration of calcium (the opposite of TXA_2 which increases cytosolic calcium) (183; 224). This is likely to decrease calcium-calmodulin complexes that are capable of binding to myosin kinase, and then phosphorylating myosin, resulting in cross bridge formation with actin and smooth muscle contraction (17). PGI_2 also inhibits smooth muscle cell proliferation, extracellular matrix production and growth factor release by increasing intracellular

cAMP (156). PGI_2 has a local negative feedback system that prevents further production of itself by inhibition of cyclooxygenase (24; 126; 183). PGI_2 production is further antagonized by oxygen free radicals, which may be important in reperfusion injury (156) and in vascular diseases where angiotensin II is increased (angiotensin II increases oxygen free radical release (79; 206)). Schror, Vane, and others have hypothesized that PGI_2 and TXA_2 work together to maintain a homeostatic balance in platelet aggregation and vessel tone because they have nearly opposite effects on platelets and vascular smooth muscle (156; 173; 183; 224). Furthermore, the distribution of PGI_2 synthase and TXA_2 synthase in the layers of the vascular wall is nearly opposite from endothelium to adventitia with PGI_2 synthase having its highest concentration in the endothelium (174; 183; 224). If in disease the balance of PGI_2 and TXA_2 production were upset, then excessive vasodilation/constriction or platelet aggregation/bleeding disorders could develop. Indeed, this is the case in some diseases where there is a tendency for thrombosis to develop and increased TXA_2 is present and/or decreased PGI_2 are present. The opposite occurs for diseases in which an increased bleeding tendency is present (224). Because PGI_2 works together with TXA_2 to maintain homeostasis and prostaglandins have been implicated in renin angiotensin system-dependent hypertension (113), its functions and actions are central to this investigation of vascular diseases in women. It follows that if estrogen increases production of TXA_2 in the presence of angiotensin II, then postmenopausal women with hypertension who are receiving hormone replacement therapy may have an increased tendency toward thrombosis (108; 176; 233) because they have an imbalance in PGI_2/TXA_2 production in their vasculature.

Most likely there is an overproduction of TXA_2 resulting in increased platelet aggregation on the vascular endothelium. In women with gynecologic cancer, production of prostaglandins shifts from PGI_2 to TXA_2. In preeclampsia and other chronic placental insufficiency syndromes, there is a deficiency in production of prostacyclin and an overproduction of TXA_2, resulting in the increased vasoconstriction that occurs in hypertensive pregnancies (295). In addition, oxygen free radicals (produced by angiotensin II) inhibit the synthesis of two very important vasodilators, PGI_2 and nitric oxide. Thus, the homeostasis between vasoconstrictors and vasodilators may be out of balance. The increased incidence of vascular diseases in premenopausal women (primary pulmonary hypertension, Raynaud's disease, oral contraceptive-induced hypertension) may be explained by such an imbalance in constrictors and dilators in the vasculature.

1.3.2 Prostaglandin D_2

Prostaglandin D_2 (PGD_2) is generated from prostaglandin H_2 endoperoxide (Fig. 2) by prostaglandin D synthase and albumin (175; 259). This prostanoid is synthesized predominately by the spleen, macrophages, dendritic cells, and mast cells (37; 183). Platelets also produce a small amount of PGD_2, but this amount increases if thromboxane synthase and therefore, TXA_2 production, is inhibited (175). PGD_2 induces sleep, regulates nociception, inhibits platelet aggregation and mediates allergic reactions (259). When PGD_2 is infused intravenously, it acts as a peripheral renal vasodilator, opposing the actions of noradrenaline, angiotensin II and antidiuretic hormone (156;

183). Intravenous PGD_2 also induces pulmonary vasoconstriction and is a potent bronchoconstrictor (183). In primary pulmonary hypertension, there is a two-fold increase in urinary excretion of the PGD_2 metabolite, PGD-M (37). Inhibition of thromboxane A_2 synthase can result in an accumulation of prostaglandin endoperoxide PGH_2, and as such increase PGD_2 synthesis by platelets and albumin. PGD_2 could then induce vasodilation and inhibit platelet aggregation, unrelated to a decrease in TXA_2 (156; 175; 224), resulting in conflicting data. Therefore, studies involving inhibition of thromboxane synthase may result in ambiguous results, and inhibition of the thromboxane A_2/prostaglandin H_2 endoperoxide receptor may be a more appropriate means of investigating the effects of TXA_2 on the vasculature.

1.3.3 Prostaglandin E_2

PGE_2 synthase is stimulated by glutathione and PGE_2 is the major arachidonic acid metabolite produced by the kidney (183). PGE_2 is present in high concentrations at sites of inflammation and has a central role in fever generation. This prostaglandin can also induce thermal hyperalgesia in rats (181). In reproduction, PGE_2 stimulates luteinizing hormone-releasing hormone secretion from the hypothalamus and may be involved in embryo implantation (181). PGE_2 has many effects on kidney function, including antagonism of antidiuretic hormone-induced water resorption and modulation of renin release (183); therefore, it may play a significant role in vascular diseases where renin release is stimulated. PGE_2 also regulates ion transport and glomerular filtration in the kidney. As with PGD_2, PGE_2 is a renal vasodilator and inhibits norepinephrine and

angiotensin II-induced vasoconstriction in the kidney (156). In addition, PGE_2 can induce platelet aggregation (175) and can either stimulate smooth muscle constriction or vasodilation in the systemic vasculature, depending on the receptor subtype stimulated. Prostaglandin E_2 (PGE_2) has 4 different receptor subtypes: EP_{1-4} that have different distributions throughout the body (181). For example, the EP_2 receptor induces vasodilation and hypotension when stimulated, whereas stimulation of the EP_1 or EP_3 receptor induces vasoconstriction. Knockout mice deficient in the EP_2 receptor fed a high-salt diet develop severe hypertension (181). PGE_2 may have an important role in some vascular diseases but further study is needed.

1.3.4 Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is expressed in the kidney, heart, lung, stomach and corpus luteum. The main function of $PGF_{2\alpha}$ and the main expression of its receptor is in the corpus luteum. This prostanoid induces luteolysis and it is also important in parturition, as mice deficient in the FP receptor do not undergo parturition even with oxytocin administration (181). Like PGE_2, $PGF_{2\alpha}$ is involved in the induction of fever, but likely plays a lesser role than does PGE_2 (181). Finally, in fibroblast cells $PGF_{2\alpha}$ can act as a mitogen (181) to stimulate their replication.

1.4 Thromboxane A_2 in Normotension and Hypertension

Thromboxane A_2 (Figure 3), a potent vasoconstrictor and platelet aggregator, is elevated in humans with a genetic propensity for coronary heart disease and is elevated

significantly more in these affected females than in males (2). In general, hypertension and increased platelet aggregability are associated with increased TXA_2 levels in humans and experimental animal models of hypertension (175; 213). Elevated plasma TXA_2 levels have been documented in patients with Prinzmetal's and vasotonic angina and cerebral ischemic syndromes and implicated in preeclampsia, pregnancy-induced hypertension and pulmonary hypertension (156; 195; 224; 282). Further, TXA_2 also has been associated with asthma, acute respiratory distress syndrome, and endotoxin-induced pulmonary ventilation-perfusion mismatch (62).

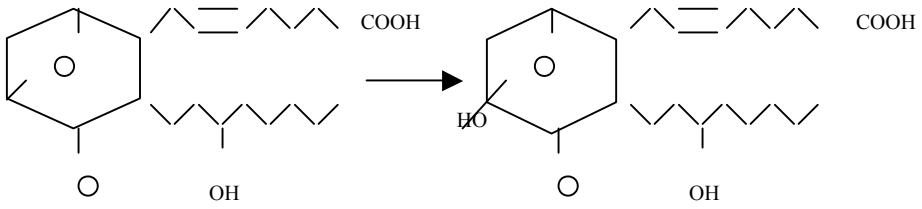


Figure 3: Thromboxane A_2 and thromboxane B_2 (Adapted from Moncada, S and Higgs EA)(175).

TXA_2, like PGI_2, is produced from the PGH_2 endoperoxide precursor (Figure 2) and has a short half-life of 30 seconds due to its spontaneous hydrolysis to the stable metabolite thromboxane B_2 (TXB_2) (183), Figure 3. Thromboxane synthase (the enzyme that catalyzes TXA_2 formation) acts on PGH_2 to synthesize TXA_2 and 12-hydroxy-heptadeca-trienoic acid and is located near dense tubular membranes within the cell (such as the endoplasmic reticulum) (183). TXB_2 is excreted in the urine (183) and has

no vascular activity. TXA_2 not only stimulates platelet aggregation and vascular smooth muscle contraction, but it also causes the release of platelet constituents, bronchoconstriction and seritonin release via the TXA_2/PGH_2 receptor (62; 175; 183). TXA_2 is synthesized in many vascular tissues including arterial and venous endothelium and smooth muscle, blood cells (platelets, neutrophils, macrophages) and cardiac myocytes (including the endocardium) (156; 175; 183). TXA_2 is the most potent platelet aggregator of the prostaglandins, but when synthesis is inhibited, PGH_2 and PGG_2 may induce aggregation in its place by binding to the TXA_2/PGH_2 endoperoxide receptor (175). TXA_2 synthesis and release is activated by a number of humoral and physical factors including cholesterol, acetycholine, histamine, angiotensin I and II, ADP, ATP, arachidonic acid, superoxide anion (O_2^-), 5-hydroxytryptamine, substance P, A23187 (calcium ionophore) and stretch (122; 175). In both humans and Wistar-Kyoto rats, TXA_2 production increases with age, which may contribute to increased vascular disease in older people (122; 175). It is unknown whether activity of TXA_2 synthase increases with age, but there is a decrease in prostacyclin synthase and subsequent PGI_2 formation, while the level of COX enzyme does not change (175). This may result in increased shunting of COX products toward the TXA_2 synthase pathway, resulting in an increase in TXA_2 synthesis.

TXA_2 acts by binding to the TXA_2/PGH_2 endoperoxide receptor to activate phospholipase C and increase intracellular Ca^{2+} and protein kinase C (through formation of inositol-1,4,5-trisphosphate (IP_3) and diacyl glycerol). TXA_2 also inhibits cAMP formation in the cytosol, resulting in activation and aggregation of platelets and

contraction of vascular smooth muscle (85; 156). Through these actions, TXA_2 promotes rupture of plaques and occlusion of vessels in atherosclerosis (156) (page 278). TXA_2 also stimulates mesangial cell contraction in the kidney, which can a decrease glomerular filtration rate (156).

TXA_2 is thought to be an important mediator of sepsis and endotoxic shock (156). Inhibition of TXA_2 synthase by dazoxiben in animals given endotoxin prevents the increase in pulmonary artery and pulmonary wedge pressures as well as the decrease in cardiac output that occur with endotoxic shock (290), therefore TXA_2 is thought to play an important role in the pathogenesis of sepsis.

With regard to the renin-angiotensin-aldosterone system, there appears to be a negative feedback mechanism between renin and TXA_2. When TXA_2 receptor antagonists or TXA_2 synthase inhibitors are given to anesthetized rats, plasma renin activity increases. In contrast, when a TXA_2 mimetic is administered, plasma renin activity declines (201; 279). In addition, angiotensin II stimulates vascular TXA_2 and PGI_2 release (148; 156; 287); therefore, it is logical that TXA_2 would decrease renin and subsequent angiotensin II formation in the plasma. In this way, during both normotension and hypertension the vasculature may regulate the renin-angiotensin system to stabilize systemic blood pressure. This system is likely overridden by the macula densa cells and vascular baroreceptors to control systemic blood pressure, but with prostaglandins and the renin-angiotensin-aldosterone system working in concert, a balance may be achieved. Inhibition of the TXA_2/PGH_2 receptor in male rats subjected to renovascular hypertension (two kidney-one clip Goldblatt hypertension) both arterial

blood pressure and mortality (286). However in one study, this occurred only in the male rats that developed malignant hypertension, and TXA_2/PGH_2 receptor antagonism/blockade had no effect on 40% of the renovascular-hypertensive rats (20).

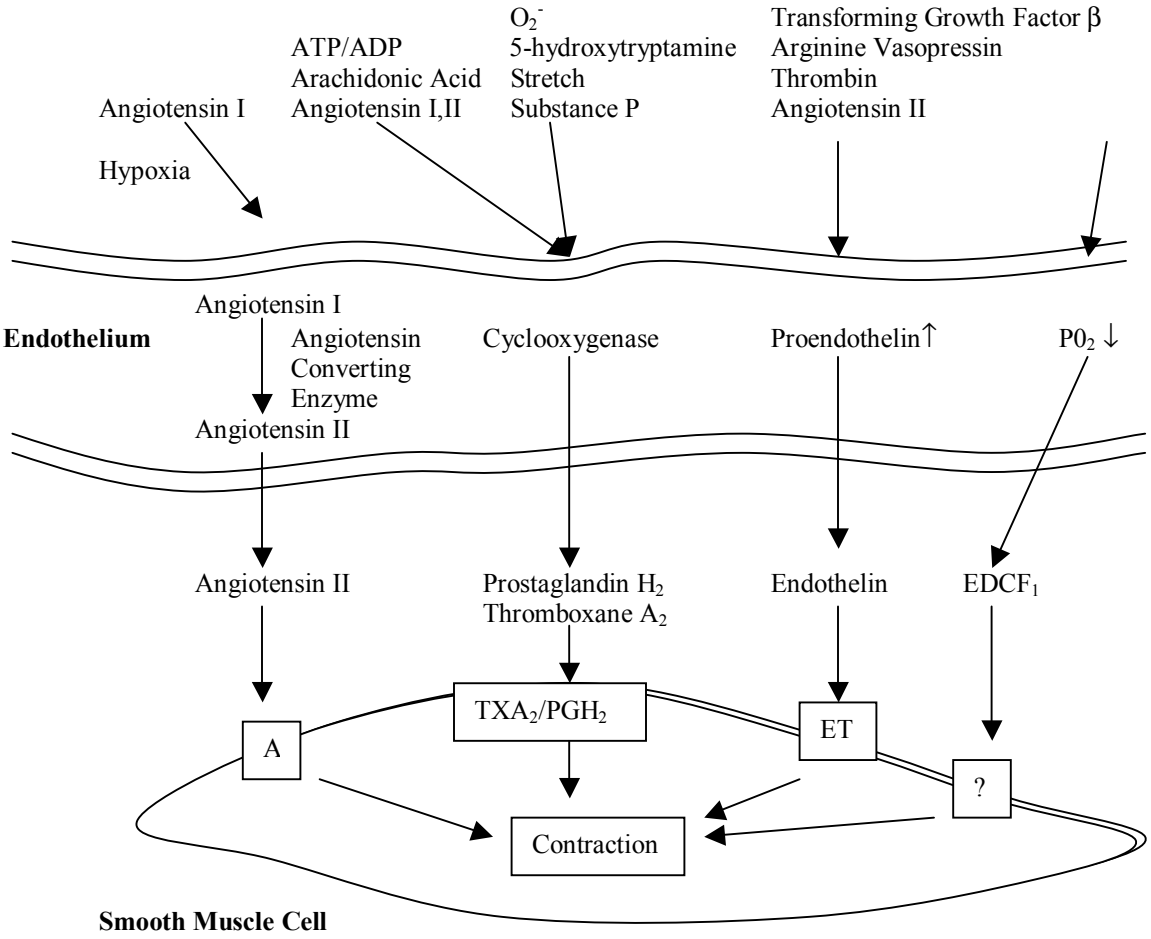


Figure 4: Endothelium-derived contracting factors produced by the vascular wall (Adapted from Katusic and Shepherd, 1991)(122). A = angiotensin II receptor, ET = endothelin receptor, TXA_2/PGH_2 = thromboxane/endoperoxide receptor, ? = unidentified receptor (122).

The TXA_2-angiotensin II feedback system may play a more important role in females than in males, since stimulation of females with vasoconstrictors such as vasopressin results in greater TXB_2 production and vasoconstriction than in males (67; 67; 244; 247). In males, other endothelium-derived contracting factors may contribute more to the development and maintenance of hypertension than the PGH_2/TXA_2 pathway (Figure 4).

Sexual dimorphism with respect to TXA_2 has been identified in normotensive rats (68). Maximum contractile responses to vasopressin and phenylephrine are higher in female rat aortae than in males (68). The endothelium of the female rat, at least in the pulmonary vasculature and aorta, potentiates the contractile response to U 46619 but it does not in the male (48; 68). Further, normotensive female rats have an increased vasoconstrictor response to angiotensin II and arachidonic acid compared to males (3; 197; 247). Approximately 30 % of vascular responsiveness in the female rat has been attributed to TXA_2/PGH_2 (68; 145; 168; 248). Therefore, it would appear that the female vasculature has increased CP release in response to vasopressors such as angiotensin II and arginine vasopressin over the male vasculature. If this is the case, then vascular diseases involving the renin-angiotensin system and/or arginine vasopressin would result in elevated TXA_2 production, at least while the above hormones were elevated in the plasma.

The response of male and female normotensive and spontaneously hypertensive rats (SHR) to the TXA_2 mimetic U 46619 differs significantly. Normotensive rats have an increase in mean arterial blood pressure when administered U 46619, however, the

endothelium potentiates contraction to U 46619 only in the normotensive female (48; 68). Both male and female SHR have a similar increase in pressure (253) in response to U 46619. Giving the COX inhibitor acetylsalicylic acid to male SHR abolishes any U 46619-induced increase in blood pressure (253) thus indicating activation of the CP pathway (PGH_2) in the male. Female SHR, on the other hand, did not exhibit any change in MAP with the administration of acetylsalicylic acid (253). Further, administration of U 46619 increases plasma TXB_2 concentrations in male SHR but it decreases TXB_2 in the plasma of female SHR (221). These results suggest that the vasoconstrictor responses in SHR to u 46619 are different in SHR males and females. The response is unlikely due to PGI_2 formation in the female since no change in 6-keto-$PGF_{1\alpha}$ plasma levels occurred with U 46619 administration (221). If stimulation of the TXA_2/PGH_2 endoperoxide receptor in males resulted in increased blood pressure and plasma levels of TXB_2, then displacement of TXB_2 from the receptor and activation of the receptor likely occurred in male SHR. The lack of response in the female (no change in BP or plasma levels of TXB_2) to U 46619 may therefore be due to a difference in the receptor in the female and does not necessarily indicate that the CP pathway is not involved in females.

There are conflicting reports in male SHR concerning the involvement of CP (74; 122; 263; 293). In male rats upregulation of the CP system has been implicated as the mediator of hypertension in SHR (74; 263). Other reports have found no evidence of involvement of TXA_2 in male SHR (293) In male SHR, the immediate precursor to TXA_2, PGH_2, may be actively taking the place of TXA_2. If the TXA2/PGH2 endoperoxide receptors are different in male versus female SHR, then there may be

increased affinity in the male receptor for PGH2 compared to the female receptor, explaining, at least in part, the differing responses of the two sexes to U 46619.

The aortic coarctation model was used here since in the acute phase in male rats the model is renin-angiotensin system-dependent and a CP-mediated mechanism of vasoconstriction is responsible, at least in part, for the elevation in blood pressure and vascular tone (56; 148). An investigation of the effects of estrogen 45 minutes after *partial* acute aortic coarctation in female rats showed that estrogen increased the sensitivity of ovariectomized rat arteries to angiotensin II stimulation (219). Henzel and Alsip (96) investigated the effects of *partial* aortic coarctation in pregnant and virgin female rats for 10 to 11 days. No elevation in mean arterial blood pressure was identified in the virgin females by 10-11 days after partial coarctation. This investigation did not follow these rats chronically (14 days or more) and did not investigate complete aortic ligation, which has the effect of maximally stimulating the renin-angiotensin-aldosterone system. It is important to remember that in the aortic coarctation model, the hypertension is renin-angiotensin system-dependent in the acute phase (148). Chronically, the vasculature has remodeled and the baroreceptors have reset so that the peripheral vascular resistance and systemic blood pressure remain elevated without the involvement of the renin angiotensin system.

TXA_2 is a potent mitogen and stimulates vascular smooth muscle cell proliferation and vascular remodeling (130; 156). If the acute phase of aortic coarctation-induced hypertension is CP-dependent to a much greater extent in females than in males, then the remodeling that occurs in the vascular wall may be different between sexes due

to the influence of TXA_2 and estrogen (which also regulates cellular proliferation). TXA_2 directly stimulates extracellular matrix protein production independent of its mitogenic effect or its ability to increase cellular protein. TXA_2 increases the steady state levels of mRNA for the proteins fibronectin, laminin, and type IV collagen (26; 165). It also decreases the level of mRNA for heparin sulfate proteoglycan (27). TXA_2 also stimulates angiogenesis and endothelial cell migration induced by basic fibroblast growth factor or vascular endothelial growth factor (185). If TXA_2 is elevated to a greater extent in females, then the vascular remodeling seen may display a more pronounced increase in extracellular matrix protein. Estrogen inhibits vascular smooth muscle cell proliferation (59; 60; 297) by increasing intracellular cAMP and adenosine (59). The presence of estrogen may inhibit vascular smooth muscle proliferation and some protein synthesis in early aortic coarctation while elevated TXA_2 levels may stimulate increases in collagen and other extracellular matrix proteins that results in a very specific remodeling unlike that seen in males.

1.5 Thromboxane A_2 Synthase Enzyme and the Thromboxane/Endoperoxide Receptor in the Aorta

Thromboxane Synthase is an enzyme that catalyzes conversion of PGH_2 to TXA_2 (62; 135). Table 3 summarizes some of the properties of the enzyme. Thromboxane synthase was originally isolated from platelets, but has since been found in neutrophils, macrophages, monocytes, endothelium and in the tissues of the colon, kidney, liver, lung and utero-placental unit (62; 282). In approximately 80% of neoplastic cells investigated,

mRNA for TXA_2 synthase was present (156). This enzyme has been seen as a possible target for pharmacological regulation of tumor growth, but the ability of PGH_2 to also stimulate the TXA_2/endoperoxide receptor negated this possibility (156). In the lung, the distribution of TXA_2 synthase closely follows that of the COX-1 and COX-2 (62).

Table 3: Properties of Thromboxane A_2 Synthase (Adapted from Marks F, et al., 1999)(156).

Molecular Mass (Da)	60,487 (cDNA sequence)
Optical Absorption Spectrum Oxidized Protein	418,537 and 5+0
Reduced plus CO	424,450 and 545
EPR Spectra	Heme-thiolate (ferric state)
Isoelectric point	4.8
Molecular Activity	$27s^{-1}$ (TXA2 + HHT)

The enzyme has similarities to the cytochrome p450-type hemoproteins and may be related to them (257) (36% homology (156) (page 91); and also forms 12-hydroxy-heptadeca-trienoic acid along with TXA_2 as a byproduct. It is located near dense tubular membranes within the cell (183). Surprisingly, TXA_2 synthase shares only 16% homology with the prostacyclin synthase enzyme, which catalyzes conversion of PGH_2 to PGI_2 (156). Although the mRNA and protein levels of the enzyme do not increase in hypertensive diseases such as preeclampsia or endotoxin induced pulmonary hypertension, post-transcriptional mechanisms for enzyme regulation may account for the possible role of TXA_2 in these diseases (62; 282). Alternate splicing of the mRNA

for TXA_2 synthase can result in either an active or inactive (lacks the Fe^{3+} binding site) enzyme (274). The enzyme is also a "suicide" enzyme in that it is inactivated by catalysis of PGH_2 to TXA_2 (114). In addition, TXA_2 synthase antagonists can protect the enzyme from inactivation and thereby induce increased TXA_2 production following reduction or removal of the TXA_2 antagonist (114). Further control of TXA_2 function also could be conferred by regulation of the COX enzymes and redirection TXA2 of precursors to other prostanoid or leukotriene pathways, see Figure 2.

TXA_2 synthase inhibitors include imidazole, dazoxiben, and pyridine derivatives (OKY 046, OKY 1555, OKY 1581), (135; 183). These inhibitors have been shown to inhibit the production of TXA_2 but not it's actions which led to the discovery that endoperoxides such as PGH_2 and PGG_2 can bind to the TXA_2/PGH_2 endoperoxide receptor to induce platelet aggregation, vasoconstriction, etc (175; 183; 224). Increases in PGI_2 and prostaglandin E_2 have been documented in rats after oral ingestion of the thromboxane A_2 synthase antagonist CGS 13080, at a dose that suppressed elevations of TXA_2 in response to calcium ionophores (135).

Only inhibitors of the TXA_2/PGH_2 receptor are able to prevent any actions induced by TXA_2 or its endoperoxide precursors (175; 201; 213). Thromboxane receptor antagonists include BM 13.177, BP 13.505, SQ 29,548, GR 32,191, ifetroban and S 18,886 (32; 213; 235). Combination TXA_2/PGH_2 receptor antagonists and TXA_2 synthetase inhibitors have been synthesized as well and include Ridogrel and azo analogue I (52-54; 175; 201). These compounds have the potential to not only prevent TXA_2 actions, but also those of its endoperoxide precursors, which may prove useful

clinically(175). On the other hand, competitive receptor antagonists that do not affect the formation of other prostanoids or eicosanoids would have the benefit of not shunting synthesis from one prostanoid to another the way that synthase inhibitors can. Therefore, the use of receptor antagonists to study the effects of TXA_2/PGH_2 receptor binding may provide more conclusive results in the research of CP actions.

The TXA_2/PGH_2 receptor is a glycoprotein and has been identified in platelets, vascular smooth muscle, vascular endothelium, kidney, brain and immature and mature thymocytes (72; 84-86; 88; 181; 278). In thymocytes, it has been suggested that the receptor may play a role in antigen-mediated immunomodulation and T-cell maturation and development, for if a receptor agonist is administered to immature thymocytes, apoptotic cell death is induced (181). Receptor binding is reduced in the presence of basic fibroblast growth factor and phosphorylation enzymes such as protein kinase A and C (128).

Two subtypes of the TXA_2/PGH_2 receptor have been suggested- a platelet subtype named α, and a vascular receptor named τ (85). Studies of atherogenesis in apo E-deficient mice support the concept of TXA_2/PGH_2 receptor subtypes. Inhibition of the receptor with S 18886 inhibited atherosclerosis by a mechanism independent of platelet-derived TXA_2 synthesis; and inhibition of the cyclooxygenase enzyme with aspirin prevented platelet-derived TXA_2 synthesis but did not prevent atherosclerosis (32). If the receptor subtypes respond differently to antagonism, the effects of receptor blockade on endothelium versus smooth muscle and male versus female tissues may differ significantly. In male rats, no difference in receptor type were found between aortic

smooth muscle cells and platelets (87), and the receptor has the same affinity for SQ 29,548 in both cell types(87). With respect to sex differences in receptor function, male vascular smooth muscle cells express increased numbers of TXA_2/PGH_2 receptors compared to females. Testosterone and not 17β-estradiol induces increased receptor density in rat aortic smooth muscle cells of normotensive rats (99; 158) and in human platelets (1). RT-PCR in our lab has identified increased receptor density in the normotensive female rat aorta. It is unknown whether the density of receptors changes in males and females in vascular disease or to what extent with induction of hypertension. Since angiotensin II stimulates TXA_2 release, one may speculate that it also increases receptors but this is unknown.

The receptor, once bound, is coupled to second messenger systems through a guanine nucleotide regulatory protein and increases intracellular free Ca^{2+}. Through second messenger systems, platelet aggregation and activation as well as smooth muscle contraction occur (84; 85). TXA_2/PGH_2 receptor antagonists such as SQ 29,548 and BM 13.505 are reported to decrease myocardial ischemia-reperfusion injury , endotoxin-induced thrombocytopenia and pulmonary hypertension (235-237). In hypertension, the receptor has been correlated with salt intake. Salt loading increases receptor expression in the kidney and brain (neurons) of rats; and rats fed a high salt diet exhibit increases in systemic blood pressure when given the TXA_2 mimetic U-46619 either intravenously or intracerebroventricularly (72; 278). This may indicate upregulation of the CP receptor in hypertensive states. In spontaneously hypertensive rats, administration of the TXA_2/PGH_2 receptor antagonist ifetroban restores normal endothelial cell function.

However, in these rats, mean arterial blood pressure remained elevated (213). In aortic coarctation-induced hypertensive male rats, vascular tone was reduced by up to 75% by ifetroban (213), indicating not only the upregulation of the CP system with aortic coarctation, but also that differences exist in the various models used to study hypertension. Blockade of the TXA_2/PGH_2 receptor in a clipped kidney in the Goldblatt model of hypertension in rats diminishes the pressor response to nitric oxide synthesis inhibition (232), indicating again a very important role for TXA_2 in hypertension, especially renin angiotensin system-dependent hypertension. Both losartan (an angiotensin I receptor antagonist) and ifetroban significantly reduce blood pressure to the same degree in early, intermediate, and late phases of Goldblatt hypertension (286). Therefore, TXA_2 and its receptor have been implicated in vascular disease, but very little information is known about its presence and/or action in vascular diseases in the presence of estrogen.

1.6 Mechanobiology of the Aorta

Mechanics involves the study of how matter responds to applied loads (109). This is of interest in the physiology of the aorta because the aorta is constantly exposed to applied loads and therefore its behavior is relevant under these conditions. To understand the behavior of the aorta in the intact animal will allow for understanding and insight into its behavior under pathological conditions such as hypertension. There is much evidence that arteries are affected by the loads applied to them, including pressure and shear stress (131; 132). Acute shear stress elicits acute nitric oxide and prostacyclin

release, and transcriptional activation of many genes (endothelial nitric oxide synthase, cyclooxygenase II, intracellular adhesion molecule-1, MCP-1, tissue factor, platelet-derived growth factor-B, transforming growth factor-β1) as well as transcription factors (nuclear factor kappa B, c-fos, c-jun, specific protein-1) (9; 103; 103; 150; 155; 172; 180; 189; 209; 231; 255; 292).

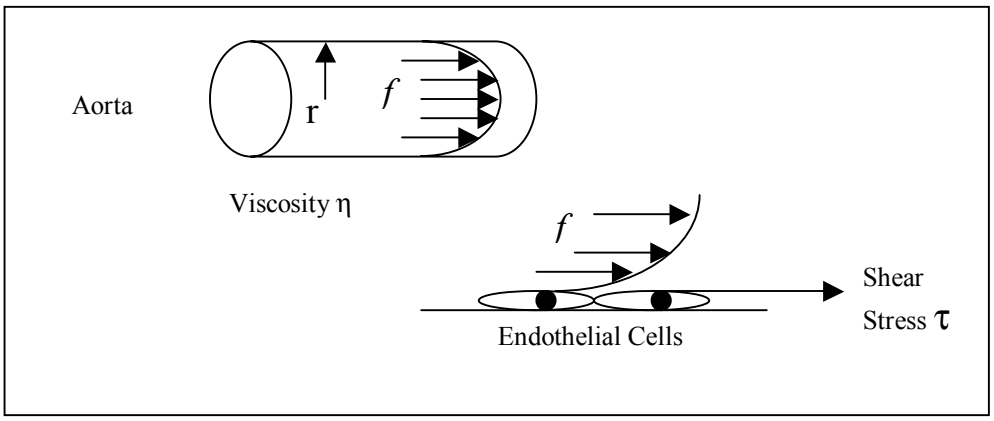


Figure 5. Wall shear stress. Wall shear stress as defined by the Hagan-Poiseuille Law assuming fully developed laminar flow. Adapted from (155).

First, let us define the forces applied to the aorta. Strain is a measure of the change in length or angle that occurs within a material when placed under a load. Stress is a measure of a force applied to an area, such as an area of aorta wall, which is the pressure placed on that area. (109). Stiffness is the change in stress that occurs when there is a change in strain (109) such as the change in pressure that occurs with a change in the diameter of a vessel that results. The two most important hemodynamic forces on the

aorta wall are tensile stress and wall shear stress (71). Tensile stress on the wall has both circumferential and longitudinal components as blood pressure is exerted on the vessel. Wall shear stress is the frictional force per unit area of vessel wall and is the tractive force of the blood acting on the wall parallel to the long axis of the wall.

In the artery, shear stress is determined by blood flow and viscosity as well as the geometry of the vessel (71), Figure 5. The equation for the shear rate is

$$\gamma = (m + 2)f/ (\pi r^3);$$

and the wall shear stress is

$$\tau = \eta\gamma;$$

where m=2 if there is laminar flow. The internal radius is r and the total blood flow is f, while the blood viscosity is η (117; 155). Therefore, if we assume fully developed laminar flow, the wall shear stress is defined by the Hagen-Poiseuille Law:

$$\text{wall shear stress} = \tau = 4\eta f/\pi r^3.$$

Vessels have been tested in the past in uniaxial strips (170; 294) and flat sheets (171) . Cylindrical specimens are preferred in biomechanical research (44; 46; 261; 269) however, because they preserve the vessel ultrastructure and therefore can better mimic in vivo loading conditions (46; 110). In cell culture, endothelial cells respond to a steady state flow by elongating and orienting their long axis in the direction of the flow as well as by increasing their stiffness by orienting their actin fibers parallel to the direction of flow (184). In vivo experiments have shown that increases in wall shear rate result in an increase in the diameter of the artery lumen whereas with decreases in shear stress, the intima thickens and the lumen diameter decreases (51; 117; 139; 140). Increased wall

shear stress likely induces a flow dependent vasodilation and remodeling (16) through induction of membrane phospholipid metabolism that then results in alterations in intracellular cell signals (18). Shear stress has been found to induce expression of endothelial enzymes such as prostacyclin synthase and endothelin converting enzyme (39) and nitric oxide production increases as flow increases (9; 26; 111; 132; 137)). Increased blood flow and resulting shear stress elicits a long-term structural expansion so that the internal radius of the artery increases to the point that shear stress returns to it's previous value of approximately 15 dyn/cm^2 (117).

Vessels respond to tensile stress in addition to shear stress. When blood flow is reduced in the carotid artery at the same time that hypertension is induced vessel wall hypertrophy due to smooth muscle cell proliferation results (256). Therefore, although there was no increase in wall shear stress (i.e. *r* remains the same), the smooth muscle cells still responded to an elevation in tensile stress (hypertension). Smooth muscle cell hypertrophy and fibrosis has been documented to result from increased wall tensile stress (elevation in blood pressure) (16).

Unlike smooth muscle cells, the endothelium responds to hypertension in the face of normal blood flow by increasing apoptosis as well as proliferation (i.e. increased cell turn over) (256). This response may be due to nitric oxide production in the endothelium. Nitric oxide production is enhanced by increased shear stress (which stimulates increased endothelial nitric oxide synthase expression (9; 208)) and nitric oxide then inhibits smooth muscle cell proliferation (226) . Therefore, it follows that if there is no change in shear stress but an increase in blood pressure as with hypertension,

nitric oxide production will not increase and there will be no inhibition of smooth muscle cell proliferation.

A key question, however, is: does prostaglandin production change with endothelial cell biomechanical responses? As stated previously, expression of prostacyclin synthase in endothelial cells is augmented in a shear stress intensity-dependent manner (9; 39; 132). An acute effect of shear stress is release of prostacyclin (9; 28; 29; 132). An initial burst of prostacyclin is produced by arteries followed by a steady state release over a 3 hour period in arterial perfusion studies (29). The increased prostacyclin would likely enhance vasodilation of the artery and along with nitric oxide promote a return of shear stress to it's previous level of approximately 15 dyn/cm^2.

With regard to CP, production is also stimulated by changes in wall shear stress. Thromboxane A_2 production is increased in the initial period of perfusion and then reaches a steady state release over the next 3 hours (28; 29). The reason for release of CP is not known, but it may act to balance the release of the potent vasodilators nitric oxide and prostacyclin. CP release is higher during perfusion at physiologic pressure and pulsatile flow (100 mm Hg, 90 ml/min, respectively) than in static incubation (28). In addition, prostanoid release has been found to have no consistent correlation when measured by static incubation versus perfusion of the vessel lumen (28; 161). This indicates a need to measure prostanoid release during perfusion as well as static incubation. Indeed, in hypertension, increased shear stress increases the release of CP such as prostaglandin H_2 and thromboxane A_2 (105), which may mean that these prostanoids are important mediators in hypertension that require further study. In the

model of hypertension used in this investigation, namely renovascular hypertension, thromboxane A_2/prostaglandin H_2 play major roles in the maintenance of the elevated blood pressure (286). In hypertension, at least, CP may do more than just balance the actions of shear stress-induced vasodilators.

Investigators have studied enhanced release of CP in male rats but estrogen may influence the regulation of shear stress-induced prostaglandin release as well. Estrogen has been found to enhance nitric oxide release stimulated by increased shear stress in female rats (104) and in hypertension as well (106). Estrogen inhibits cultured vascular smooth muscle cell mitogenesis produced by cyclic mechanical strain (151). If nitric oxide synthesis is chronically inhibited, then estrogen continues to enhance shear stress induced dilation but through endothelium-derived hyperpolarizing factor and not via prostacyclin (106). The mechanism behind estrogen's ability to inhibit smooth muscle cell hypertrophy is likely mediated through its ability to stimulate endothelial nitric oxide synthase. Interestingly, the effect of estrogen on CP production with changes in shear stress has not been investigated. It is likely, however, that estrogen, along with changes in shear stress and tensile stress, contributes to the vascular remodeling that occurs in hypertension, perhaps through activation of CP. Both male and female human aortae decrease distensibility with increasing age but these changes (i.e. an increase in stiffness) occur much earlier in men than in women (240). The subjects in this study were healthy and not hypertensive and may not reflect what occurs in forms of hypertension where ANG II is involved.

1.7 Aortic Coarctation-Induced Hypertension Model in the Rat

The aortic coarctation model of hypertension was used in the rat in this investigation. First developed by Rojo-Ortega and Genest (211) and characterized by Fernandes, et al. (64), this model involves complete ligation of the abdominal aorta between the right and left renal arteries, cranial to the superior mesenteric artery.

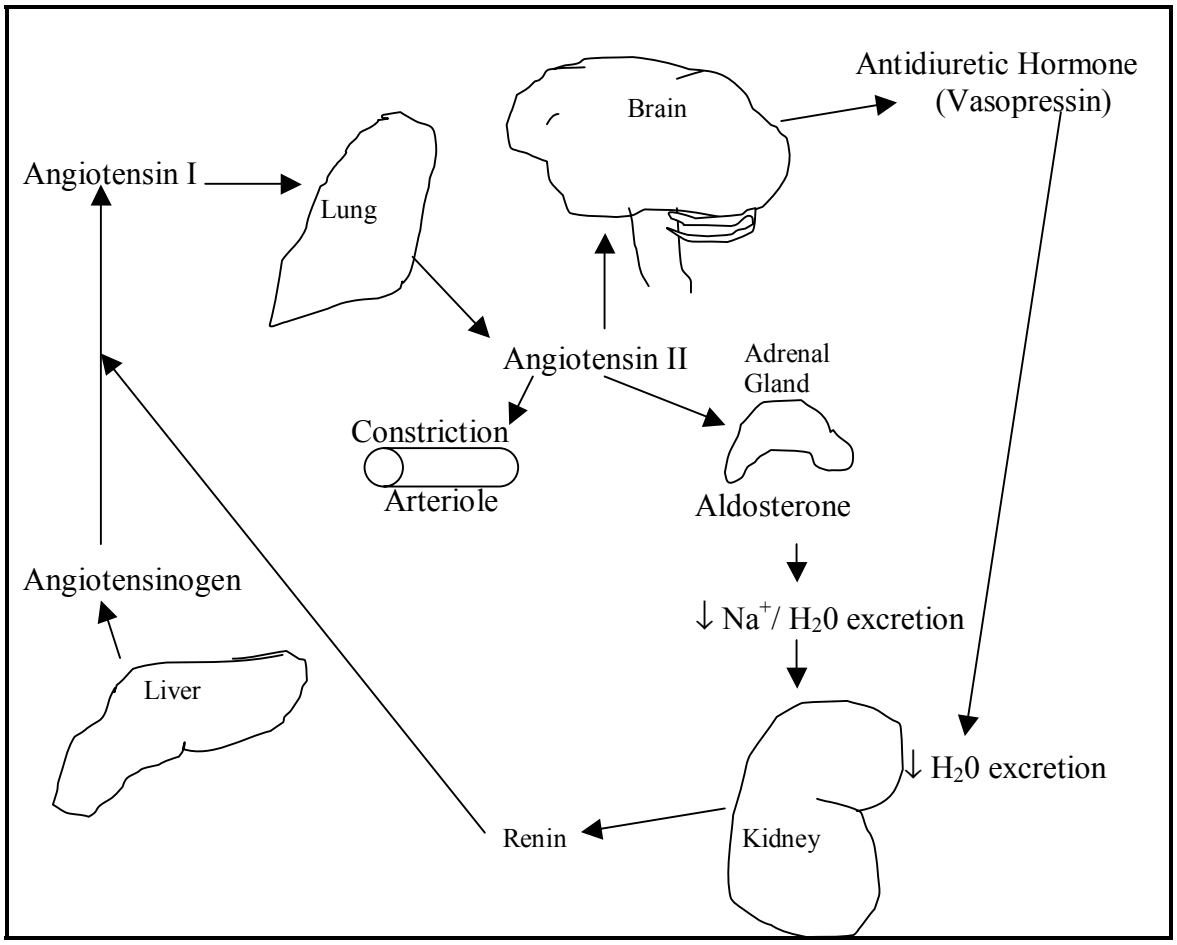


Figure 6. The renin-angiotensin-aldosterone system (Adapted from Berne, RM and Levy MN, eds. 1998)(17).

Thus, the left renal artery becomes ischemic, which in turn stimulates renin release from the juxtaglomerular renin-secreting granular cells of that kidney (64) (277). Renin in turn stimulates conversion of angiotensinogen to angiotensin I, which is converted to angiotensin II in the lungs (17). Through angiotensin II (ANG II), aldosterone and antidiuretic hormone (Figure 6), acute hypertension is induced in the rat in the upper half of the body, cranial to the aortic ligature (64) (220). See Figures 7 and 8. There is a peak in renin and mean arterial pressure at approximately 5 days post-aortic coarctation. Therefore, the renin-angiotensin-aldosterone system is responsible for the acute phase hypertension that occurs, while a later chronic phase develops through vascular remodeling and likely changes in baroreceptor set point. Once the chronic phase appears, the hypertension will persist indefinitely. By 30 days post-aortic coarctation, renin blood levels have returned to pre-coarctation levels, yet hypertension will persist cranial to the aortic ligature and normotension is present caudal to the ligature (64). The administration of captopril, an angiotensin I converting enzyme inhibitor, will significantly reduce the acute increase in blood pressure that follows after aortic coarctation (8; 141). No change in arterial blood pressure in the cranial half of the rat will result and no increase in blood renin levels will occur if total infarction or removal of the left kidney is performed (64; 113). This model, then, relies upon the renin angiotensin system for the induction of hypertension.

Evidence for the involvement of not only the renin angiotensin system but also the eicosanoids was first reported by EK Jackson et al in 1981 (113). Injections of indomethacin, an inhibitor of the cyclooxygenase enzyme and prostaglandin

biosynthesis, at six days post-aortic coarctation, decreased both mean arterial blood pressure and plasma renin activity in rats (113). Further, chronically-coarcted rats (4 weeks after coarctation) also exhibit a persistent vascular response to CP that interact with nitric oxide to maintain a homeostatic, although elevated systemic blood pressure. When the TXA_2/PGH_2 receptor is antagonized, there is a decrease in the pressor response to nitric oxide synthesis inhibition, indicating that at least part of the elevated blood pressure is maintained by CP (232). It is likely there is a change in the balance of vasodilators such as prostaglandin I_2 and nitric oxide and vasoconstrictors such as thromboxane A_2 and prostaglandin H_2.

The pathology of essential hypertension has been deemed polygenic and endothelial dysfunction has been suggested as a contributing factor (229). If substances produced by the vascular wall contribute to resetting of the arterial baroreceptors, then hypertension would remain after the renin-angiotensin-aldosterone system is no longer involved. Endothelial dysfunction could cause increases in peripheral vascular resistance (229) due to relative or absolute increases in vascular contracting factors, resetting of the arterial baroreceptors, remodeling of the vascular wall through stimulation by mitogens such as thromboxane A_2, and/or acute increases in renin release (229). Once vascular remodeling has occurred to the point that vascular resistance is increased, then hypertension would persist despite the return of renin and ANG II to baseline levels, and regardless of whether the arterial baroreceptors have reset or not.

The form of vascular remodeling that occurs in this model of hypertension has been investigated extensively in male rats. Thoracic aortic vascular smooth muscle cells

undergo hyperplasia with a 25-fold increase in DNA synthesis and a 25% increase in smooth muscle cell number. Inhibition of the ANG II type 1 receptor results in an attenuation of arterial medial thickening in coarcted rats and spontaneously hypertensive rats (188) (239; 296). The endothelium, on the other hand, has a 23-fold increase in cell turnover, but no change in absolute cell number (191). Although the endothelium does not undergo hyperplasia, there is a marked increase in cytoplasmic microfilaments seen in the endothelial cells that contain actin (69; 70); however, the function of this apparatus in the endothelium is unknown.

The characteristic vascular hypertrophy of the vascular smooth muscle that develops with this renin-angiotensin-dependent model is important because it decreases arterial compliance and increases impedance, which perpetuates the hypertension and eventually leads to heart failure due to pressure-overload left ventricular hypertrophy (144). In addition to vascular cell changes the aortic basement membrane loses chondroitin sulfate proteoglycans and gains fibronectin and laminin components (83; 152). These basement membrane changes occur in association with induction of the renin-angiotensin-aldosterone system and to a much greater extent than in nonrenin-angiotensin system-dependent models of hypertension (83). This indicates that the rat aortic coarctation model, which depends upon renal ischemia and induction of the renin-angiotensin system, represents a specific form of hypertension with its own vascular responses. Hypertensive male rats exhibit an increased vascular reactivity to ANG II via increased thromboxane A_2 release during the first two weeks following aortic coarctation (148) and an increase in total vascular resistance can be induced in normotensive male

rats by infusion of U-46619, a thromboxane/endoperoxide receptor agonist (73). It is likely that the renin-angiotensin system induces vascular remodeling directly via the actions of ANG II and/or through CP in aortic coarctation, which leads to maintenance of the hypertension after the renin and ANG II levels have declined to normal (Figs. 7 and 8). Although these findings are limited to the specific model of hypertension called aortic coarctation, they may be significant in some forms of hypertension where the renin-angiotensin system is activated. Elevated ANG II levels have been documented in essential hypertension in humans (107; 198; 258). Further, release of vasoconstrictor prostanoids, such as thromboxane A_2 by the vasculature of humans with essential hypertension reduce endothelium-dependent relaxations (230). Therefore, mechanisms of vascular remodeling similar to those that occur in aortic coarctation may also occur in essential hypertension. Thus, continued investigations using this model are warranted.

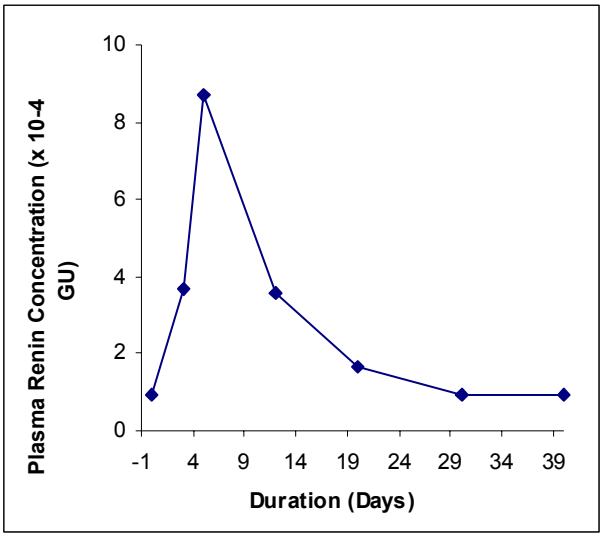


Figure 7: Plasma renin concentrations of the male rat following aortic coarctation (Adapted from Fernandes et al., 1976 (64)).

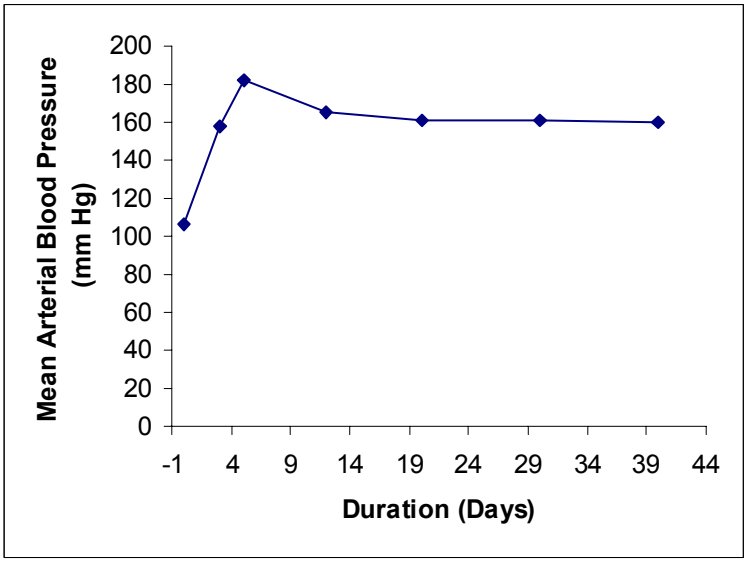


Figure 8: Mean arterial blood pressure of the male rat following aortic coarctation (Adapted from Fernandes et al., 1976 (64)).

The relationship between the renin-angiotensin system and CP in coarctation-induced hypertension has been investigated. Coarcted male rats exhibit dose-dependent increases in perfusion pressure response to ANG II that are 3-fold greater compared to normotensive controls. Moreover, U-46619 (TXA_2/endoperoxide receptor agonist) increases perfusion pressure to a similar extent in both coarcted-hypertensive and normotensive rats (47). Other studies of thromboxane A_2 receptor antagonists and thromboxane synthase inhibitors have reported decreases in arterial blood pressure in renin-angiotensin-dependent models of hypertension in response to these compounds (20; 31; 55; 148). Although these experiments have determined that CP such as thromboxane A_2 and prostaglandin H_2 play important roles in the elevation of the arterial blood pressure following aortic coarctation, inhibition of CP synthesis or CP receptor antagonism decreases but does not return blood pressure to pre-coarctation levels. Therefore, a second unknown vasoactive agent has been proposed (31). Boussairi et al. documented two responses to TXA_2/PGH_2 receptor inhibition in male Goldblatt hypertensive (two kidney-one clip) rats. In one group of male rats, there was no response to receptor blockade and in the other (termed "malignant hypertensive group") both blood pressure and mortality were decreased (20). This may indicate that two different responses to renal ischemia exist in these rats that may result in two very different forms of hypertension; or it may indicate that the degree of renal ischemia induced alters the vascular responses seen. The degree of ischemia induced may vary with the amount of ligation of the aorta, and this may explain the apparent discrepancies in blood pressure responses observed in coarctation-induced hypertension.

Inhibition of the prostanoid biosynthesis pathway enzymes cyclooxygenase or thromboxane synthase, and/or inhibition of the thromboxane A_2/endoperoxide receptor, reduces basal arterial tone by up to 75%, decreases renin production, and decreases mean arterial pressure in aortic coarcted rats (56; 124; 149; 273; 286). Various antagonists used in these studies included the cyclooxygenase inhibitors indomethacin (56) and SC 58,236 (273), the thromboxane A_2 synthase inhibitors: CGS 13,080 (149) and U63557A (124), and the thromboxane A_2/endoperoxide receptor antagonists: SQ 29,548 (149) and ifetroban (56; 286). Further, urinary excretion of thromboxane B_2 (thromboxane A_2 metabolite) is increased in rats with ANG II administration (153), and the rate of conversion of prostaglandin endoperoxide H_2 to prostaglandin I_2 (prostacyclin) in hypertensive rats is less than 50% that of normotensive rats (146). In people with essential hypertension, urinary 6-keto-prostaglandin $F_{2\alpha}$ (stable metabolite of prostaglandin I_2) excretion is decreased. Importantly, this excretion is significantly lower in normotensive and hypertensive females compared to their male counterparts (80). This indicates that in ANG II-dependent forms of hypertension, prostaglandin synthesis is shifted away from the vasodilator prostanoids such as prostaglandin I_2 and toward the CP such as thromboxane A_2. This may explain, at least in part, why mean arterial blood pressure remains elevated after renin and ANG II levels return to baseline.

Aortic coarctation in male rats not only induces vascular remodeling and altered ANG II sensitivity through enhanced CP synthesis, but also disrupts nitric oxide synthesis (14; 82; 95). ANG II stimulates the formation of reactive oxygen species (79; 206) which in turn inactivates the nitric oxide pathway (14; 82). Although endothelial

nitric oxide synthase and neuronal nitric oxide synthase are upregulated in aortic coarctation and the pressor response to nitric oxide synthase inhibition with NG-nitro-L-arginine methyl ester is enhanced in rats after aortic coarctation (13; 14; 200), the contribution of nitric oxide to arterial blood pressure in this model of hypertension is significantly decreased (due to ANG II-stimulated reactive oxygen species). The impairment of nitric oxide-dependent vasodilation mechanisms is particularly relevant in these investigations, because 17β-estradiol induces rapid release of nitric oxide and activation of guanyl cyclase in endothelial cells (21; 93; 162; 202; 215; 216). 17β-estradiol increases endothelial nitric oxide synthase expression as well (125; 162; 216; 217; 275). If the actions of nitric oxide are impaired by ANG II-stimulated reactive oxygen species, then this model may be more appropriate to the study of hypertension in females.

In the aortic coarctation model of hypertension, the CP may also play an important role in the regulation of the renin-angiotensin system. This control could then influence ANG II-induced production of oxygen free radicals and the ability of nitric oxide to inhibit vasoconstriction. The aortic coarctation model is therefore useful in the investigation of hypertension at the vascular level in the female (a vascular system with enhanced ability to produce nitric oxide) and the role that CP play in the development of hypertension in females. Use of a renin-angiotensin system-dependent model of hypertension in females is also appropriate because estrogen elevates gene expression and blood levels of angiotensinogen (92; 214).

Previous use of the aortic coarctation model of hypertension in female rats has been quite limited. An investigation of the effects of estrogen 45 minutes after partial acute aortic coarctation revealed that estrogen increased the sensitivity of ovariectomized rat arteries to ANG II stimulation (220), but the effects of chronic coarctation were not determined. Henzel and Alsip investigated the effects of partial aortic coarctation in pregnant and virgin female rats for 10 to 11 days. In their study, significant increases in mean arterial blood pressure occurred in pregnant rats but not in coarcted virgin rats. This discrepancy is likely due to: 1) partial aortic occlusion rather than complete ligation; and 2) study of the animals at 10 to 11 days did not allow sufficient time for peak arterial blood pressure to develop.

The aortic coarctation model in exploration of the effects of estrogen on the vasculature is appropriate since it is dependent on the renin-angiotensin-aldosterone system and estrogen clearly has the ability to regulate ANG II in females.

1.8 Statement of Objectives and Investigation Rationale

1.8.1 Objectives

As discussed in Section 1.4, the renin-angiotensin system and CP (PGH_2/TXA_2) have been implicated in aortic coarctation-induced hypertension in the rat and primary pulmonary and essential hypertension (8; 38; 107; 113; 141; 147; 230; 286). However, in the male rat, there is still debate about the mechanism of induction of hypertension and a second unknown vasoactive agent has been proposed (31). In the female rat, little is known about the roles of any of the vasoactive agents such as ANG II and TXA_2 in

hypertension. Given the greater activity of the TXA_2 pathway in the normotensive female rat (67), it seems likely that vascular reactivity to ANG II in aortic coarctation-induced hypertension would be potentiated to a much greater extent in the female than the male. However, sex differences in the vasculature of this hypertension model have not been studies previously. Therefore, the objectives of the proposed project are: **1**) to determine male and female differences in the role of TXA_2/PGH_2 in the acute and long-term regulation of vascular tone during development of aortic coarctation-induced hypertension in the rat, and **2**) to elucidate the role of ovarian steroid hormones in the regulation of TXA_2-pathway function during the development of aortic coarctation-induced hypertension in the female rat. Thus, the central hypothesis to be tested is: Ovarian steroid hormones upregulate CP function in the female rat aorta during the development of aortic coarctation-induced hypertension, thereby altering the functional and structural responses of the aorta to a greater extent in female than in male rats. If so, then CP-mediated changes in vascular reactivity, vascular structure, and systemic blood pressure during the development of aortic coarctation-induced hypertension would play a larger role in female than in male rats.

1.8.2 Rationale

Studies of the pulmonary vasculature have established that constrictor metabolites of arachidonic acid play a greater role in the regulation of pulmonary vascular tone in females than in males, and that estrogen is an important regulator of this mechanism. Further, disturbances in this mechanism may be responsible for the

greater incidence of primary pulmonary hypertension in women than in men (48; 197; 272; 282). Recent studies by Stallone et al. suggest that significant male-female differences exist in CP-mediated regulation of systemic (peripheral) vascular tone and that this mechanism appears to be upregulated by the ovarian steroid hormones (67; 67); however, little else is known about male-female differences in constrictor metabolites of arachidonic acid in the systemic vasculature. Very little is also known about the role that these substances play in normal vascular regulation or in peripheral vascular diseases such as Raynaud's disease or hypertension in women. TXA_2 is also known to be a potent mitogen and in this way may also contribute to vascular wall remodeling during the development of HT (159; 192; 196; 222). Thus, the goals of this research are to determine male-female differences in the role of CP (TXA_2) in the regulation of vascular tone and vascular remodeling in a model of hypertension known to involve enhanced release and actions of TXA_2, aortic coarctation-induced hypertension; and to determine the role that ovarian steroid hormones (estrogen) play in the regulation of this mechanism in normotension and hypertension.

CHAPTER II

MATERIALS AND METHODS

2.1 Animal Preparation

Male (M) and female (F) Sprague-Dawley rats were obtained from Harlan, Inc (Houston, TX) at 4 weeks of age (females) and 11 weeks of age (males). The rats were housed in pairs in standard plastic laboratory rat cages and were segregated by sex at the Texas A&M University Laboratory Animal Research and Resources (LARR) facilities. Animals were fed a soy- and alfalfa-free diet (Global diet, formulation 2016, Harlan Teklan Inc., Houston, TX) and tap water was provided ad libitum. The temperature (21-26°C) and photoperiod (12 hours dark: 12 hours light) were controlled. At 5 weeks of age, one group of F rats were ovariectomized bilaterally (OVX) under general anesthesia. General anesthesia consisted of preanesthetic with atropine sulphate (0.05 mg/kg, subcutaneously) and anesthesia with ketamine HCl (50 mg/kg) and chloral hydrate (150 mg/kg) administered intraperitoneally. At 8 to 12 weeks of age, some of the OVX rats received two time-release pellets placed subcutaneously over the right paralumbar area, each containing 0.05 mg 17β-estradiol or a placebo (Innovative Research, Sarasota, FL). Previous trial studies by the manufacturer and our laboratory have shown that this dose produces physiological plasma levels of estrogen in the rat (57; 58). After 2-4 weeks of estrogen replacement (14-16 weeks of age), estradiol and placebo (OVX) rats underwent aortic coarctation surgery.

To ensure adequate supplementation of estrogen in the ovariectomized estrogen-replaced rats (OE), after euthanasia, plasma from the OE and OVX rats was collected and stored at -80°C until analysis of estrogen levels. Estrogen levels were determined via a double antibody RIA for 17β-estradiol (Diagnostic Products Corp., Los Angeles, CA), which was validated previously on unextracted rat plasma. At the time of experimentation, the rats in all experimental groups (M, F, OVX and OE) were age matched at 12-16 weeks old. Because the reactivity of the F rat aorta does not vary with respect to the phase of estrous cycle when exposed to vasoconstrictors such as vasopressin and phenylephrine, intact F rats were studied without regard to phase of the estrous cycle (246; 247). All surgical and experimental procedures used in these studies were reviewed and approved by the Texas A&M University Laboratory Animal Care and Use Committee (ULACC).

2.2 Induction of Aortic Coarctation-Induced Hypertension

Experimental groups consisted M, F, ovariectomized female (OVX), and ovariectomized female supplemented with estrogen (OE) and each group was divided into either aortic coarctation-induced hypertension (HT) or sham-operated normotensive-control (NT). Age-matched rats from all experimental groups were anesthetized using a preanesthetic of atropine sulfate (0.05 mg/kg, subcutaneous injection) and an anesthetic combination of ketamine hydrochloride (50 mg/kg) and chloral hydrate (150 mg/kg), administered by intraperitoneal injection. After stable anesthesia was achieved, the abdominal cavity was exposed and a silk ligature (4-0)

placed around the aorta between the origins of the left and right renal arteries, caudal to the superior mesenteric artery. The aorta was ligated completely to produce coarctation-induced hypertension. Control rats were sham operated: a ventral midline incision was made and the aorta visualized and isolated, but not ligated (64; 148). Following aortic coarctation, a chronic arterial catheter (polyrenethane, Braintree Scientific Inc, Braintree, MA) was placed in the right carotid artery and tunneled subcutaneously to exit at the dorsal neck to monitor arterial blood pressure in some groups of rats. Catheters were flushed every other day with heparinized saline and closed with metal stylets. After flushing, a heparin lock was placed in the catheter lumen by administering 0.02-0.03 ml of 50% dextrose with 200 Units per ml Heparin. The heparin lock was removed prior to flushing the catheter the next day.

For the experiments involving SQ 29,548, a right jugular vein catheter was placed at the same time as and in a similar manner to the carotid arterial catheter and tunneled subcutaneously to exit at the same location as the carotid artery catheter on the dorsal aspect of the rat's neck. The jugular catheter was flushed and a heparin lock placed in the catheter when not in use as described above.

2.3 Monitoring of Arterial Blood Pressure

Prior to aortic coarctation, M, F and OVX rats were acclimated for 1-2 weeks to gentle restraint in a specially designed plastic cage that allowed the rats to freely move forwards and backwards but prevented them from turning around or climbing. The rats were acclimated in the room in which blood pressure experiments were to occur for one

hour each day. Starting on the second day after aortic coarctation surgery, rats were placed in the restraint cage. The heparin lock was removed from the catheter. It was flushed with heparinized saline (5 Units heparin sulfate per 1 ml 0.09% normal saline), connected to PE-50 tubing (0.58 mm polyethylene tubing, Becton Dickinson and Co., Parippany, NJ) that was filled with heparinized saline, and connected to a pressure transducer (Model DTX, Becton Dickinson and Co., Parsippany, NJ). The pressure transducer readings were recorded to a chart recorder (Gould 2600S) for a continuous record of blood pressure. Before recording blood pressure, each rat was allowed to acclimate to the restraint cage for a period of 5 to 20 minutes. Arterial blood pressure (MAP) and heart rate (HR) were recorded at approximately the same time every other day for 14 days. After recording MAP and HR each catheter was flushed with heparinized saline and a heparin lock placed in the catheter to maintain patency. The pressure transducer and chart recorder were calibrated using a manometer at least twice a week.

2.4 Acute Inhibition of Thromboxane/Endoperoxide Receptor *in vivo*

Acute inhibition of the TXA_2/PGH_2 receptor was accomplished by intravenous infusion of the receptor inhibitor SQ 28,548 in fully conscious adult rats. HT and NT M, F, OVX and OE rats were implanted with carotid artery and jugular vein catheters. At 14 days post aortic coarctation or sham surgery, the rats were placed in the blood pressure monitoring restraint cages and their arterial blood pressures and heart rates recorded after an acclimation time in the cage of 10 to 20 minutes. The jugular vein catheter of

each rat was attached to PE-50 tubing and a syringe pump. Following arterial blood pressure recording the SQ 29,548 vehicle (0.9% saline with 14 mM Na_2C0_3) was infused into the jugular vein for 20 minutes at a rate of 1 ml/hour. During infusion of the vehicle and subsequent infusion of SQ 29,548, arterial blood pressure and heart rate were recorded (Gould 2600S). Following vehicle infusion, each rat was given a bolus of 2 mg/kg SQ 29,548 IV, followed immediately by a continuous intravenous infusion of SQ 29,548 at 2 mg/kg/hour. The arterial catheter was flushed with heparinized saline as needed to keep the line patent during the continuous rate infusions. At the end of the SQ 29,548 bolus and every 10 minutes during the vehicle and SQ 29,548 infusions mean arterial blood pressure and heart rate were recorded (5 replicates at each time point). The average mean arterial blood pressure and heart rate recorded for each time point.

2.5 Inhibition of Thromboxane Synthase and the Thromboxane/Endoperoxide Receptor during the Early Phase of Aortic Coarctation-Induced Hypertension

In order to determine the effects of CP on the development of aortic coarctation-induced hypertension, rats received the TXA_2/PGH_2 receptor inhibitor and TXA_2 synthase antagonist. Four to 5 animals from each treatment group received oral Ridogrel (12.5 mg/kg) twice daily for the first 14 days post-aortic coarctation or sham surgery. During the initial aortic coarctation or sham surgery, a right carotid artery catheter was implanted and tunneled subcutaneously to exit at the dorsal neck of each rat. Mean arterial blood pressure and heart rate were recorded every other day from the rats as described above. Each rat had been acclimated to the pressure reading cage prior

to the study and pressures were recorded until a stable pressure was reached (5 to 20 minutes). Data over the 14-day period were analyzed and compared to the initial mean arterial blood pressures and heart rates obtained from rats that did not receive Ridogrel.

2.6 Release of Prostanoids from Aortic Rings *in vitro*

M-HT, F-HT and M-NT M, F-NT rats were prepared without catheters to determine vascular prostanoid release from the thoracic aorta. Fourteen days after coarctation or sham surgery, the rats were sacrificed by rapid decapitation and the thoracic aortae removed rapidly and placed in chilled (4ºC) Krebs' Henseleit bicarbonate (KHB) solution (concentration in mM: 118.0 NaCl, 25.0 $NaHCO_3$, 10.0 glucose, 4.74 KCl, 2.50 $CaCl_2$, 1.18 $MgSO_4$, and 1.18 KH_2PO_4, pH = 7.40, osmolality = 292 ± 1.0 mosmol/kg H_2O), gassed with 95% O_2/5% CO_2. The aortae were cleaned of all adipose and connective tissue, with care being taken to avoid damage to the endothelium. The vessels were then cut into 3-mm length rings and placed in chilled, gassed KHB for 30 minutes. The rings were then transferred to 12x75 mm plastic culture tubes containing 2 ml of fresh KHB, gradually warmed to 37°C, and then preincubated for 30 minutes while continually gassed with 95% O_2/5% CO_2. The KHB was aspirated from each tube and 1.0 ml of fresh KHB or KHB with 1 x 10^{-6} M ANG II was added to the tubes and incubated at 37°C for 45 minutes while gassed as above. Following incubation, the KHB was removed from the tubes, aliquoted, and frozen at -80°C until specific radioimmunoassays (RIA) for TXB_2, the stable metabolite for TXA_2, and $PGF_{1\alpha}$, the stable metabolite of PGI_2, were performed, using antisera

developed by Leffler and Busija (142) and RIA methods described by Stallone and Weis (246; 276).

2.7 Release of Prostanoids from Aortae *in vitro* under Constant Pulsatile Pressure and Flow Conditions with and without Angiotensin II Stimulation

Rats from the experimental groups: F-HT, F-NT, OVX-HT, OVX-NT, OE-HT, OE-NT, were decapitated 10-14 days after sham or aortic ligation surgery. The thoracic aortae were removed rapidly and placed in chilled Krebs' Henseleit bicarbonate (KHB) solution (at 4ºC), gassed with 95% O_2/5% CO_2. Prior to removal, a 2 to 3 cm segment of aorta was measured at the ends where the cuts were to be made, and the *in-situ* length was recorded. The aorta was then excised and cleaned of all adipose and connective tissue, with care being taken to avoid damage to the endothelium. The vessel segments were also prepared by ligation of the intercostal arteries with 6-0 silk. The segments were mounted on glass vessel cannulae, on a custom computer-controlled apparatus used to evaluate the aorta biomechanically (Figure 9). The perfusate was recirculated through the vessel using a peristaltic pump, with waveforms occurring at approximately 250 per second. The flow rate was maintained at approximately 10 ml/min or the flow rate that yielded a wall shear stress of 15 dyn/cm^2. An adjustable clamp was placed over the tubing located between the reservoir and the vessel so that downstream resistance could be adjusted in order to change the perfusate pressure at the vessel.

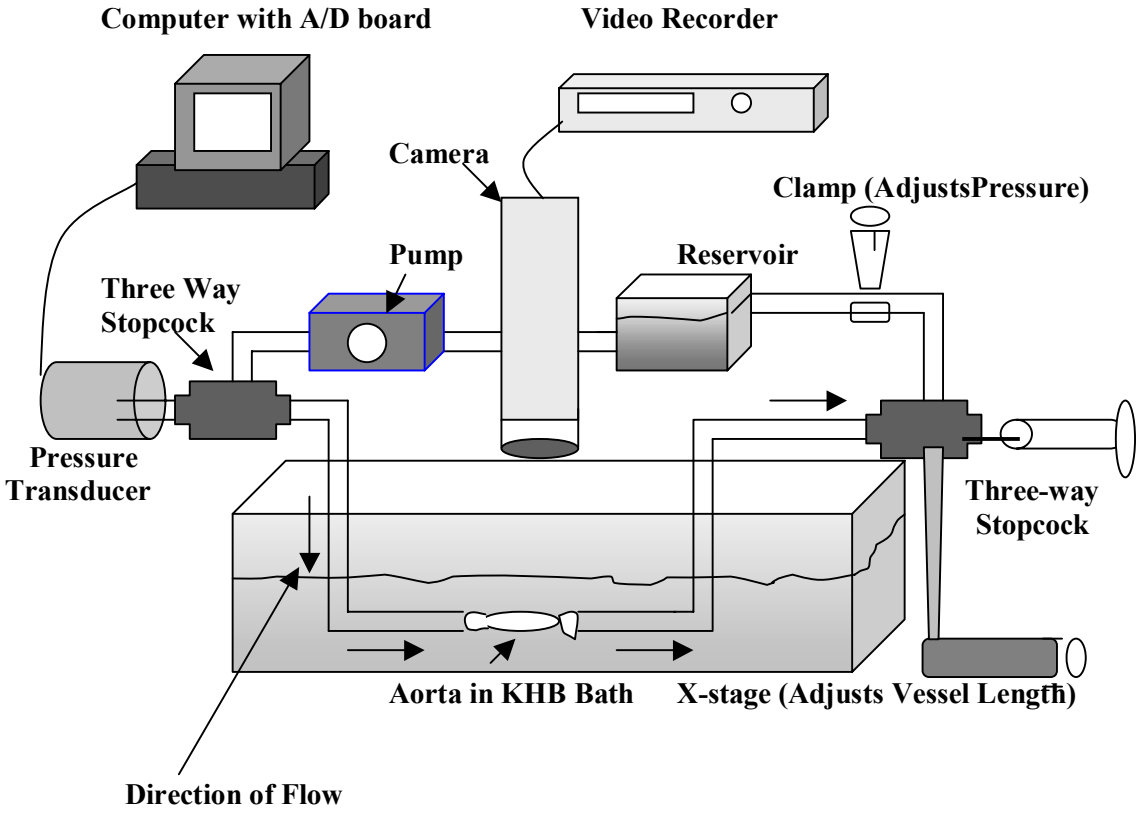


Figure 9: Schema of the test apparatus. Aortic segments are mounted at the in-situ length, using two opposing cannulae, one of which is connected to a platform that rides on an x-stage. The specimen is perfused with warmed/oxygenated KHB at a constant rate which yields a mean wall shear stress of 15 dyn/cm^2 and mean pressure (100 mm Hg or 150 mm Hg), using a digitally controlled pump. Axial force f, luminal pressure P, and outer diameter b (via a video data acquisition system) are measured on-line using a 12 bit analog digital converter and computer. The mean wall shear stress $\tau = 4\mu Q/\pi a^3$, where μ is the viscosity, Q the volumetric flow rate and a the inner diameter. Mean radial, circumferential, and axial stresses are $\sigma = -Pa / (a + b)$, $\sigma_\theta = Pa / (b - a)$, $\sigma_z = F / \pi (b^2 - a^2)$. Inner and outer diameter are related by $b^2 - B^2 = \pi (a^2 - A^2) / \lambda\theta_o$ where A and B are stress-free values, λ is the *in-situ* stretch and θ_o is the opening angle (118).

The connector of one end of the cannulation apparatus was on a movable slide to adjust the overall length of the aorta to the *in-situ* value recorded at the time of removal from the rat. The length was adjusted and set to the *in situ* vessel length. To maintain a seal to pressurize the vessel, the ends of the vessel were secured to the cannula by ligatures tied over the ends with 6-0 silk suture. The other end connector was attached to a 3-way stopcock with one port attached to a pressure transducer to measure the perfusion pressure attained as the KHB solution flowed through the aortic segment (set to 100 or 150 mm Hg).

A video camera mounted on a stage above the cannulated vessel was used to record changes in axial and circumferential strains in response to pressurization of the aortic segment and agonist challenge (Figure 9). Changes in diameter in response to ANG II were recorded. Single frames were captured from the videos using Adobe Premiere 5.1 (Adobe Systems Inc, San Jose, CA). For each experiment a micrometer was also recorded and later used to calibrate the computer program Scicon Image β 3b (Scion Corp., Frederick, MD) to pixels per mm.

The approximately 6.8 mls of KHB that continuously recirculated through the vessel segment was collected (after 30 minutes per condition) from the reservoir located before the pump but after the clamp used to adjust resistance and pressure to flow. Basal release of TXA_2 and PGI_2 were determined by perfusion of the cannulated vessel segment at 100 mm Hg. The aortic segment was bathed in a warm KHB solution (37°) that was continually gassed with 95% O_2 and 5% CO_2. The KHB used to perfuse the aortic segment was similarly warmed and gassed as well. The aortic segment was

allowed to stabilize for 30 minutes at a perfusion pressure of approximately 60 mm Hg. During constant flow conditions (approximately 10 ml/min), the aortic segment was pressurized at 100 mm Hg and the perfusate recycled through the system for 30 minutes, while 300 µl of perfusate was collected from the reservoir at 1, 3, 10, and 30 minutes. The perfusate was placed on ice until storage at –80 °C. The collected solutions were analyzed for prostacyclin (PGI_2) and TXA_2 by RIA of their stable metabolites, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and TXB_2, respectively. Immediately after perfusion at 100 mm Hg, the aorta was perfused with fresh KHB for an additional 30 minutes at a pressure of 150-mm Hg and the perfusate collected for TXA_2/ PGI_2 analysis at 1, 3, 10, and 30 minutes. The aortic segment was allowed to equilibrate at 100 and 150 mm Hg before the perfusate was collected. PGI_2 and TXA_2 release was reported as picograms per mg dry weight of aortic segment. The pressures of 100 and 150 mm Hg were chosen to mimic MAP's found in normotension (100 mm Hg) and hypertension (150 mm Hg).

After the pressure tests at 100 and 150 mm Hg, the aortae were maintained at constant flow (approximately 10 ml/min) and pressure (150 mm Hg) conditions and then challenged with a high dose of the α_1-adrenergic agonist phenylephrine (PE, 25.0 nmol) to stabilize the vasoconstrictor and vasodilator mechanisms. The bolus of PE was administered into the 6.8 ml reservoir. A 15-minute recovery period was allowed between injections of PE and all subsequent vasoconstrictors, during which the aorta was perfused at a pressure of 150 mm Hg. After the first PE challenge, a second dose of PE

(25.0 nmol) was administered exactly as the first, followed by another 15-minute recovery period.

After the perfusate was changed to fresh KHB, the aortic segment was perfused with KHB at 150 mm Hg and 3 doses of ANG II (0.005, 0.02 and 0.1 nmol) were administered (15 minutes recovery between doses). At the end of each 15 minute time period, and before administration of the next dose of ANG II, 0.4 mls of perfusate was removed and placed on ice until frozen at -80°C. The perfusate was later analyzed for TXB_2 and $PGF_{1\alpha}$ content by RIA. Previous studies have demonstrated that prostanoid output peaks within 2 minutes after administration of vasoconstrictor agonists, and that administration of PE and ANG II vehicles have no effect on perfusion pressure or prostanoid output (167; 246). The perfusate samples collected did not change the pressure or shear rate present in the aortic segment, since sample collection resulted in a decrease in the reservoir volume only. When the calculations were made for TXA_2 and PGI_2 concentrations, the decreases in recirculating volume were taken into account in the concentration calculations. Dose-response relationships to determine the change in diameter of the vessel with each dose of ANG II was then established from the data.

The perfusate was replaced after each 30-minute perfusion period, including the stabilization at 60 mm Hg, the 100 mm Hg test, the 150 mm Hg test and after each PE challenge, but not between doses of ANG II. The perfusate was changed by filling a syringe connected to the perfusion circuit with fresh KHB, clamping first the tubing leading to the vessel and detaching the pump, then flushing through the line fresh KHB through to the reservoir. Then the tubing towards the reservoir was clamped and the line

and vessel flushed with fresh KHB through to the reservoir with care taken to avoid exceeding a pressure of 80 mm Hg at the vessel. Changing the perfusate took less than 4 minutes each time.

After the experiment, the aorta was carefully removed from the cannulae, the sutures removed, and the vessel placed in a 12x75 mm plastic culture tube. The aorta was allowed to dry several days at room temperature, then dried at 85°F for 3 hours. After drying, the aorta segments were placed in a room temperature dessicator overnight. The following day, the weight of each dried aorta was recorded to the nearest 0.001 mg. TXB_2 and $PGF_{1\alpha}$ release were calculated as pg/mg aorta dry weight/30 minutes for the 100 mm Hg and 150 mm Hg pressure tests and as pg/mg aorta dry weight/15 minutes for the ANG II challenge tests.

2.8 Radioimmunoassay of Perfusate Prostanoids

The radioimmunoassays for TXB_2 and $PGF_{1\alpha}$ used antisera developed by Leffler and Busija (142). An equilibrium RIA was performed by incubating prostanoid standards (1.95-1,000 pg) or unextracted perfusate samples with [^{3}H] TXB_2 or [^{3}H] 6-keto-$PGF_{1\alpha}$ and with the corresponding antiserum overnight at 4°C. Bound and free fractions of [^{3}H] TXB_2 or [^{3}H] $PGF_{1\alpha}$ were separated by the charcoal-dextran method. Bound radioactivity was determined by liquid scintillation spectroscopy. Cross-reactivity of the 6-keto-$PGF_{1\alpha}$ is <0.1% with the TXB_2 antibody. The limit of detection for these RIAs was 1.95 pg/tube and the intra-assay and interassay coefficients of variation were 5.0 and 7.6% for TXB_2 and $PGF_{1\alpha}$, respectively (142; 246; 276).

2.9 Immunohistochemistry of the Aortic Thromboxane A_2 Synthase Enzyme

Thoracic aortae from all 8 treatment groups (M-HT, M-NT, F-HT, F-NT, OVX-HT, OVX-NT, OE-HT, OE-NT) were subjected to immunohistochemical analysis to detect changes in vascular wall thromboxane synthase enzyme content among the sexes and experimental groups. Four μm thick sections of aorta that had previously been embedded in paraffin (see Section 2.7) were rehydrated in ethanol and deionized water. The sections were rinsed in PBS buffer (120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer), PH 7.4 at 25°C. Antigen retrieval was achieved by microwaving the tissue for a total of 13 minutes in citrate buffer (5.0 mM, pH 6.0). The sections were allowed to cool in the citrate buffer for 20-30 minutes. They were incubated with normal equine serum (RTU Vectastain Kit-Universal Elite ABC kit, Vector Laboratories, Burlingame, CA). After 30 minutes of equine serum incubation, the sections were incubated for 15 minutes each with avidin then biotin (Avidin/Biotin Blocking Kit, Vector Laboratories). The primary antibody (monoclonal mouse TXA_2 synthase antibody, diluted 1:500 with common antibody diluent, BioGenex, San Ramon, CA) was incubated on the tissues overnight. The secondary antibody (universal biotinylated equine anti-rabbit and mouse IgG antibody, Vector Laboratories) was incubated on the sections for 30 minutes the next morning. The sections were counter stained with hematoxylin (Gill No. 3, Sigma Diagnostics, St. Louis, MO). Intensity of antibody staining was determined by a blinded investigator in our laboratory. Three sections of aorta per animal were scored on a scale of 0 to 4; with sections that had no staining receiving a score of 0 and those with most intense

staining receiving a score of 4. Each animal had three consecutive sections analyzed and each section was scored independently 3 different times by the blinded investigator. The average of the scores was determined for each section, then each animal, and finally for each group, to determine intensity of staining. The results were reported as the mean score ± standard error.

2.10 RT-PCR of Aortic Thromboxane A_2 Synthase Enzyme

2.10.1 Isolation of Total RNA from Aorta

Rats from all 8 experimental groups (M-HT, M-NT, F-HT, F-NT, OVX-HT, OVX-NT, OE-HT, OE-NT) underwent aortic coarctation or sham surgery 14 days prior to sacrifice by decapitation. After the thoracic aorta was removed from each animal and cleaned of all adipose and connective tissue, the wall was incised longitudinally. The endothelium was then removed from the underlying internal elastic lamina and smooth muscle with a cotton swab. Endothelial cells were placed in a 1.5 ml RNAse-free Eppendorf tube containing 1 ml Trizol (Invitrogen Corp., Carlsbad, CA). The smooth muscle was placed in a second tube also containing 1 ml Trizol. Both samples were kept on ice until homogenized. After the samples were homogenized (1 minute for endothelium, 5 minutes for smooth muscle, Polytron Model MR 2100, Kinematica Ab, Switzerland), they were incubated at room temperature for 5 minutes. Two hundred µl of chloroform (Fisher Scientific, Fair Lawn NJ) was added to each tube of homogenized tissue, shaken vigorously by hand for 15 seconds, and then incubated at room temperature for 3 minutes. After the samples were centrifuged at 10,000g for 15 minutes

at 4°C, the aqueous RNA-containing top layer was removed (400-500 µl) and transferred to a 500 µl Eppendorf tube. Glycogen (5 µl, 25 µg/ml) and sodium acetate (50 µl of 5 M) were added to the RNA solution. After vortexing the tube, 400-500 µl of isopropyl alcohol was added. These samples were then stored at -20°C overnight.

The following day, the samples were centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was removed without disturbing the pellet and 1 ml of 75% ethanol was added to the pellet, then vortexed. The samples were centrifuged again at 8,000g for 10 minutes at 4°C. The supernatant was removed, being careful not to disturb the pellet at the bottom of the tube. The tube was allowed to air dry for 1–2 minutes at room temperature. DEPC (diethyl pyrocarbonate) water (12-17 µl) was added to the pellet and mixed with a pipette tip. The RNA concentration was determined at 260 and 280 nm with 2 µl of RNA solution diluted with 98 µl of deionized water, using a universal microplate spectrophotometer (µQuant model, Bio-Tek Instruments, Inc, Winooski, VT). The remainder of the sample was stored at –80 °C until further use.

2.10.2 Reverse Transcriptase Reaction

To 1 µl of RNA in a 1.5 ml RNAse-free Eppendorf tube, 9 µl of DEPC water, and 1 µl of the 3'oligonucleotide primer for thromboxane synthase were added. The 3' oligonucleotide sequence for thromboxane synthase was: 5' GAA GCA TCA CAA ACA TTT ATTC and for 5' sequence was 5'-TCC ACA GGT GTT ACT GCT GT. Constituitively expressed glyceraldehydes phospho-dehydrogenase (GAPDH) was used

as an exogenous control to correct for potential variation in cDNA synthesis, RNA loading or amplification reactions (Doroudi 2000). The GAPDH primers (Sigma-Genosys Inc, The Woodlands, TX) used were 3': 5'-CAC AGT CTT CTG AGT GGC AGT GAT-3'; and 5': 5'-GTG AAG GTC GTG TCA ACG GAT TT-3'. The samples were heated to 75-80°C for 5 minutes. After cooling, the following reagents were added on ice: 4 µl 5x TBE buffer (50% diluted 10x tris boric acid buffer: 27 g trishydroxymethylaminomethane base, 13.75 g boric acid, 2.25 g ethylenediaminetetraacetic acid dihydrate (EDTA)), 2µl 10 mM dNTP, 1 µl DTT (0.1 M), 1 µl RNAse Out, 1 µl Thermoscript RT (Invitrogen Corp); then incubated at 60°C for 60 minutes. The reverse transcriptase reaction was terminated by heating the sample to 85°C for 5 minutes. The RNA was removed with the addition of 1.0 µl RNAse (Invitrogen Corp).

2.10.3 Polymerase Chain Reaction

The cDNA sample (2 µl) was used for each reaction (thromboxane synthase and GAPDH). The following reagents were added: 35 µl water, 5 µl 10x PCR buffer without MgCl2, 2 µl of 10 mM dNTP, 3 µl MgCl2, 1 µl Taq (Expand high fidelity system, Roche Diagnostics Corp, Indianapolis, IN), 1 µl 5' oligonucleotide and 1 µl 3' oligonucleotide thromboxane synthase (diluted stock 10x). PCR cycles were performed on the above solution with an initial denaturation period of 3-4 minutes at 94°C and a final extension of 7 minutes. Each step during the cycles was 30 minutes and the tubes were not added to the thermocycler (Thermocycler T3, Biometra, Gottinger, Germany)

until the temperature in the cycler had reached 90°C. The housekeeping gene GAPDH was cycled at 94, 60, and 72°C for 33 cycles and thromboxane synthase cycled at 94, 54, 72 °C for 30 cycles.

2.10.4 Gel Electrophoresis

The 1.8% agarose gel was placed in a container and submerged in ethidium bromide for staining. One µl of stain was added to 3 µl of each amplified cDNA sample and pippetted into each well. The first well on the left was always filled with 3 µl of DNA ladder (X174 RF DNA/Hae III hexamer fragments, Invitrogen Inc). An electrical gradient across the gel was created at 100 mVolts. The gel was allowed to run for 20 to 30 minutes, then the gel was removed and scanned. Relative density of staining was determined with Quantity One SWPC Imaging Analysis software, Bio Rad Laboratories, Hercules, CA.

2.11 Aorta Morphology

Thoracic aortae from all 8 treatment groups (M-HT, M-NT, F-HT, F-NT, OVX-HT, OVX-NT, OE-HT, OE-NT) were analyzed histologically for morphologic differences between sexes and changes in the vascular wall with hypertension. Rings of aorta (1-2 mm) were obtained from the rats immediately after decapitation and placed in chilled (4°C) KHB. The aortae were cleaned of all adipose and connective tissue, with care being taken to avoid damage to the endothelium. One end of the aortic segment was cannulated with a 23-gauge needle cannula and the end tied to the

needle with 4-0 silk. The vessels were perfused with 10% neutral buffered formalin using a syringe and silicon tubing. Once the vessel was free of air and contained only formalin, the end of the vessel opposite to the cannulated end was ligated with 4-0 silk suture. The needle cannula with the aorta segment and attached tubing was placed in a beaker of formalin with the vessel completely submerged. The tubing was connected to a 60 ml syringe and hung 43 cm above the aorta in the beaker. The aorta was immersed in formalin and the syringe was filled with 40 mls of formalin and the syringe plunger was removed. The formalin in the syringe was allowed to flow through the aorta and its intercostal arteries overnight in order to fix the vessel in an expanded position equal to approximately 80 mm Hg pressure. After fixation, the aorta was removed from the cannula and placed in 70% ethanol until embedded in paraffin. Four-µm thick sections of aorta were placed on glass slides and stained with hematoxylin and eosin. The stained sections were photographed and with an image analyzer and National Institutes of Health image analysis software the morphology of the aorta sections was determined. The area between the internal and external elastic laminae was defined as the media. The media cross-sectional area was determined at 20 X and wall thickness, wall area and wall to lumen ratio were determined as well (188) (142; 159). Results were reported as mm/g body weight (wall thickness), mm^2/g body weight (media area, wall area), and lumen to wall area ratio.

2.12 Collagen and Elastin Density

An adjacent section of aorta to those obtained for aortic morphology was also stained with Movat's solution to determine collagen and elastin density. Densities were measured with a computerized morphometric system, the Optimus at 2.5 x 1.6X and the percentage of the total tissue area occupied by collagen or elastin per cross-section calculated (25). Results were reported as the percentage of elastin or collagen per 4 μm thick tissue section and the collagen: elastin ratio was calculated from these data.

2.13 Chemicals and Reagents

Phenylephrine hydrochloride (Sigma Chemical, St. Louis, MO) was prepared fresh daily in KHB solution. ANG II (BACHEM-California, Torrence, CA) was prepared daily from stock solutions (10^{-3} M) maintained at -80°C and diluted with KHB solution. PE and ANG II solutions were kept on ice during the experiments. Ridogrel was a gift of Janssen Research Foundation (Beerse, Belgium). SQ 29,548 was a gift of Bristol Myers Squibb Co. (Princeton, NJ) and prepared daily (0.5 mg/ml in 0.9% saline with 14 mM Na_2C0_3).

The immunohistochemistry reagents were purchased from Vector Laboratories, Inc. (Burlingame, CA). The TXA_2 synthase monoclonal antibody was a gift of Dr. William B. Campbell at the University of Wisconsin, Milwaukee, WI.

The TXA_2 synthase primer for the RT-PCR experiment was obtained from Sigma Genosys (The Woodlands, TX). Trizol and Thermoscript RT-PCR were obtained from

Gibco BRT (Grand Island, NY). The PCR nucleotide mix and Expand High Fidelity PCR System were from Roche, Inc (Indianapolis, IN). All other chemical compounds were obtained from Sigma Chemical or Fischer Scientific (Fair Lawn, NJ) and were of reagent grade quality.

2.14 Data Analysis

All data were expressed as the mean ± standard error with "n" indicating the number of animals studied for each sex and/or experimental treatment group. Prostanoid output is expressed as picograms of 6-keto-PGI_2 or TXB_2 per mg dry weight of aorta. To detect significant differences among means of experimental groups, data groups were analyzed by sex and experimental treatment. using 2-way analysis of variance. 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 (127); and differences between means were accepted as significant if $P<0.05$.

CHAPTER III

RESULTS

3.1 Plasma Estrogen Concentrations

Plasma estrogen levels were significantly higher in F and OE rats than in OVX rats (one-way ANOVA; $P<0.001$). There were no differences in plasma estrogen between F and OE rats ($P>0.2$). See Table 4.

Table 4: Plasma estrogen concentrations.

Treatment Group	Plasma 17-β-estradiol (pg/ml)
F-NT	8.4 ± 3.4^{a}
F-HT	6.3 ± 1.6^{a}
OVX-NT	0.6 ± 0.5^{b}
OVX-HT	0.8 ± 0.3^{b}
OE-HT	10.5 ± 1.8^{a}
OE-NT	15.6 ± 3.9^{a}

Concentration was determined in each F, OVX, and OE rat. Upon sacrifice, trunk blood was collected and the resulting plasma was stored at –80°C until radioimmunoassay for 17-β-estradiol. Data are reported as the means ± SE of plasma estrogen levels in pg/ml. F-NT (female normotensive-control), OVX-NT (ovariectomized female normotensive-control), OE-NT (ovariectomized estrogen-replaced female normotensive-control), F-HT (female hypertensive), OVX-HT (ovariectomized female hypertensive), OE-HT (ovariectomized estrogen-replaced hypertensive).

3.2 Effects of Aortic Coarctation on Arterial Blood Pressure and Heart Rate

The heart rate and MAP were recorded every other day for 14 days following aortic coarctation. See Table 5 for heart rates. A two-way ANOVA over time and between groups was significant ($P<0.05$).

Very few differences in heart rate were present over time within a treatment group. All NT groups had similar heart rates throughout the study period ($P>0.05$). All HT groups had slower heart rates on day 2 than later in the study period ($P<0.05$). F-NT had a faster heart rate on day 4 than on days 10 through 14. OVX-HT had a slower heart rate on day 6 than on days 10 and 12.

Table 5. Heart rate (beats per minute) following aortic coarctation or sham surgery.

Heart Rate (beats per minute)						
Group	**F-HT**	**M-HT**	**OVX-HT**	**F-NT**	**M-NT**	**OVX-NT**
Day 2	408 ± 36	383 ± 23	371 ± 28	488 ± 10	435 ± 44	430 ± 13
Day 4	435 ± 21	446 ± 7	459 ± 42	515 ± 11	428 ± 31	495 ± 23
Day 6	459 ± 20	473 ± 23	443 ± 13	475 ± 20	450 ± 17	495 ± 9
Day 8	476 ± 12	458 ± 10	476 ± 13	480 ± 25	430 ± 5	510 *
Day 10	470 ± 14	446 ± 22	500 ± 10	460 ± 20	480 *	480 *
Day 12	478 ± 15	446 ± 15	491 ± 9	430 ± 17	428 ± 53	470 ± 57
Day 14	468 ± 14	437 ± 15	460 ± 18	461 ± 17	428 ± 8	450 ± 15

Results are reported as means ± SE. * n=1, all others n=3-7, n= no. of animals per group.

There were some minor differences in heart rates among the groups on a given day. On day 2, M-HT and OVX-HT had slower heart rates than F-NT, while on day 4 F-HT and M-HT had slower heart rates than F-NT ($P<0.05$). No differences in HR were seen on days 12 and 14 between any of the groups (one-way ANOVA, $P>0.05$). The data suggest that sex influenced the heart rate responses to aortic coarctation during the first 4 days following surgery, but not later when MAP was significantly different among the treatment groups (see below).

Following aortic coarctation, all animals exhibited significant increases in MAP compared to sham-operated normotensive-controls ($P<0.01$, Fig. 10). In contrast, there were no changes in MAP in any of the normotensive-control groups over the 14 day period following sham surgery ($P>0.05$).

Only F-HT exhibited a continuous increase in MAP over time (Fig. 10). MAP increased in F-HT from day 2 (145 ± 7 mm Hg) to day 14 (186 ± 7). MAP in M-HT increased from day 0 to 6 then declined and stabilized at a lower MAP than day 6. In OVX-HT, MAP changed little until day 8 then exhibited minor insignificant variations until day 14 (Table 6).

During the 14 days post-coarctation, no differences in pressure were identified between M-HT and OVX-HT rats ($P>0.05$). There were some striking differences in MAP among some of the other groups during the latter part of the two week study period. Prior to and including day 6, M-HT exhibited greater arterial pressure compared to F-HT, but this did not reach significance (Table 6). By day 8, the MAP reversed and F-HT was then higher than M-HT and remained elevated for the remainder of the study.

The MAP of F rats was significantly higher than M rats at days 10, 12 and 14 post-aortic coarctation ($P< 0.05$), (Table 6, Fig. 10). By day 14, the mean MAP in F-HT (186.1 ± 7.0 mm Hg) was 14% higher compared to M-HT (160 ± 4 mm Hg). The MAP of OVX-HT was slightly higher than F-HT on day 2; however, MAP's of both groups were similar on day 6, and by day 14, the MAP of F-HT was 35 mm Hg higher than that of OVX-HT ($P=0.02$, Fig. 10).

Estrogen replacement of OVX rats restored MAP in OE-HT (171 ± 3 mm Hg) rats ($P<0.05$) to that of F-HT rats. OE-HT exhibited a higher MAP than OVX-HT ($P<0.05$) that did not differ from F-HT as shown in Figure 11 ($P>0.05$). The striking differences in blood pressure on day 14 were not present in heart rate, as all the groups had similar heart rates on day 14 post-aortic coarctation or sham surgery.

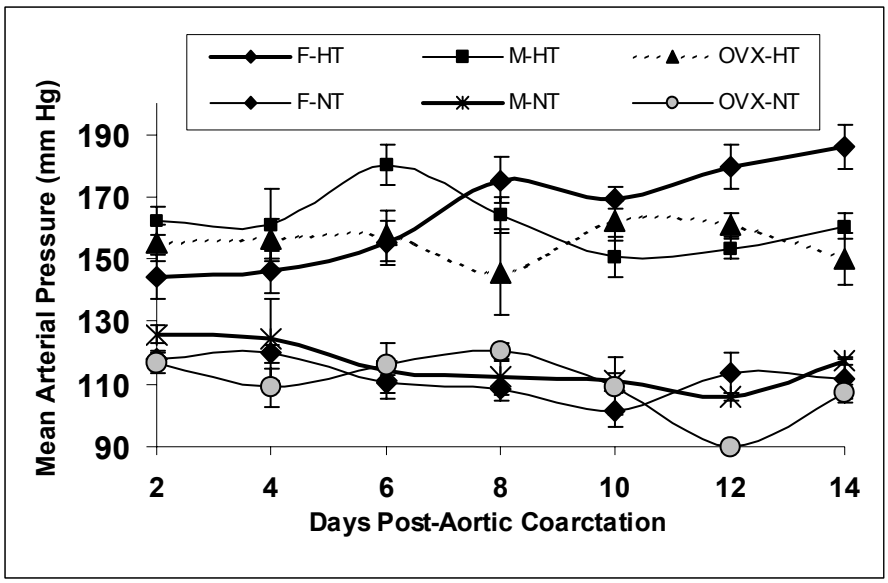


Figure 10. Time course of mean arterial pressure following aortic coarctation. In M (male), F (female), OVX (ovariectomized female), and OE (ovariectomized female estrogen –replaced) rats. Mean ± SE (n =5-9 animals per group)

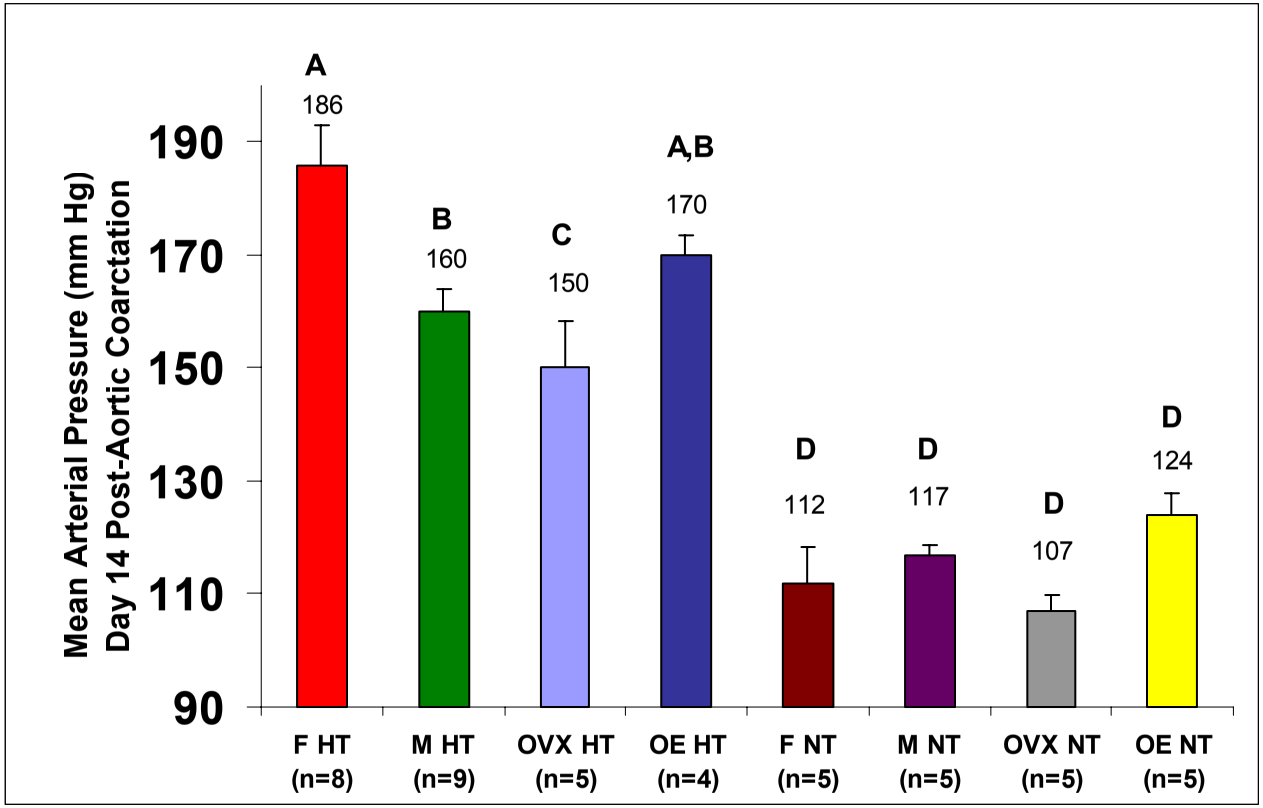


Figure 11. Mean arterial pressure at day 14 post-aortic coarctation. Bars represent means ± SE, n = 4-9 (n, no. of animals). A-D: Mean values without a common script are significantly different, $P<0.05$.

Table 6. Time course of mean arterial pressure following aortic coarctation of M, F, OVX and OE coarcted (HT) and sham (NT) rats.

Mean Arterial Pressure Following Aortic Coarctation								
Sex	**Experimental Group**	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10 ***	**Day 12 ***	**Day 14 ***
F	**Hypertensive n = 8**	145 ± 7.0^{a}	$146 \pm 7.0_{a}$	155 ± 7.0^{a}	175 ± 7.3^{a}	170 ± 3.5^{b}	180 ± 7.2^{b}	186 ± 7.0^{b}
M	**Hypertensive n = 9**	162 ± 4.6^{a}	161 ± 11.4^{a}	180 ± 6.4^{a}	164 ± 5.8^{a}	151 ± 6.5^{a}	154 ± 3.3^{a}	161 ± 4.0^{a}
OVX	**Hypertensive n = 5**	155 ± 5.7^{a}	156 ± 6.5^{a}	158 ± 8.1^{a}	146 ± 3.7^{a}	162 ± 6.3^{ab}	161 ± 4.3^{ab}	150 ± 8.2^{a}
F	**Normotensive-Control n = 5**	118 ± 1.8^{c}	120 ± 3.0^{c}	110 ± 2.7^{c}	109 ± 3.8^{c}	102 ± 5.4^{c}	114 ± 6.3^{c}	112 ± 6.4^{c}
M	**Normotensive-Control n = 5**	126 ± 2.8^{c}	124 ± 13.0^{c}	114 ± 8.8^{c}	113 ± 5.8^{c}	111 ± 2.5^{c}	106 ± 1.5^{c}	118 ± 1.5^{c}
OVX	**Normotensive-Control n = 5**	117 ± 3.5^{c}	109 ± 5.8^{c}	117^{c} (n=2)	120 ± 2.8^{c}	110 ± 9.0^{c}	90^{c} (n=2)	107 ± 2.8^{c}

Results are expressed as means ± SE, n= 5-9 unless otherwise stated (n, no. of animals). A-C: Within columns (days) and rows (experimental groups) mean values without a common superscript are significantly different, $P<0.05$.

3.3 Effects of Acute Thromboxane/Endoperoxide Receptor Antagonism with SQ 29,548 on Aortic Coarctation-Induced Hypertension

Heart rates before infusion, after the SQ 29,548 (SQ) bolus and after the hour-long SQ infusion are summarized in Table 7 and Figure 12. No differences in heart rate were observed between HT and NT groups prior to infusion of SQ. However, heart rate decreased significantly following infusion (2-way ANOVA, $P<0.05$) in all groups. All NT groups exhibited similar heart rates following SQ infusion, while heart rates of HT groups were more variable. Heart rate was significantly reduced in M-HT compared to F-HT, OVX-HT and OE-HT ($P<0.04$). Comparisons of heart rate between NT and HT groups revealed that HR differed significantly only between OE-HT and OE-NT ($P<0.01$).

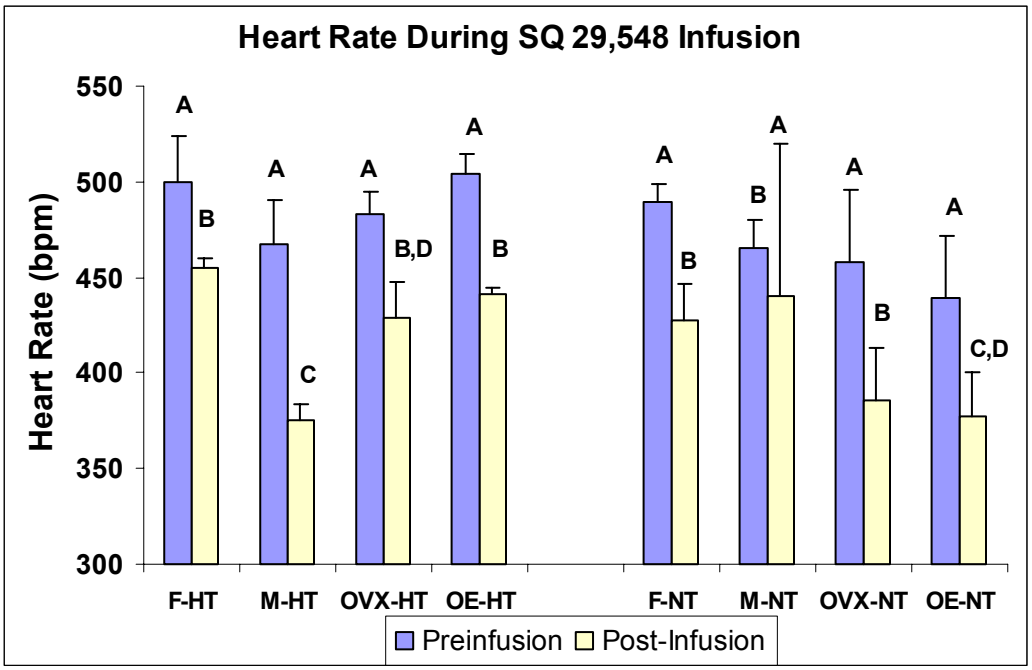


Figure 12: Heart rate prior to and after SQ 29,548 infusion. Bars represent means ± SE, n = 5-9 (n, no. of animals). A-D: Mean values without a common script are significantly different, $P<0.05$.

Table 7: Heart rate prior to, during, and after intravenous infusion of SQ 29,548.

Heart Rate (beats per minute) During Infusion of SQ 29,548			
Group	**Pre-Infusion**	**Post-bolus SQ 29,548 (2 mg/kg iv)**	**Post-Infusion (2 mg/kg/hour iv)**
F-HT	500 ± 24^{a}	493 ± 22^{a}	455 ± 5^{a}
M-HT	468 ± 23^{a}	420 ± 45^{a}	375 ± 9^{b}
OVX-HT	483 ± 12^{a}	462 ± 17^{a}	439 ± 19^{a}
OE-HT	504 ± 10^{a}	477 ± 18^{a}	441 ± 4^{c}
F-NT	489 ± 10^{a}	420 ± 17^{b}	$428 \pm 19^{a,d}$
M-NT	$465 \pm 15^{a,e}$	510 ± 30^{a}	$440 \pm 80^{b,d,e}$
OVX-NT	458 ± 38^{a}	461 ± 34^{a}	$386 \pm 27^{a,d}$
OE-NT	$439 \pm 33^{a,f}$	$431 \pm 35^{a,f}$	$378 \pm 23^{d,f}$

Results are means ± SE, n = 5-9 (n, no. of animals). A-F: Within columns and rows for each group and experimental treatment (pre-infusion, post-bolus, and post-infusion), mean values for heart rates without common superscript are significantly different, $P<0.05$.

In general, infusion of SQ exerted a significant attenuating effect ($P<0.013$) on MAP of the various groups of rats. Inter-group variation did not have an effect on the response of rats to SQ ($P=0.54$), Table 8. A One-way ANOVA applied to the maximal change in MAP with SQ infusion was significant ($P<0.007$). With respect to NT groups, F-NT and OE-NT rats experienced significant decreases in MAP during the infusion of SQ (paired one-tailed t test, $P<0.05$); however, M-NT and OVX-NT did not ($P>0.05$). M-NT had a 5.5 ± 3.8 mm Hg decrease in MAP while the other NT groups exhibited at least a 3-fold greater decline in pressure compared to pre-infusion MAP's (Figure 13b).

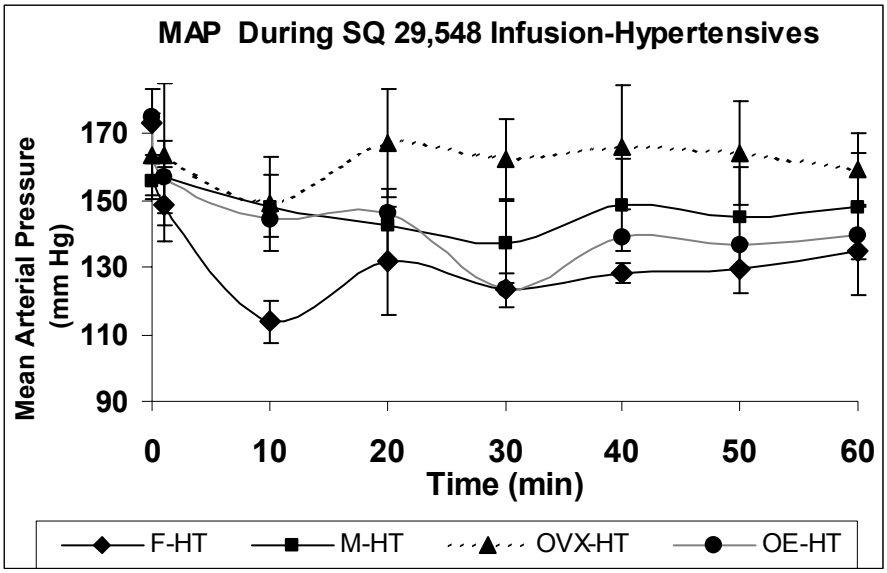
Table 8: Maximal decrease in mean arterial pressure following SQ 29,548 administration.

Maximal Change in Mean Arterial Pressure with Intravenous Administration of SQ 29,548 Mean ± SE		
Sex	**Group**	**Change in Pressure (mm Hg)**
Female	Hypertensive	59.0 ± 8.1^{a}
Male	Hypertensive	20.3 ± 8.4^{b}
OVX	Hypertensive	$22.0 \pm 10.7^{b,c}$
OE	Hypertensive	$49.8 \pm 13.1^{a,c}$
Female	Normotensive-Control	$18.7 \pm 3.6^{b,d}$
Male	Normotensive-Control	5.5 ± 3.8^{b}
OVX	Normotensive-Control	$20.0 \pm 4.6^{b,d,e}$
OE	Normotensive-Control	$18.0 \pm 9.0^{a,b,e}$

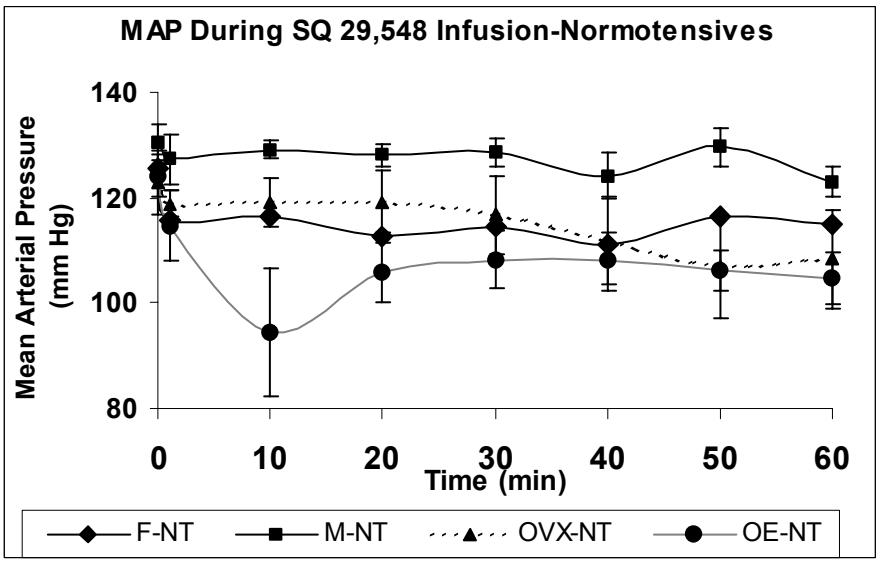
Data are reported as means ± SE, n = 5-9 (n, no. of animals). A-E: For each treatment group, mean values without common superscript are significantly different, $P<0.05$.

All groups of HT rats exhibited a decrease in MAP with infusion of SQ (Figure 13a). MAP decreased by 50 mm Hg or more in OE-HT and F-HT rats with infusion and by 20 to 22 mm Hg in M-HT and OVX-HT rats, respectively ($P<0.05$). The responses to intravenous SQ infusion were clearly greater in HT groups. Table 8 shows the maximal decreases in MAP that occurred for each group following intravenous SQ. F-HT displayed a dramatic fall in MAP with intravenous SQ infusion ($P<0.005$) to 59 ± 8.1 mm Hg. OE-HT had a similar significant decrease in MAP ($P<0.05$), but the decrease was not as rapid as with F-HT as shown in Figure 13a. In contrast, MAP of M-HT and OVX-HT did not change significantly during the infusion of SQ ($P>0.05$). The decline in MAP in F-HT was greater than in either M-HT or OVX-HT rats ($P<0.02$, Fig. 14). OE-HT also exhibited a significant reduction in MAP compared to M-HT ($P<0.05$), but not compared

to OVX-HT (P=0.08). These data indicate that the cardiovascular responses to SQ are affected by sex of the rat as well as by aortic coarctation.



(a)



(b)

Figure 13: Time course of mean arterial pressure during intravenous infusion of SQ 29,548; at 14 days a: post-aortic coarctation or b: sham surgery (normotensive control). SQ 29,548 was administered as a bolus from 0 to 1 minute at a dose of 2 mg/kg, then given as a constant rate infusion from 1 minute to 60 minutes at a rate of 2 mg/kg/hour. Data points represent means ± SE, n = 5-9 (n, no. of animals).

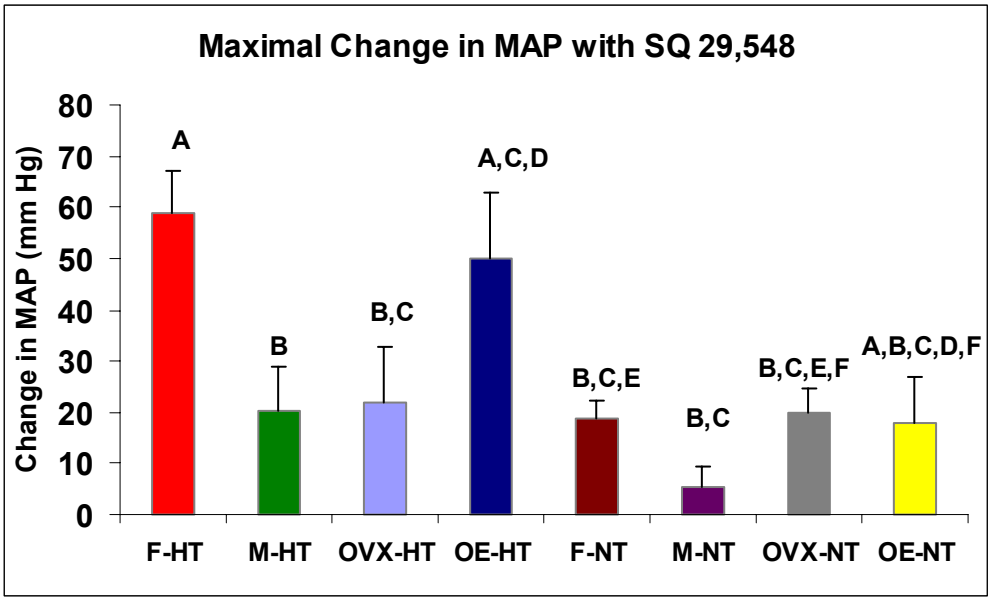


Figure 14: Maximal decrease in mean arterial pressure (MAP) following intravenous infusion of SQ 29,548 fourteen days after aortic coarctation or sham surgery. Bars represent means ± SE, n = 4-5 (n, no. of animals). A-E: Mean values without common script are significantly different, $P<0.05$.

3.4 Effects of Combined Thromboxane A_2 Synthase Inhibition and Thromboxane/Endoperoxide Receptor Antagonism with Ridogrel on Aortic Coarctation-Induced Hypertension

Heart rate and blood pressure were recorded every other day during the 14 days of Ridogrel treatment following aortic coarctation or sham surgery. Table 9 summarizes changes in heart rate during the experiment. A two-way ANOVA detected differences in heart rate over time and among the experimental groups ($P<0.05$). Very few differences in heart rate occurred over time within each treatment group; however, differences did occur within the first 4 days following aortic coarctation. All groups except OVX-NT had slower heart rates on day 2 than on later days in the study ($P<0.05$). F-HT, OVX-HT and

OE-HT (but not M-HT) had slower heart rates on day 4 than on later days in the study (after day 6). This suggested that M-HT responded less to Ridogrel early after aortic coarctation. From day 6 to day 14, heart rates had stabilized in all groups and no further significant changes in their heart rates were detected ($P>0.05$). Differences in heart rates among groups on a given day were significant in all groups except in M-HT and M-NT. M-HT and M-NT had similar heart rates to other treatment groups throughout the study ($P>0.05$). In contrast, F-HT had a slower heart rate than any of the NT groups on days 2, 4, 6, and 12 ($P<0.05$). OE-HT also had slower heart rates than any of the NT groups on days 4 and 6.

Table 9: Mean heart rate during Ridogrel treatment.

Time Course of Heart Rate (mean ± SE beats per minute) During Ridogrel Treatment Following Aortic Coarctation (HT) or Sham Surgery (NT)							
Group	**F-HT**	**M-HT**	**OVX-HT**	**OE-HT**	**F-NT**	**OVX-NT**	**OE-NT**
Day 2	383 ± 28	398 ± 38	401 ± 35	396 ± 31	458 ± 13	465 ± 29	450 ± 14
Day 4	414 ± 24	430 ± 36	370 ± 26	372 ± 22	480 #	465 ± 11	506 ± 20
Day 6	468 ± 7	468 ± 29	480 #	435 ± 29	503 ± 14	476 ± 9	506 ± 9
Day 8	500 ± 20	505 ± 13	450 ± 40	448 ± 17	480 #	484 ± 22	503 ± 26
Day 10	420 ± 42	460 ± 20	480 #	480 #	470 ± 26	480 ± 1	518 ± 8
Day 12	450 ± 17	490 ± 10	465 ± 15	476 ± 16	470 ± 26	458 ± 38	514 ± 13
Day 14	473 ± 19	470 ± 10	446 ± 9	462 ± 12	480 #	420 (n=1)	510 ± 30

Heart rate was recorded in beats per minute at same time that MAP was recorded. Data are means ± SE, n=3-9 (n, no. of animals). # Heart rates for individuals in that group that day were the same.

Table 10: Mean arterial pressure following aortic coarctation or sham surgery in rats treated with Ridogrel or vehicle-control.

Mean Arterial Pressure at 14 Days Post-Aortic Coarctation With or Without Ridogrel Administration (Mean ± SE)		
Group	**Mean Arterial Pressure (mm Hg)**	
	With Ridogrel	**Vehicle Treatment**
Female Hypertensive n=4	132 ± 2.8^{a}	193 ± 9.3^{b}
Male Hypertensive n=4-9	159 ± 8.1^{c}	161 ± 4.0^{c}
OVX Hypertensive n=4-5	147 ± 6.2^{cd}	150 ± 9.1^{cd}
OE Hypertensive n=4-5	134 ± 6.0^{ad}	173 ± 8.1^{b}
Female Normotensive Control n=4	124 ± 2.3^{e}	109 ± 6.7^{e}
OVX Normotensive Control n=4	110 ± 7.4^{e}	105 ± 3.2^{e}
OE Normotensive Control n=3-4	121 ± 3.3^{e}	117 ± 5.7^{e}

Data are reported as means ± SE; n, no. of animals. A-E: Within columns (experimental groups) and rows (Ridogrel treatment versus no Ridogrel treatment), mean values without common superscript are significantly different, $P<0.05$.

Table 10 summarizes the mean arterial pressures recorded over14 days following aortic coarctation (HT) or sham surgery (NT). There were no significant daily variations in MAP among any of the treatment groups with Ridogrel administration (2-way ANOVA, $P>0.05$). Comparison of Ridogrel-treated groups to vehicle-treated groups revealed that there was a significant difference (one-way ANOVA, $P<0.0001$). The NT groups did not show any changes in MAP due to Ridogrel treatment ($P>0.05$). Ridogrel administration significantly reduced MAP only in HT groups that had estrogen present (i.e., F-HT and OE-HT). Ridogrel significantly reduced mean arterial pressure in F-HT

(P<0.008) and OE-HT (P<0.01) rats compared F-HT and OE-HT that received only vehicle (Table 10). There were no changes in MAP in M-HT or OVX-HT rats (P>0.05) with Ridogrel treatment, compared to vehicle treatment (Table 10, Fig.15). In addition, F-HT rats given Ridogrel exhibited lower MAP than M-HT and OVX-HT groups that also received Ridogrel (P<0.05). OE-HT showed lower MAP than M-HT (P<0.02) as well. At 14 days post-aortic coarctation, MAP in HT rats was similar to that of NT rats only in the groups with estrogen (F and OE, P≥0.05), indicating that estrogen replacement restored OVX responses to Ridogrel.

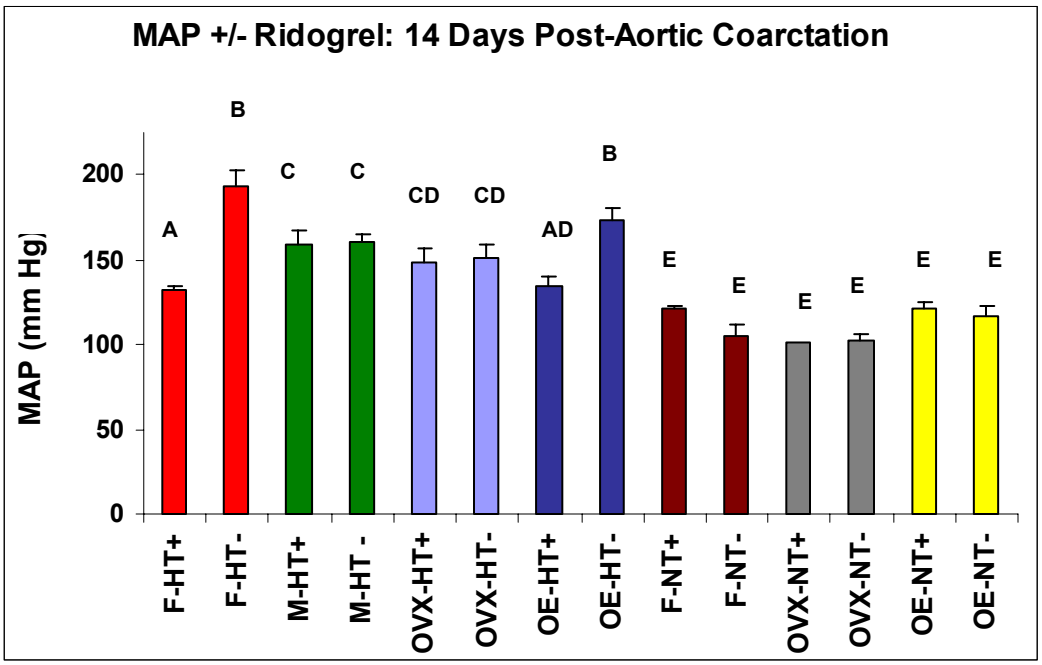


Figure 15: Mean arterial pressure 14 days after aortic coarctation or sham surgery (NT) with (+) and without (-) Ridogrel. Bars represent means ± SE, n = 3-9 (n, no. of animals). A-E: Bars without common script are significantly different, P<0.05.

When the differences in MAP among rats that received Ridogrel were compared to those that received only vehicle at 14 days post-aortic coarctation, only those with

estrogen present responded with a decline in MAP (Fig. 16). F-HT MAP declined by an average of 61.5 mm Hg ($P<0.008$) compared to only 1.4 mm Hg in OVX-HT rats ($P>0.05$). Estrogen supplementation of previously ovariectomized rats restored the response to Ridogrel, resulting in a 39 mm Hg decline in MAP in the Ridogrel-treated OE-HT rats ($P<0.01$).

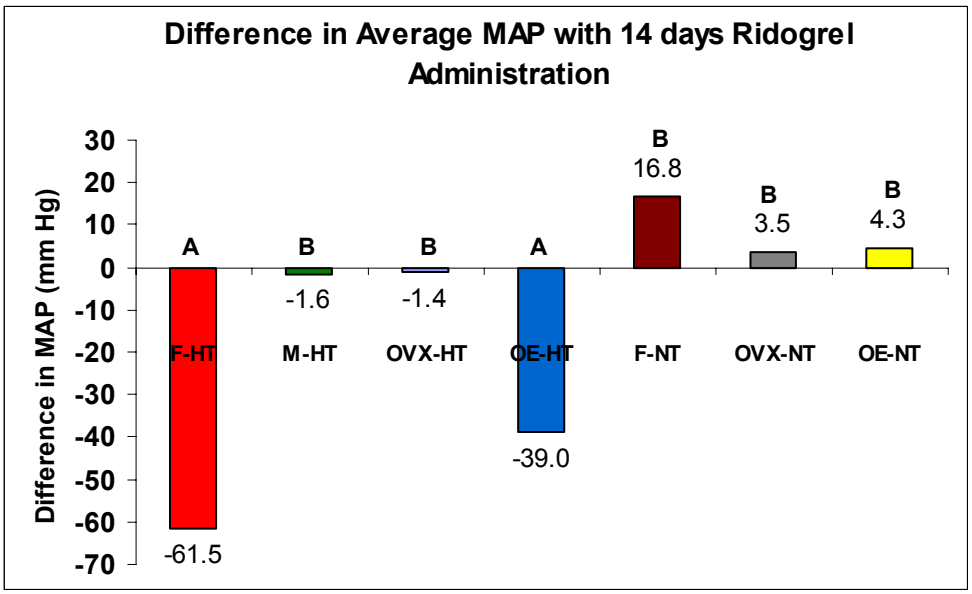


Figure 16: Difference in mean arterial pressure with Ridogrel administration 14 days after aortic coarctation or sham surgery (NT). Bars represent means of average MAP, n = 3-9 (n, no. of animals). A-B: Bars with different script are significantly different ($P<0.05$).

3.5 Effects of Aortic Coarctation on Prostanoid Release

3.5.1 Thromboxane A_2

Basal release of TXB_2 (stable metabolite of TXA_2) from aortic rings was significantly different among the treatment groups (one-way ANOVA, $P<0.0001$). Basal release of TXB_2 was similar between M and F only in NT aortae. With aortic coarctation, basal production of TXB_2 was 2-fold higher in F-HT than in M-HT

($P<0.05$, Table 11 and Fig. 17). Although TXB_2 release increased in both sexes, the increase with coarctation was 7-fold in F-HT but only 4-fold in M-HT.

Stimulation of TXB_2 release by ANG II in thoracic aortae was significantly different in F versus M (1-way ANOVA, $P<0.0001$). In NT aortae, ANG II-stimulated production in F-NT was twice that of M-NT ($P<0.05$). Hypertensive aortae also exhibited sexual dimorphism in ANG II-induced TXB_2 release; thus, release of TXB_2 was 28% higher in F-HT than in M-HT. These data reveal that during the development of hypertension, both basal and ANG II-stimulated TXB_2 release were upregulated to a much greater extent in F-HT than M-HT.

Table 11: Basal and ANG II-stimulated release of TXB_2 from thoracic aortic rings.

Sex	Experimental Groups	Basal TXB_2 (pg/mg) Mean ± Standard Error	ANG II-Stimulated TXB_2 Release (pg/mg) Mean ± Standard Error
M	Normotensive-Control	17.9 ± 1.0^{a}	41.2 ± 2.8^{b}
F	Normotensive-Control	16.9 ± 1.0^{a}	67.8 ± 7.3^{c}
M	Aortic Coarctation-Induced Hypertension	65.2 ± 12.4^{c}	132.0 ± 25.2^{d}
F	Aortic Coarctation-Induced Hypertension	114.7 ± 14.7^{d}	182.7 ± 14.5^{e}

The amount of TXB_2 released is pg/mg dry weight of thoracic aorta/45 minutes. Data are reported as means ± SE, n = 4-7 (n, no. of animals). A-E: Within columns (basal versus ANG II-stimulation) and rows (experimental groups), mean values without common superscript are significantly different, $P<0.05$.

In the continuous, pulsatile flow-pressurized aorta experiments, TXB_2 release was similar in all groups of NT rat aortae at both 100 and 150 mm Hg (Table 12). Two-way ANOVA at 100 mm Hg indicated that no differences were present in any group or over time

(P>0.05); however, at 150 mm Hg significant differences were identified among the groups (P<0.009) and over time (P<0.05).

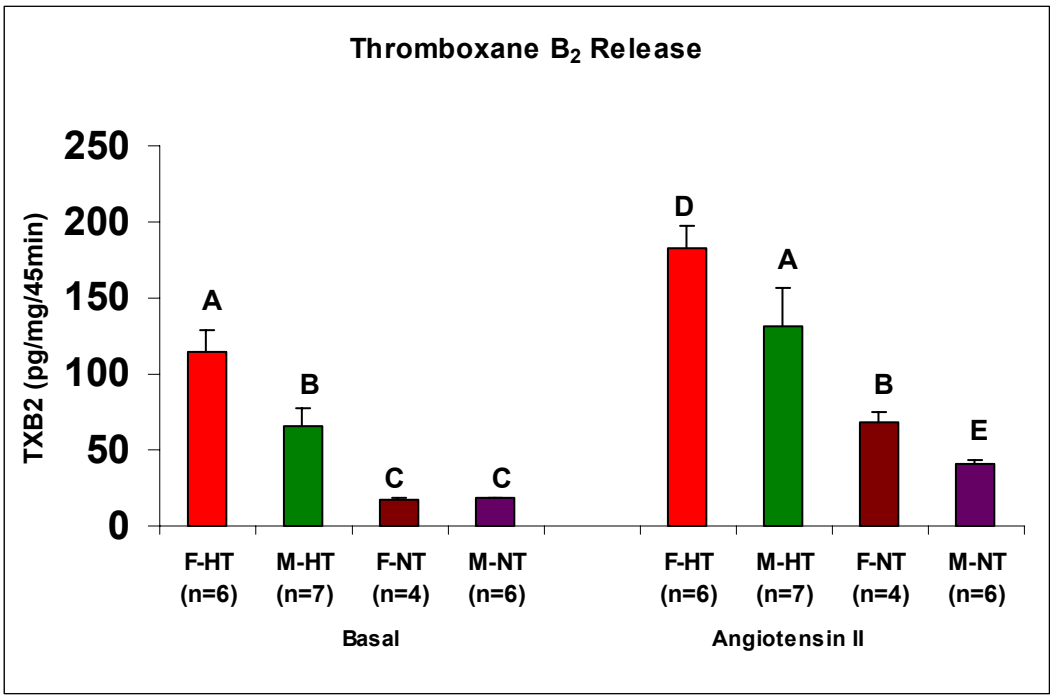


Figure 17: Basal and ANG II-stimulated release of thromboxane B_2 from aortic rings. Rings were incubated in vitro in absence (basal) or presence of 10^{-6} M ANG II (stimulated). TXB_2 release is expressed per mg aorta dry weight per 45-minute incubation period. Bars represent means ± SE (n, no. of animals). A-E: Bars without common script are significantly different, P<0.05.

TXB_2 was elevated 4-fold or more in the aortae of F-HT and OE-HT rats, compared to their normotensive-control counterparts at either 100 mm Hg or 150 mm Hg (Figs. 18 and 19). At normotensive pressure (100 mm Hg), F-HT released significantly more TXB_2 than OVX-HT at 10 and 30 minutes of perfusion, $P \leq 0.01$, Table 12 and Fig. 18. At hypertensive conditions (150 mm Hg mean pressure), F-HT released significantly more TXB_2 than OVX-HT after 30 minutes of continuous perfusion (P=0.02, Fig. 18).

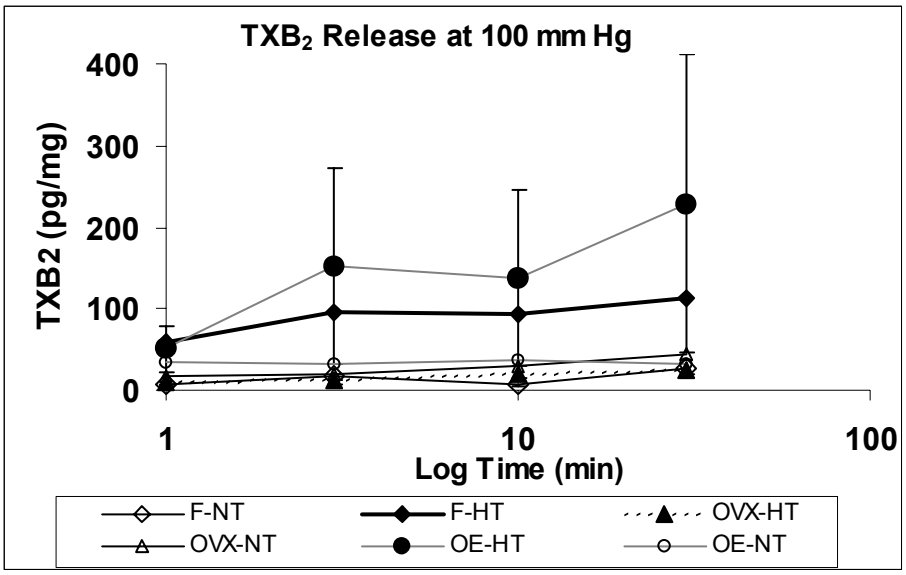


Figure 18: Thromboxane B_2 release by isolated, perfused thoracic aortae during constant flow and at 100 mm Hg pressure over a 30 minute time period. Data points are means ± SE (pg/mg aorta dry weight), n = 4-7 (n, no. of animals).

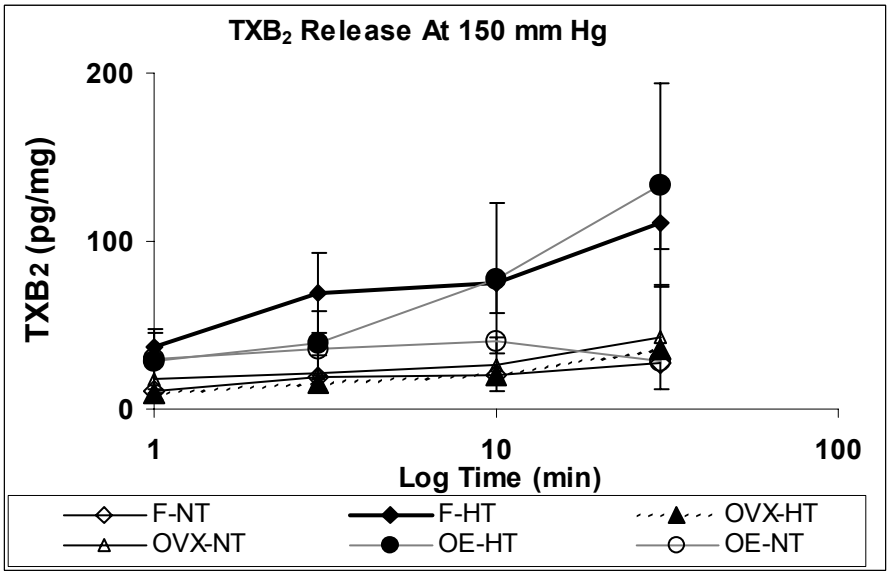


Figure 19: Thromboxane B_2 release by isolated, perfused thoracic aortae during constant flow and at 150 mm Hg mean pressure over a 30 minute time period. Data points are means ± SE (pg/mg aorta dry weight), n = 4-7 (n, no. of animals).

Ovariectomy clearly impaired the release of TXB_2 by the thoracic aorta in hypertension, as OVX-HT released half as much TXB_2 as did F-HT at either 100 or 150

mm Hg. Estrogen replacement restored the release of TXB_2, since aortae from OE-HT rats released the same or greater amounts of TXB_2 as did F-HT (Table 12).

The effects of estrogen on ANG II-stimulated TXB_2 release during continuous perfusion and 150 mm Hg pressure were similar to those observed under basal conditions. ANG II-stimulated TXB_2 release in NT rats was similar (Table 12, Fig, 20). In F-HT and OE-HT aortae, ANG II-stimulated TXB_2 production was elevated 3-fold over their respective NT counterparts, whereas OVX-HT increased TXB_2 production only 1.5-fold over OVX-NT, Fig. 20. OVX-HT aortae appeared to release less TXB_2 when stimulated with ANG II, compared to F-HT and OE-HT, although the difference was not significant (P=0.08), Table 12 and Fig, 20. The dose of ANG II significantly affected TXB_2 production (2-way ANOVA, P<0.02).

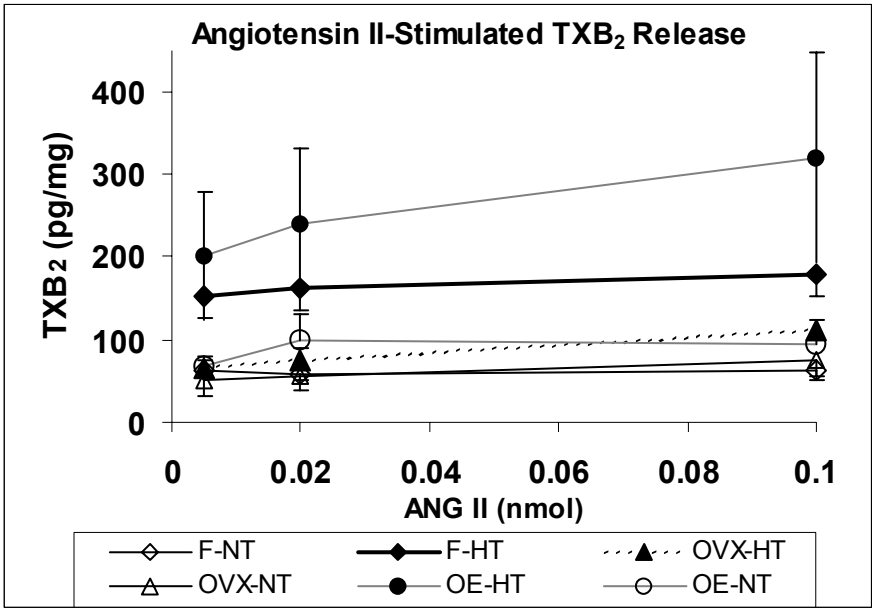


Figure 20: ANG II-stimulated TXB_2 release from isolated, perfused thoracic aortae at constant flow and a mean pulse pressure of 150 mm Hg. Increasing dosages of ANG II were given every 15 minutes with production of TXB_2 determined 15 minutes after each dose of ANG II. Data points are means ± SE (pg/mg aorta dry weight/15 minutes), n = 4-7 (n, no. of animals).

Table 12: TXB_2 release from thoracic aortic rings pressurized with recirculating KHB under constant flow conditions.

SEX	Experimental Group	Continuous Pressure and Flow: TXB_2 Release (pg/mg aorta dry weight/ 15 or 30 min)				
		100 mm Hg (per 30 min)	150 mm Hg (per 30 min)	ANG II 0.005 nmol (per 15 min)	ANG II 0.02 nmol (per 15 min)	ANG II 0.10 nmol (per 15 min)
F	Hypertensive, n=5	114 ± 26^{a}	110 ± 14.8^{a}	152 ± 27.1^{a}	163 ± 27.1^{a}	179 ± 26.1^{a}
OVX	Hypertensive, n = 4	25 ± 5.3^{b}	36 ± 5.8^{b}	66 ± 9.7^{c}	74 ± 14.4^{cd}	112 ± 12.9^{d}
OE	Hypertensive, n = 7	229 ± 182.1^{ab}	133 ± 60.7^{ab}	201 ± 77.3^{ac}	240 ± 91.3^{acd}	320 ± 126.3^{ad}
F	Normotensive-Control, n=5	27 ± 11.3^{b}	27 ± 11.4^{b}	62 ± 7.0^{c}	57 ± 6.2^{c}	64 ± 8.5^{c}
OVX	Normotensive-Control, n=4	45 ± 43.6^{abe}	43 ± 30.9^{abe}	51 ± 19.1^{ce}	56 ± 18.7^{cde}	75 ± 24.5^{acde}
OE	Normotensive-Control, n = 6	32 ± 3.3^{bef}	29 ± 2.0^{bef}	69 ± 10.8^{ceh}	100 ± 31.7^{acdeh}	94 ± 7.4^{acdeh}

Amount of TXB_2 released at 100 and 150 mm Hg is over a 30 minute period. Amount of TXB_2 produced after ANG II administration is over a 15, 30, or 45 minute period of continuous perfusion, each dose of ANG II was followed by a 15-minute recovery period. Data are reported as means ± SE (n, no. of animals). A-E: Mean values without common superscript are significantly different for experimental groups versus treatments, $P<0.05$.

3.5.2 Prostacyclin (PGI_2)

Basal release of 6-keto-prostaglandin $F_{1\alpha}$ (stable metabolite of PGI_2) from aortic rings was similar in NT groups. With aortic coarctation, basal production of 6-keto-prostaglandin $F_{1\alpha}$ was 1.5-fold higher in F-HT than in M-HT ($P<0.05$, Table 13 and Fig. 21). Basal release by F-HT was elevated 5-fold, while M-HT release was elevated only 3-fold, compared to their respective NT counterparts.

There were no differences seen in F-NT or M-NT release of 6-keto-$PGF_{1\alpha}$ with ANG II-stimulation ($P>0.05$). ANG II-stimulated $PGF_{1\alpha}$ release was significantly higher (2-fold) in F-HT than in M-HT ($P< 0.05$, Fig. 21, Table 13). This is in contrast to TXB_2 release in F-NT and M-NT aortae, where both hypertensive and normotensive-control groups responded to ANG II-stimulation with increased TXB_2 release (Fig. 17). ANG II stimulated TXB_2 production but not 6-keto-$PGF_{1\alpha}$ in the rat aorta in normotension.

Table 13: Basal and ANG II-stimulated release of 6-keto-prostaglandin $F_{1\alpha}$ from aortic rings incubated for 45 minutes.

Sex	Experimental Groups	Basal 6-keto-PGF1α (pg/mg) Mean ± SE	ANG II-Stimulated 6-keto-PGF1α Release (pg/mg) Mean ± SE
M	Normotensive-Control	1540 ± 148^{a}	1577 ± 340^{a}
F	Normotensive-Control	1462 ± 163^{a}	1470 ± 131^{a}
M	Aortic Coarctation-Induced Hypertension	4809 ± 1218^{b}	4534 ± 607^{bc}
F	Aortic Coarctation-Induced Hypertension	7559 ± 943^{be}	9089 ± 1043^{e}

Results are expressed as means ± SE (pg/mg aorta dry weight/45 minutes), n = 4-7 (n, no. of animals). A-E: Within columns (basal versus ANG II stimulated) and rows (treatment groups), mean values without common superscript are significantly different, $P<0.05$.

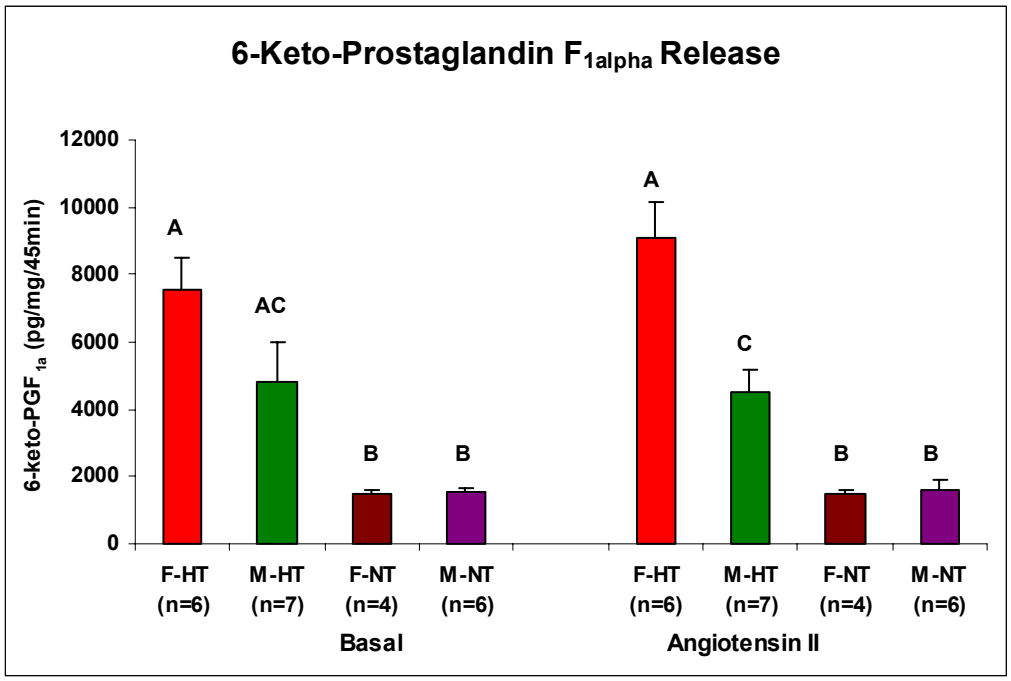


Figure 21. Basal and ANG II-stimulated 6-keto-prostaglandin $F_{1\alpha}$ release from aortic rings. Data are in pg/mg aorta dry weight/ 45 min incubation period. Bars represent means ± SE (n, no. of animals). A-C: Bars without common scripts are significantly different, $P<0.05$.

In the continuous, pulsatile flow-pressurized aorta experiments, 6-keto-$PGF_{1\alpha}$ production was similar among all NT aortae at both 100 and 150 mm Hg ($P>0.05$). 6-keto-$PGF_{1\alpha}$ was elevated 3-fold or more in the F-HT and OE-HT aortae compared to their normotensive-control counterparts at either 100 mm Hg or 150 mm Hg (Fig. 22). Under normotensive pressure (100 mm Hg), F-HT produced significantly more 6-keto-$PGF_{1\alpha}$ than OVX-HT ($P<0.05$). Under hypertensive conditions (150 mm Hg mean pressure), F-HT produced significantly more 6-keto-$PGF_{1\alpha}$ than OVX-HT after 30 minutes of continuous perfusion ($P<0.05$). Similar to the effects on TXB_2, ovariectomy clearly impaired the release of 6-keto-$PGF_{1\alpha}$ by the thoracic aorta in hypertension, as OVX-HT released less than half the amount of 6-keto-$PGF_{1\alpha}$ released by F-HT at either

100 or 150 mm Hg. Estrogen replacement restored thoracic aortic production of 6-keto-$PGF_{1\alpha}$, since OE-HT released similar amounts of 6-keto-$PGF_{1\alpha}$ as F-HT (Table 14).

Treatment group (F-HT, OVX-HT, OE-HT, F-NT, OVX-NT, OE-NT) had a significant effect on 6-keto-$PGF_{1\alpha}$ ($P<0.0002$) whereas dose of ANG II did not ($P>0.05$, 2-way ANOVA). At the highest ANG II dose (0.1 nmol), 6-keto-$PGF_{1\alpha}$ release was significantly different between the treatment groups via one-way ANOVA ($P=0.02$), Fig. 23. F-HT released more 6-keto-$PGF_{1\alpha}$ than did F-NT in response to 0.1 nmol ANG II; however, ovariectomy abolished this response. (Table 14). Estrogen again restored the increased production of 6-keto-$PGF_{1\alpha}$ to 0.1 nmol ANG II, with OE-HT producing more 6-keto-$PGF_{1\alpha}$ than OE-NT ($P<0.05$) as shown in Fig. 23.

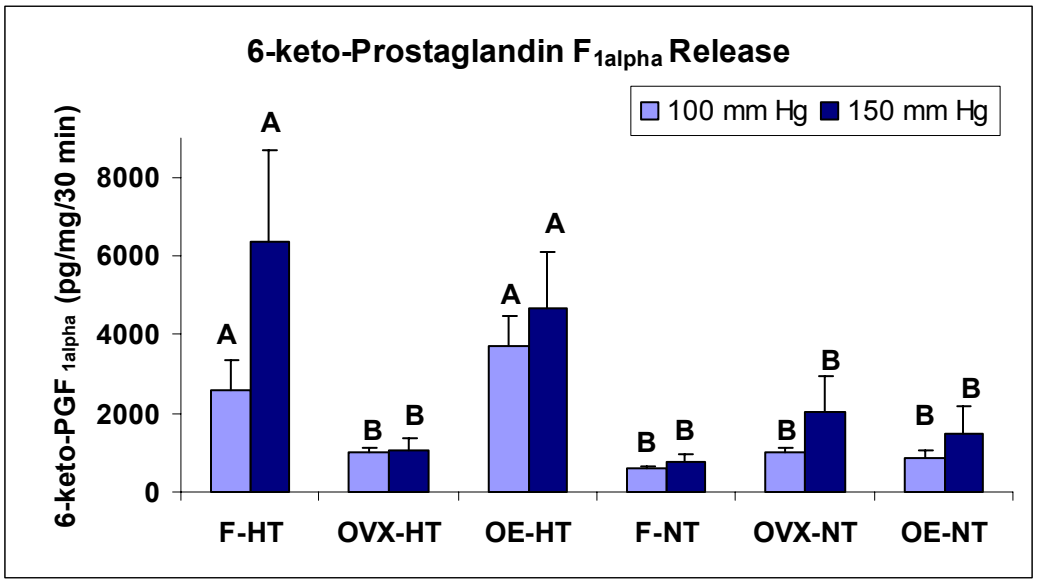


Figure 22: 6-keto-Prostaglandin $F_{1\alpha}$ release during constant flow and pressure (either 100 mm Hg or 150 mm Hg) in pg/mg aorta dry weight/30 minutes. Bars represent means ± SE, n = 4-7 (n, no. of animals). A-B: Bars without common script are significantly different, $P<0.05$.

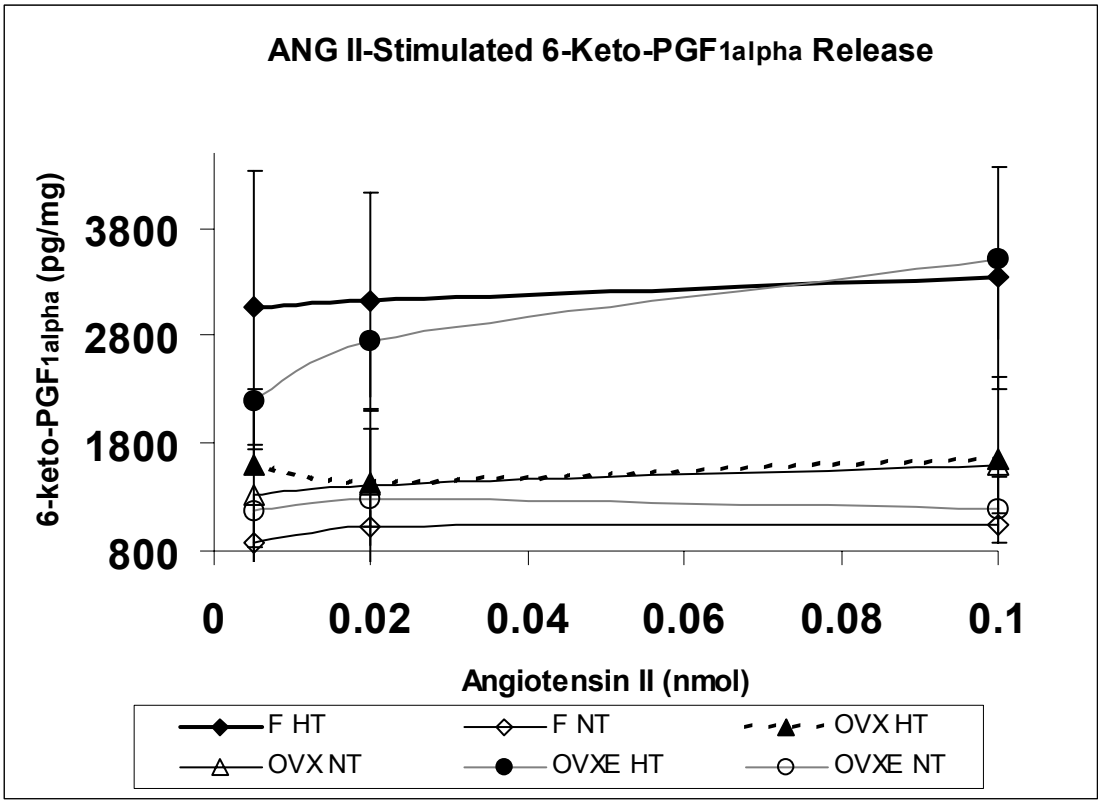


Figure 23: 6-keto-prostaglandin $F_{1\alpha}$ release during constant flow and pressure (150 mm Hg) from thoracic aortae stimulated with increasing doses of ANG II. Data points are means ± SE (pg/mg aorta dry weight/15 minutes) (post-ANG II administration to perfusate), n = 4-7 (n, no. of animals).

Table 14: Release of 6-keto-prostaglandin $F_{1\alpha}$ from isolated, perfused rat thoracic aortae during constant pressure and flow.

Sex	Experimental Group	Continuous Pressure and Flow: 6-keto-PGF1α Release (pg/mg aorta dry weight/ 15-30 min)				
		100 mm Hg (per 30 min)	**150 mm Hg (per 30 min)**	**ANG II 0.005 nmol (per 15 min)**	**ANG II 0.02 nmol (per 15 min)**	**ANG II 0.10 nmol (per 15 min)**
F	Hypertensive, n=5	$2,599 \pm 782^{a}$	$6,345 \pm 2344^{a}$	$3,064 \pm 1274^{a}$	$3,125 \pm 1003^{a}$	$3,339 \pm 1032^{ad}$
OVX	Hypertensive n= 4	$1,000 \pm 134^{b}$	$1,065 \pm 313^{b}$	$1,600 \pm 701^{ab}$	$1,441 \pm 661^{ab}$	$1,661 \pm 753^{bd}$
OE	Hypertensive n = 7	$3,442 \pm 755^{a}$	$4,205 \pm 1424^{a}$	$2,018 \pm 450^{a}$	$2,518 \pm 519^{ae}$	$3,200 \pm 750^{ad}$
F	Normotensive -Control, n=3-5	635 ± 31^{bc}	776 ± 197^{bc}	866 ± 285^{abc}	$1,016 \pm 153^{acf}$	$1,039 \pm 90^{cf}$
OVX	Normotensive -Control, n=4	$1,001 \pm 130^{bc}$	$2,045 \pm 906^{bc}$	$1,320 \pm 488^{abc}$	$1,423 \pm 402^{bf}$	$1,606 \pm 452^{bdf}$
OE	Normotensive -Control, n= 6	866 ± 208^{bc}	$1,491 \pm 681^{bc}$	$1,168 \pm 577^{abc}$	$1,284 \pm 650^{bf}$	$1,182 \pm 313^{bdf}$

Values are means ± SE (pg/mg aorta dry weight/15 or 30 minute time period), n, no. of animals. A-E: Mean values without common superscript are significantly different (experimental groups versus treatments, $P<0.05$).

3.6 Effects of Aortic Coarctation on the Pressure-Diameter Relationship of the Thoracic Aorta in Response to Angiotensin II

The aortic diameter was determined off-line from videotapes of the *in vitro* constant pressure/flow experiments with the thoracic aorta. A micrometer was recorded on the video at the beginning of each experiment in order to determine diameters accurately. Pressures and diameters were compared for each group: F-HT, OVX-HT, OE-HT, F-NT, OVX-NT, and OE-NT. Pressurization of the aortae to different degrees of distention resulted in increases in the diameters of the vessels. The increases in diameter were significantly less in F-HT and OE-HT aortae ($P<0.05$, Fig. 24).

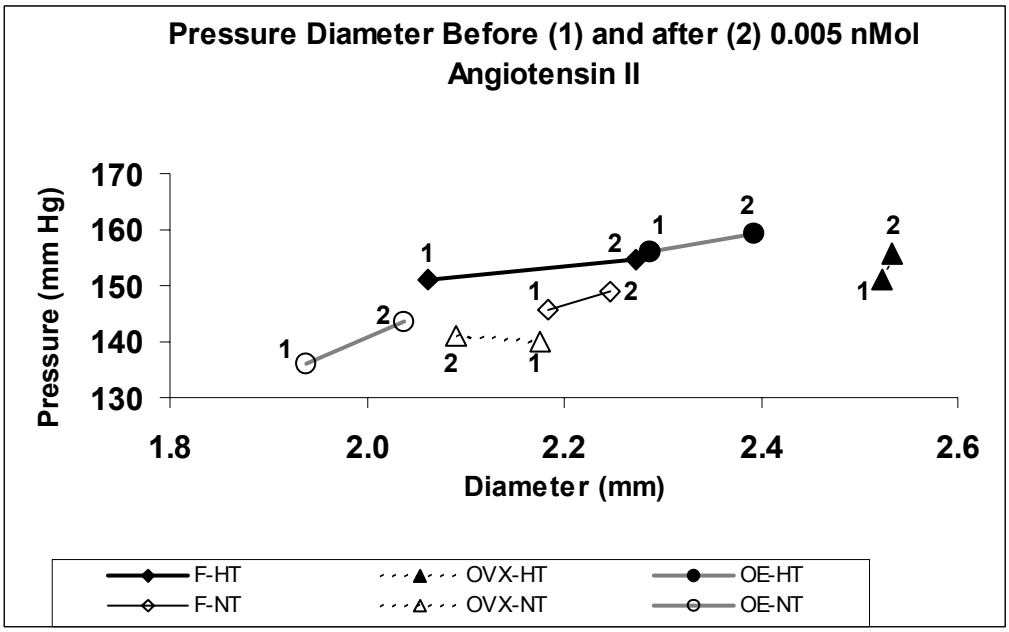


Figure 24: Pressure-diameter relationships of *in vitro* continuous flow experiments in the thoracic aortae of NT and HT rats. Each aorta was pressurized to 150 mm Hg before ANG II administration and the diameter recorded (1). ANG II (0.005 nmol) was administered and the pressure and diameter recorded using a computer and video camera (2).

Aortae from OVX-HT rats had the largest increase in diameter with initial pressurization (Fig. 24), with little change occurring with ANG II stimulation. All groups exhibited increases in diameter with ANG II stimulation; however, after the data were normalized, two-way ANOVA did not detect any significant differences among doses or groups (P>0.05, Fig. 25). No differences were identified between any of the groups with the 0.005 or 0.02 nmol doses of ANG II (P>0.05). However, at the highest dose of ANG II (0.1 nmol), OE-HT and OE-NT aortae had increases in diameter compared to F-HT/ F-NT and OVX-HT/OVX-NT (P<0.05, Fig 25).

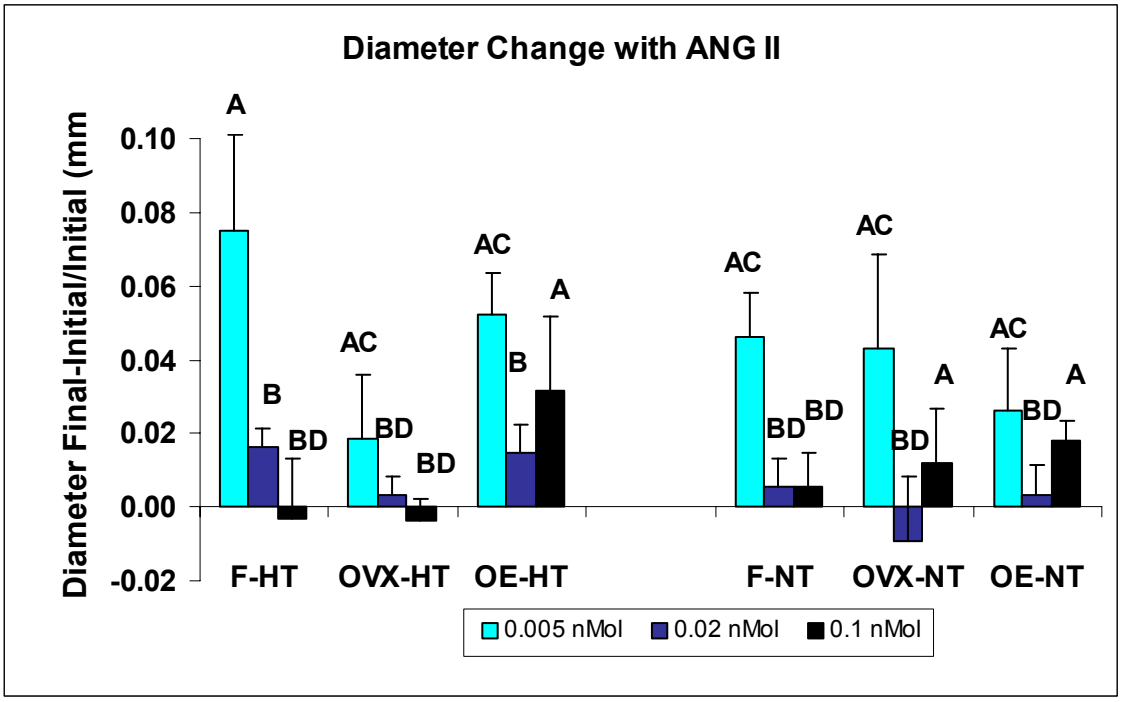


Figure 25: Aortic diameter 15 minutes after each dose of ANG II (0.005, 0.02, and 0.1 nmol). The data were normalized by subtracting the initial diameter (before each ANG II dose) from the end diameter and dividing the result by the initial diameter. A-D: For each experimental treatment, mean values without common superscript are significantly different, P<0.05.

Peak pressures attained by the aortae at increasing doses of ANG II are shown in Fig. 26. ANG II stimulation affected peak pressures attained by the aortae with increasing dose (2-way ANOVA, dose x experimental group, $P<0.003$). OE-HT aortae developed the highest peak pressure (191 ± 10), while F-NT developed the lowest (161 ± 7). Estrogen replacement restored the responses to ANG II and the pressure of OE-HT was elevated compared to F-HT at 0.005 and 0.1 n mol ANG II ($P<0.03$).

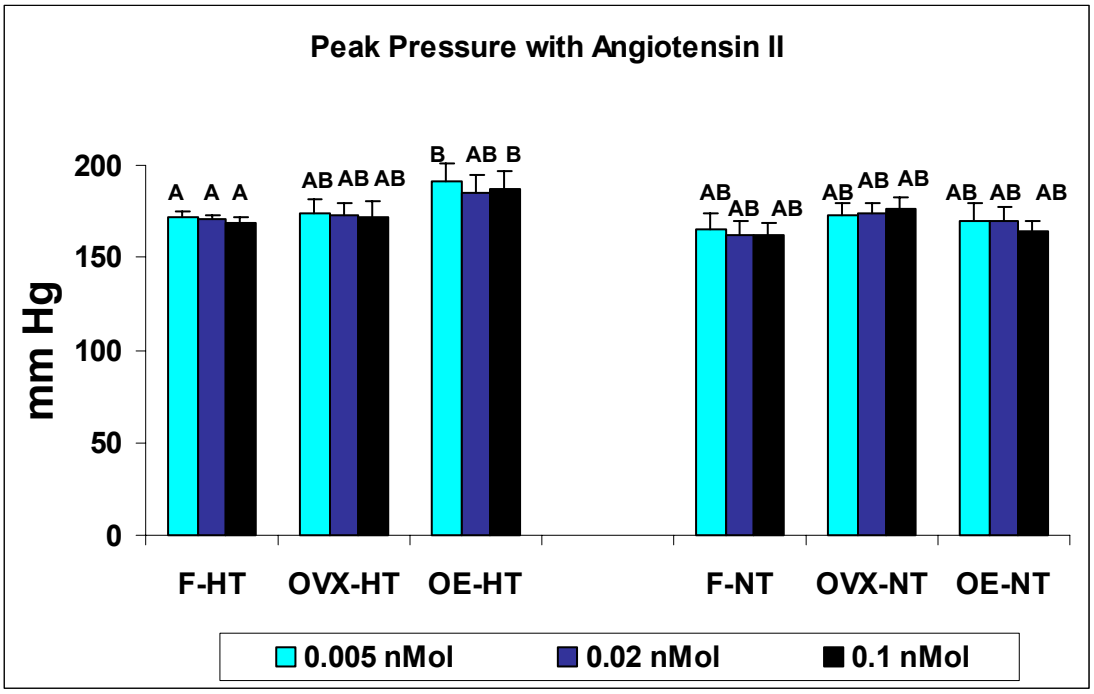


Figure 26: Dose responses of thoracic aortae under constant flow conditions and challenged with increasing doses of ANG II (0.005, 0.02, and 0.1 nmol). Prior to ANG II administration, the pressure was set to approximately 150 mm Hg. Bars represent means ± SE, n = 3-7 (n, no. of animals). A-B: Bars without common script are significantly different, $P<0.05$.

Figure 27 shows the changes in diameter that occurred when the aortae were pressurized to 150 mm Hg (but prior to ANG II stimulation) under constant flow. In general, there was a significant change in diameter when the aortae were pressurized (one-way ANOVA, $P<0.008$). NT aortae had similar increases in diameter with pressurization to 150 mm Hg; however, OE-NT exhibited smaller changes in diameter than did F-NT ($P<0.05$). In the hypertensive groups, F-HT and OE-HT demonstrated significantly smaller increases in diameter with pressurization than their respective NT control groups ($P<0.02$). This is in contrast to OVX-HT, which showed similar increases in diameter as OVX-NT. Ovariectomy resulted in a significant increase in diameter with pressurization (OVX-HT) compared to F-HT ($P<0.04$). The effect of OVX was eliminated with estrogen replacement (i.e., OE-HT had less of an increase in diameter than OVX-HT).

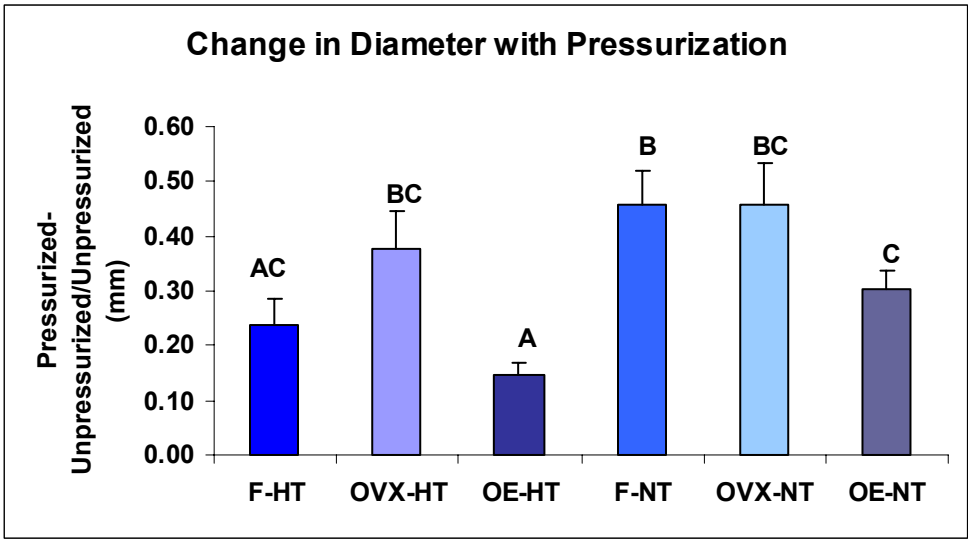


Figure 27: Changes in diameter with pressurization to 150 mm Hg. The diameter of the aorta was determined before pressurization and during constant flow and pressure (150 mm Hg) and before ANG II was administered to determine changes in diameter with loading. The data were normalized by subtracting the initial diameter (before pressurization) from the pressurized diameter and the result divided by the initial diameter. Bars represent means ± SE, n = 3-7 (n, no. of animals). A-C: Bars without common script are significantly different, $P<0.05$.

3.7 Effects of Aortic Coarctation on Expression of Thromboxane Synthase Enzyme

3.7.1 Immunohistochemistry

The presence of the TXA_2 synthase enzyme *in situ* was investigated using immunohistochemistry of thoracic aortic segments (one-way ANOVA, $P<0.02$). All NT groups had similar *in-situ* staining intensity for thromboxane synthase enzyme ($P>0.05$), Figs. 28 and 29. Induction of hypertension increased staining for the enzyme significantly in F-HT and OE-HT ($P<0.04$), whereas M-HT and OVX-HT did not differ compared to their respective NT controls, despite the development of hypertension ($P>0.05$). Ovariectomy not only abolished any response to aortic coarctation, but it also reduced the content of the enzyme in OVX-NT aortae as well. Estrogen replacement restored TXA_2 synthase enzyme levels in both OE-HT and OE-NT.

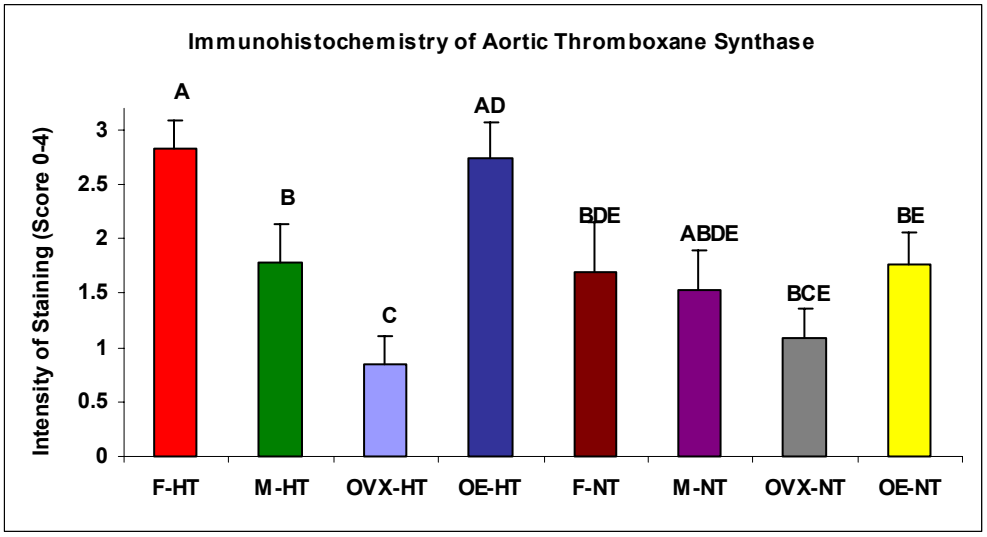


Figure 28: Intensity of staining of thoracic aortae for the thromboxane synthase enzyme using immunohistochemistry. Staining intensity was scored on a relative scale, from 4 (most intense) to 0 (least intense). Bars represent means ± SE, n = 4-10 (n, no. of animals). A-E: Bars without common script are significantly different, $P<0.05$.

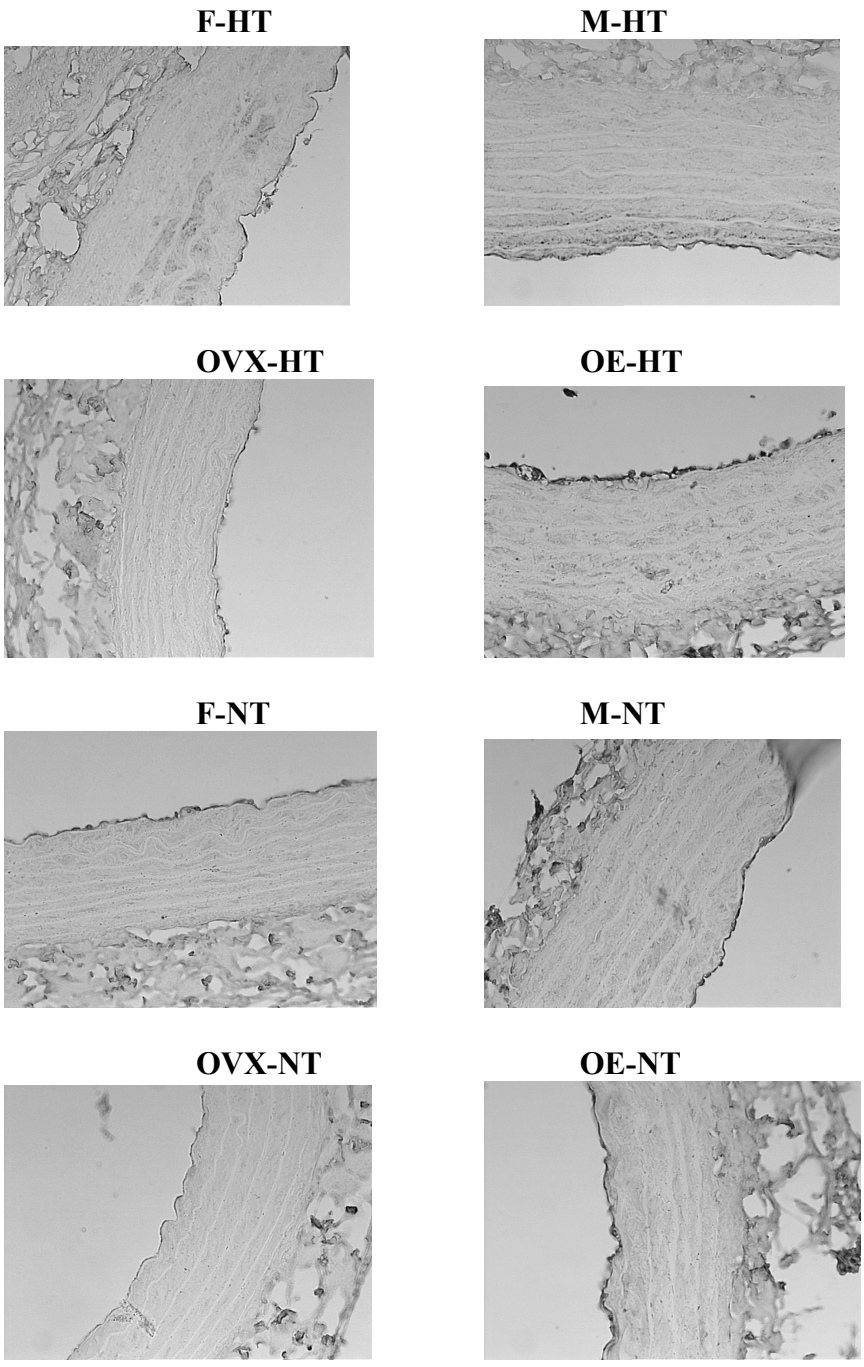


Figure 29: Immunohistochemistry of 4 μm sections of thoracic aortae.

3.7.2 RT-PCR Results

The expression of thromboxane synthase enzyme (TXS) RNA was determined by RT-PCR of TXS mRNA and expressed relative to the mRNA of the housekeeping gene GAPDH as shown in Figure 30. A marked sexual dimorphism was present in the expression of TXS mRNA in both NT and HT groups (one-way ANOVA $P<0.015$). In the NT groups, both F-NT and OE-NT exhibited significantly more enzyme mRNA in their endothelium than M-NT ($P<0.05$). In the HT groups, F-HT expressed significantly more mRNA than did M-HT ($P<0.03$, Table 15). Ovariectomy abolished the differences in mRNA expression between F-HT and M-HT, as OVX-HT and M-HT were similar ($P>0.05$).

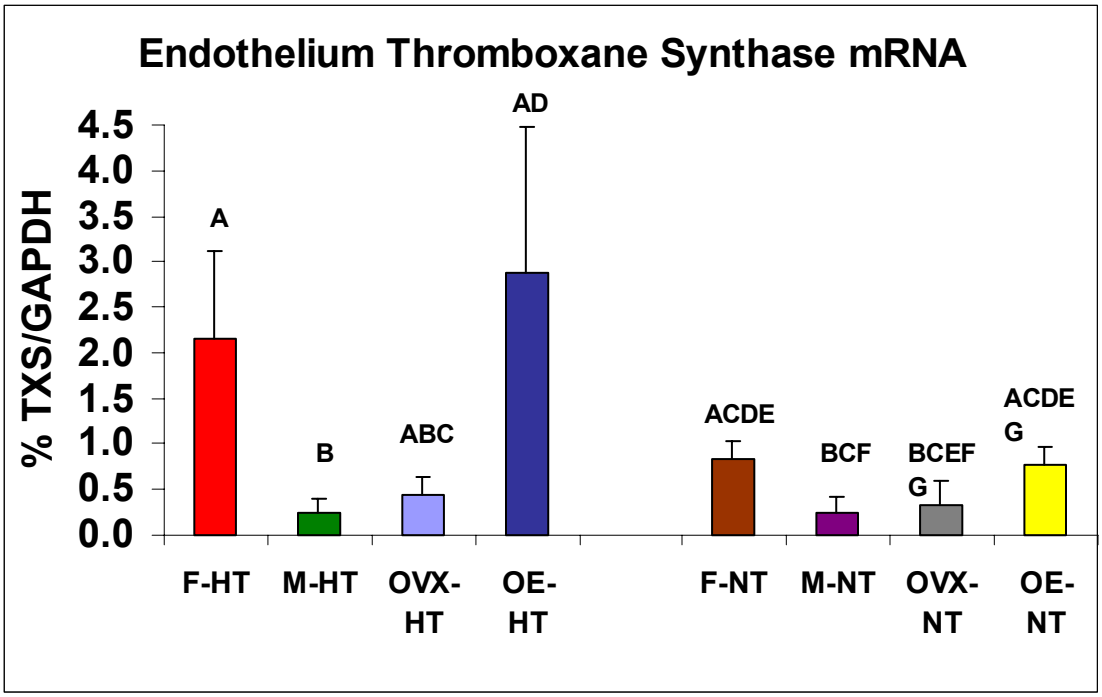


Figure 30: RT-PCR of thromboxane synthase enzyme expression in the endothelium of the thoracic aortae of HT and NT rats. mRNA is expressed as a ratio with GAPDH mRNA. Bars represent means ± SE, n = 4-5 (n, no. of animals). A-G: Bars without common script are significantly different, $P<0.05$.

Estrogen replacement restored enzyme mRNA expression, and OE-HT had significantly more mRNA than M-HT or OVX-HT (P =0.02).

Table 15: Thromboxane synthase mRNA content of aortic endothelium and vascular smooth muscle, normalized by expression of mRNA of the housekeeping gene GAPDH, obtained by RT-PCR.

Aorta Thromboxane Synthase mRNA via RT-PCR		
Group	**Endothelium % Thromboxane Synthase/GAPDH**	**Smooth Muscle % Thromboxane Synthase/GAPDH**
Female Hypertensive n=4	2.16 ± 0.97^{a}	2.27 ± 1.34^{a}
Male Hypertensive n=4	0.25 ± 0.14^{b}	0.38 ± 0.19^{b}
OVX Hypertensive n=4	0.44 ± 0.19^{abc}	0.88 ± 0.45^{ac}
OE Hypertensive n=5	2.87 ± 1.62^{ad}	1.55 ± 0.15^{abd}
Female Normotensive Control n=5	0.83 ± 0.20^{acde}	0.60 ± 0.16^{ace}
Male Normotensive Control n=4	0.23 ± 0.17^{bcf}	0.13 ± 0.05^{bcf}
OVX Normotensive Control n=5	0.33 ± 0.25^{bcefg}	0.24 ± 0.45^{bcfg}
OE Normotensive Control n=4	0.76 ± 0.20^{acdegh}	0.52 ± 0.06^{ceh}

Data are reported as means ± SE; n, no. of animals. A-H: Within rows (treatment groups) and columns (endothelium versus smooth muscle), mean values without common superscript are significantly different, P<0.05.

In vascular smooth muscle, Fig. 31 below, striking sexual dimorphism in thromboxane synthase enzyme expression was observed, similar to that found in the endothelium (one-way ANOVA, P<0.003). F-NT expressed higher levels of mRNA for the thromboxane synthase enzyme than M-NT or OVX-NT aortae (P≤0.03). Estrogen

supplementation restored the mRNA expression that was lost in OVX-NT aortae (OE-NT, $P<0.05$, Table 15). In addition, estrogen supplementation in the OE-HT group induced higher levels of mRNA than the OE-NT counterpart ($P=0.008$).

As with the endothelium in hypertension, vascular smooth muscle expressed significantly more thromboxane synthase mRNA in F-HT than in M-HT ($P=0.03$) and this effect was abolished by ovariectomy (OVX-HT, Table 15 and Fig. 32). OE-HT expressed more mRNA than M-HT, providing further evidence that estrogen enhances the expression of thromboxane synthase mRNA in females during hypertension.

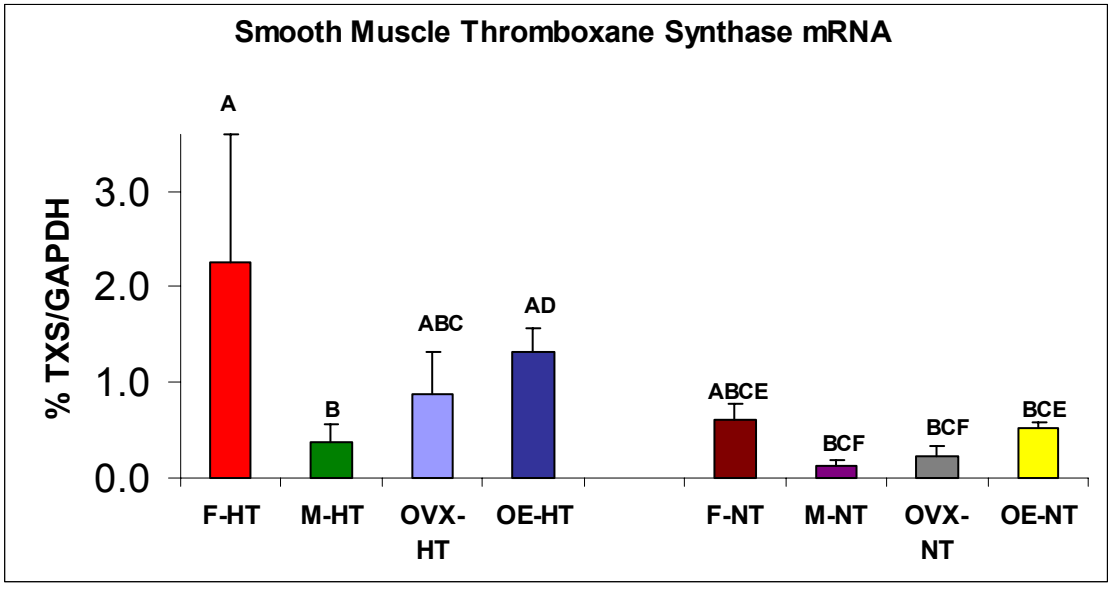


Figure 31: RT-PCR of thromboxane synthase enzyme expression in the vascular smooth muscle of the thoracic aortae of hypertensive and normotensive-control rats. Bars represent means ± SE, n = 4-5 (n, no. of animals). A-F: Bars without common script are significantly different, $P<0.05$.

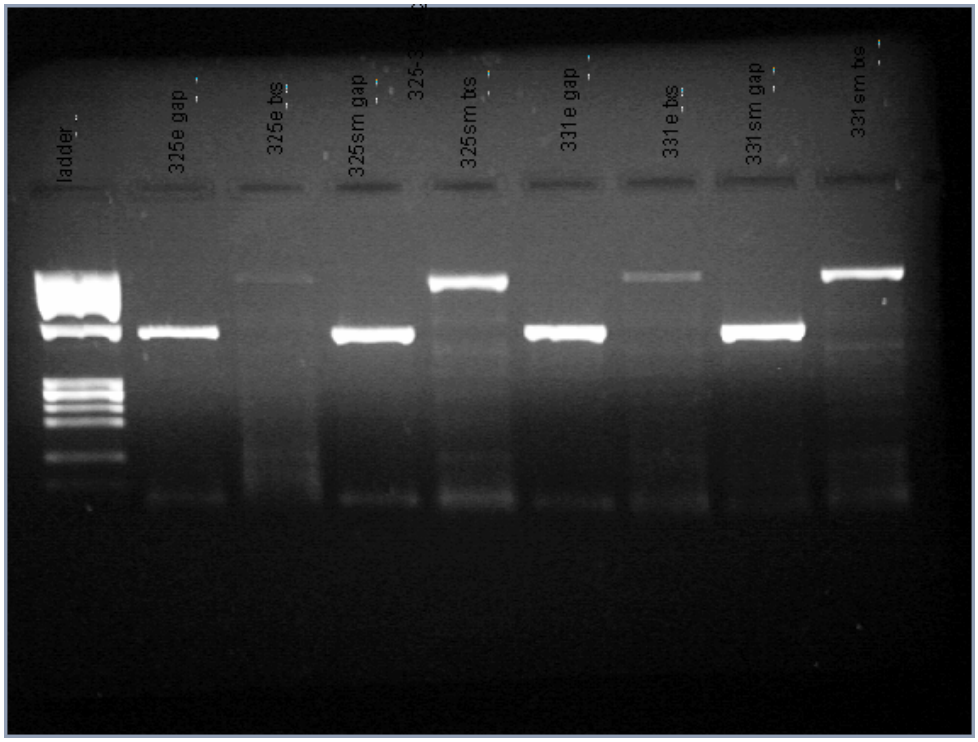


Figure 32: Representative example of gel from RT-PCR analysis of TXA_2 synthase and GAPDH in endothelial and vascular smooth muscle cells. Each lane from left to right is: DNA fragment ladder, endothelial GAPDH from OVX-HT, endothelial TXA_2 synthase from OVX-HT, vascular smooth muscle GAPDH from OVX-HT, vascular smooth muscle TXA_2 synthase from OVX-HT, endothelial GAPDH from OE-HT, endothelial TXA_2 synthase from OE-HT, vascular smooth muscle GAPDH from OE-HT, vascular smooth muscle TXA_2 synthase from OE-HT.

3.8 Effects of Aortic Coarctation on Aortic Morphology

3.8.1 Aortic Wall Thickness, Area, and Wall: Lumen Area Ratio

Aortic wall thickness is the cross-sectional wall thickness (mm) and aortic wall area is the area of the vessel wall in cross-section (mm^2), while aortic wall to lumen ratio is the ratio of the areas of the wall and lumen. All values were normalized by body weights of the individual rats in grams. See Table 16 for statistical results.

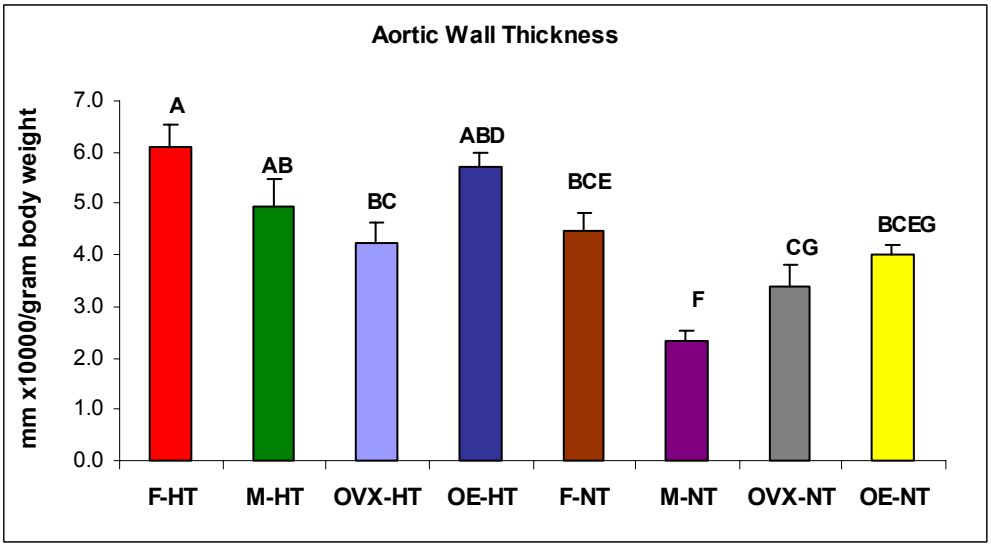


Figure 33: Aortic wall cross-sectional thickness in mm x 10^{-4}. Bars represent means ± SE, n = 4-13 (n, no. of animals). A-G: Bars without common script are significantly different, $P<0.05$.

A significant sexual dimorphism was identified in relative aortic wall thickness in both NT and HT groups as shown in Fig. 33 (one-way ANOVA, $P<0.002$). In normotension, the aortic wall was thicker in F-NT than in M-NT and this difference was abolished by ovariectomy (OVX-NT). Estrogen replacement restored the difference in wall thickness in OE-NT aortae (Table 16). Remarkably, M-NT had the thinnest aortic wall compared to any of the other NT or HT groups ($P<0.05$).

In hypertension, wall thickness of the OVX-HT aortae was less than either F-HT or OE-HT ($P<0.05$). Wall thickness of M-HT and F-HT aortae were significantly greater than their respective NT groups ($P<0.05$). This response to aortic coarctation was abolished by ovariectomy (OVX-HT versus OVX-NT, $P>0.05$). Estrogen replacement in OVX rats restored the increase in wall thickness in response to HT in OE-HT aortae (OE-HT versus OE-NT, $P<0.05$).

Table 16: Aortic morphology of HT and NT rats following aortic coarctation or sham surgery.

Aortic Wall Thickness, Area, and Ratio of Wall to Luminal Areas				
Group	**Ridogrel Administered ? (+/-)**	**Aorta Wall Thickness (mm x 10^{-4})**	**Aorta Wall Area (mm^2 x 10^{-4})**	**Wall Area: Luminal Area (ratio)**
Female Hypertensive n= 13	+	6.0 ± 0.3^{abd}	28.9 ± 1.5^{abcd}	0.0019 ± 0.0001^{adei}
	-	6.1 ± 0.4^{a}	30.8 ± 1.5^{a}	0.0019 ± 0.0002^{a}
Male Hypertensive n= 5	+	4.6 ± 0.4^{abce}	25.6 ± 2.0^{abcdij}	$0.0013 \pm 0.0001^{abceghi}$
	-	4.9 ± 0.5^{ab}	26.0 ± 2.8^{ab}	0.0014 ± 0.0002^{ab}
OVX Hypertensive n= 6	+	4.6 ± 0.2^{abceghjk}	24.6 ± 0.1^{abcdijk}	$0.0014 \pm 0.0001^{abcdeghijk}$
	-	4.2 ± 0.4^{bc}	23.4 ± 3.2^{bc}	0.0012 ± 0.0001^{bc}
OE Hypertensive n= 5	+	5.7 ± 0.2^{abd}	31.4 ± 2.1^{abcdijk}	0.0018 ± 0.0001^{abdi}
	-	5.7 ± 0.2^{abd}	28.9 ± 1.8^{abcd}	0.0017 ± 0.0001^{bd}
Female Normotensive Control n=12	+	4.0 ± 0.5^{bceghkl}	22.4 ± 1.4^{bceghijkl}	$0.0014 \pm 0.0001^{abceghjkl}$
	-	4.5 ± 0.3^{bce}	20.8 ± 1.0^{bce}	0.0013 ± 0.0001^{bcde}
Male Normotensive Control n= 4	+	N/A	N/A	N/A
	-	2.3 ± 0.2^{f}	14.7 ± 0.9^{f}	0.0007 ± 0.0007^{f}
OVX Normotensive Control n= 4	+	3.9 ± 0.3^{bceghl}	19.6 ± 0.6^{bceghl}	$0.0011 \pm 0.0000^{bcghj}$
	-	3.4 ± 0.4^{cg}	16.6 ± 1.9^{cfg}	$0.0011 \pm 0.0002^{bcefg}$
OE Normotensive Control n= 4	+	$4.9 \pm 0.4^{abcdeghijkl}$	23.5 ± 0.6^{bcdeijk}	$0.0015 \pm 0.0001^{abceghl}$
	-	4.0 ± 0.2^{bcegh}	19.2 ± 1.2^{cegh}	$0.0012 \pm 0.0001^{bcegh}$

Values are means ± SE, n, no. of animals. A-L: Within columns for each morphologic parameter, mean values of treatment groups without common superscript are significantly different, $P<0.05$

A clear sexual dimorphism was identified in both NT and HT groups for relative aortic wall area, as shown in Fig. 34 (one-way ANOVA, $P<0.0001$). In normotension, F-NT exhibited a larger wall area than M-NT and this difference was abolished by ovariectomy (OVX-NT), but restored by estrogen replacement in OE-NT aortae (Table 16). M-NT and OVX-NT had the smallest aortic wall areas compared to any of the other NT or HT groups ($P<0.05$).

Aortic wall area increased with hypertension, and both M-HT and F-HT exhibited significant increases in aortic wall area ($P<0.05$) and abolished the M-F difference in aortic wall area in HT rat aortae. This response to aortic coarctation was abolished by ovariectomy (OVX-HT versus OVX-NT, $P>0.05$), and estrogen replacement of OVX rats restored the increase in wall area in OE-HT rats ($P<0.05$).

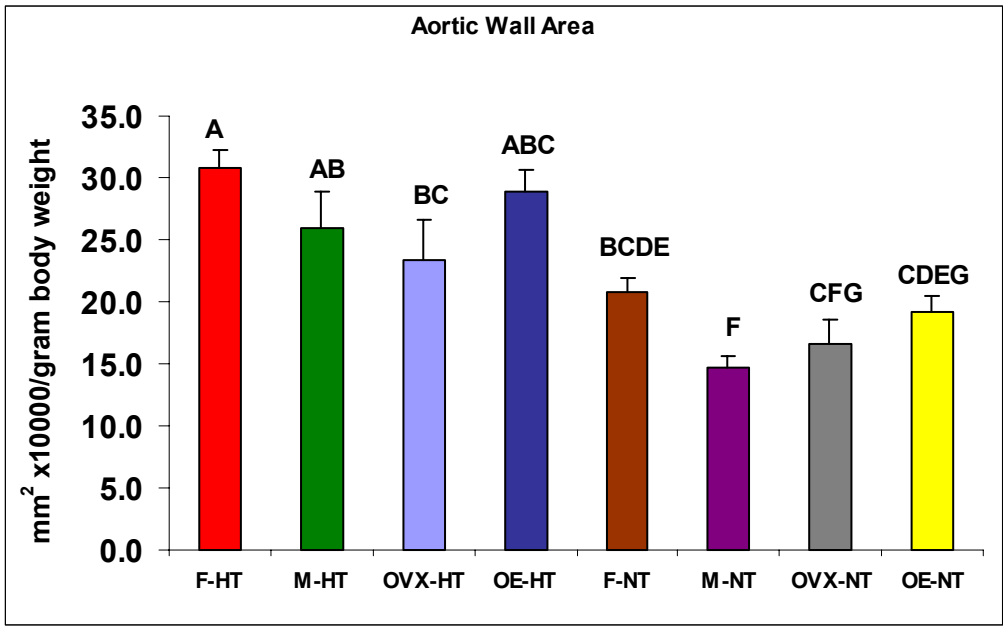


Figure 34: Cross-sectional aortic wall area in $mm^2 \times 10^{-4}$ per gram body weight. Bars represent means ± SE, n = 4-13 (n, no. of animals). A-G: Bars without common script are significantly different, $P<0.05$.

Figure 35 shows the wall: luminal area ratios, which also exhibited sexual dimorphism (one-way ANOVA, $P<0.0001$). Among the NT groups, M-NT exhibited the smallest wall to luminal ratio ($P<0.05$, Fig. 35). In the HT groups, OVX-HT had the smallest ratio ($P<0.05$). Wall to luminal area ratios increased significantly in M-HT and F-HT compared to their NT counterparts ($P<0.02$). This effect was abolished in females by ovariectomy, as OVX-HT had a similar ratio to OVX-NT ($P>0.05$). The increase in ratio seen in F-HT was restored in OE-HT by estrogen supplementation ($P<0.02$). F-HT and OE-HT ratios were greater than OVX-HT ($P<0.05$, Fig. 35).

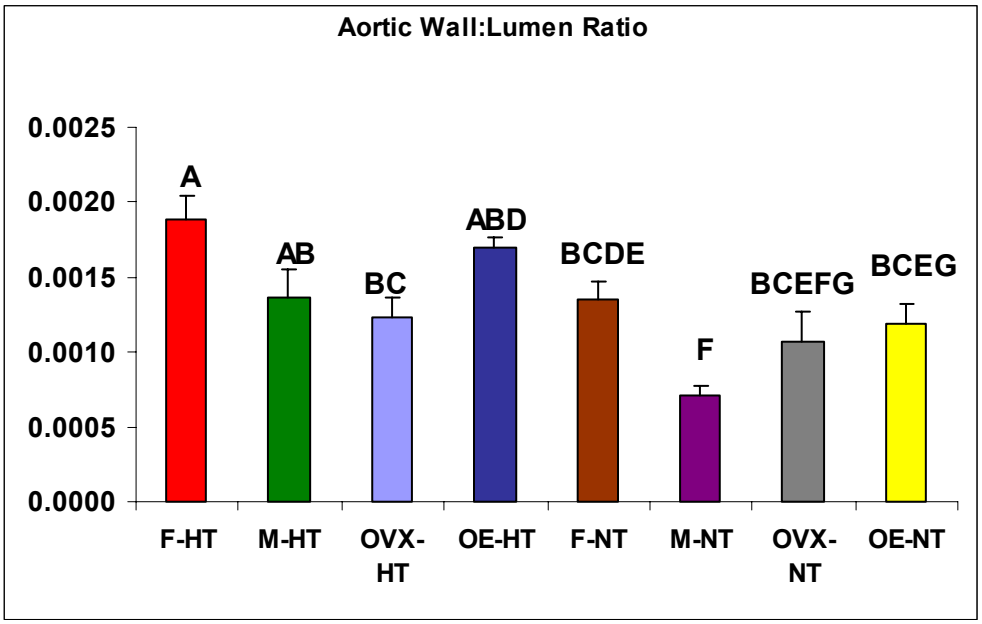
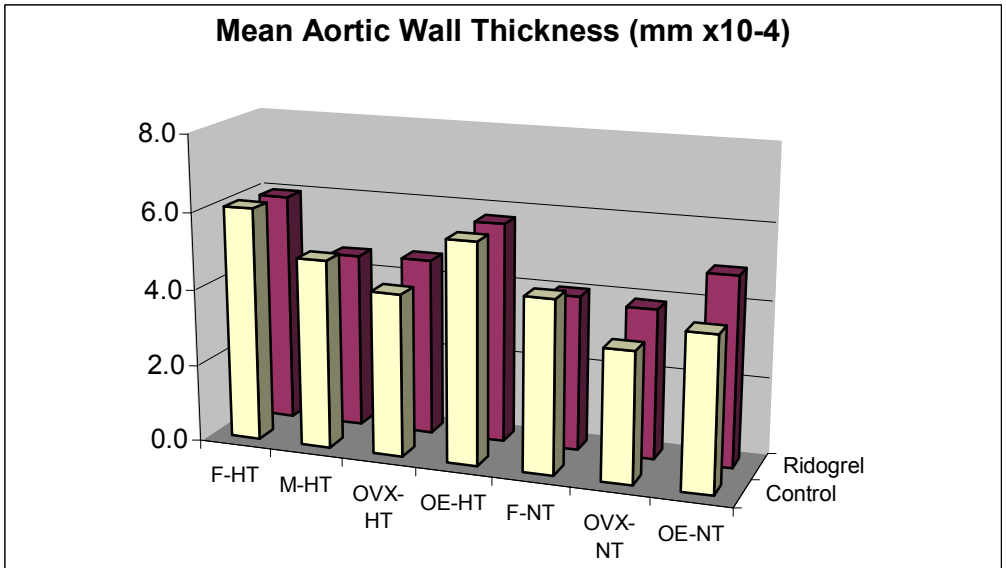


Figure 35: Aortic wall: lumen area ratios. Bars represent means ± SE, n = 4-13 (n, no. of animals). A-G: Bars without common script are significantly different, $P<0.05$.

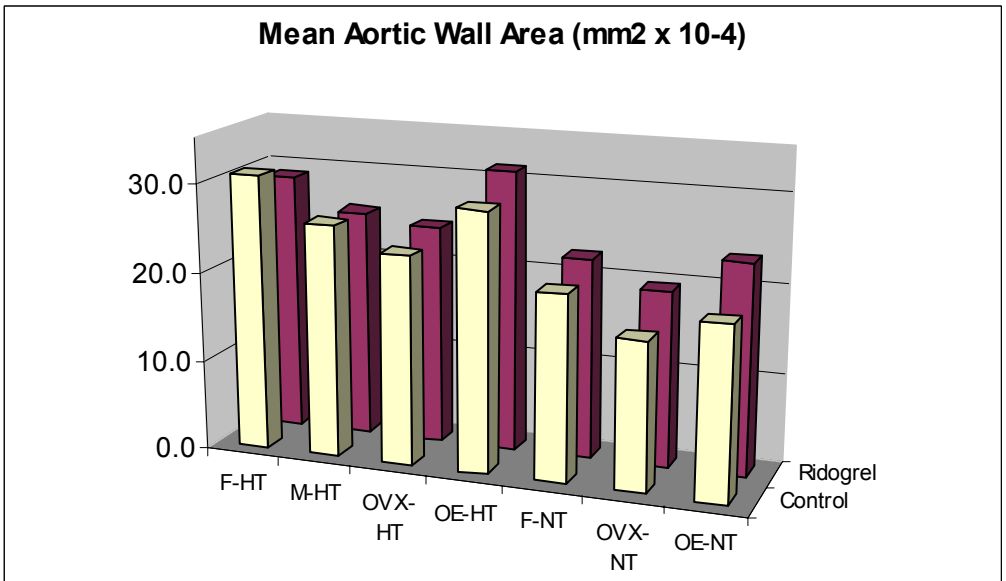
3.8.2 The Effect of Ridogrel on Aortic Wall Thickness, Area, and Wall to Luminal Area Ratio

The contrasting effects of Ridogrel and vehicle-control treatments on aortic thickness are shown in Figure 36a. Ridogrel treatment did not abolish the sexual dimorphism found in aortic thickness (one-way ANOVA, $P<0.001$). With the exception of OE-NT, aortic thickness of NT groups was not affected by Ridogrel administration ($P>0.05$), Tables 16 and 17 and Fig. 36a. OE-NT exhibited an increase in aorta thickness (mm^2/gram body weight) with the administration of Ridogrel ($P<0.04$). Similar to the vehicle-control treated groups (see Section3.8.1 above and Fig. 36a), F-HT and OE-HT exhibited aortic walls thicker than those of M-HT and OVX-HT rats ($P<0.04$). F-HT, OVX-HT, and OE-HT rats developed increased wall thicknesses with aortic coarctation despite Ridogrel administration ($P<0.05$, Fig. 36a). Estrogen replacement restored the sexual dimorphism in aortic wall thickness in OE-HT that was reduced by OVX and was not affected by TXB_2 receptor and synthase inhibition with Ridogrel.



	F-HT	M-HT	OVX-HT	OE-HT	F-NT	OVX-NT	OE-NT
Control	6.1	4.9	4.2	5.7	4.5	3.4	4.0
Ridogrel	6.0	4.6	4.6	5.7	4.0	3.9	4.9

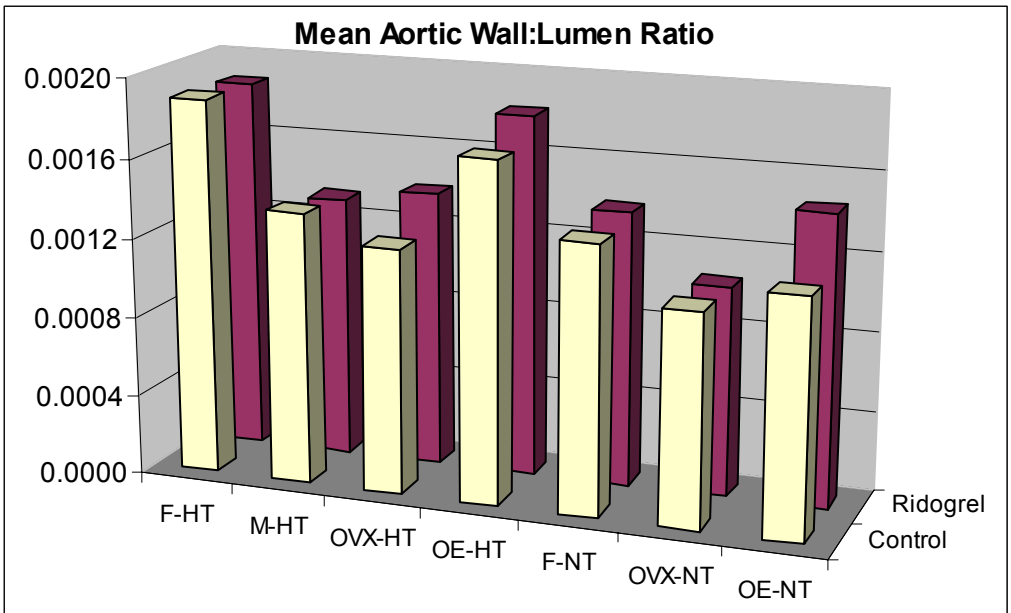
(a)



	F-HT	M-HT	OVX-HT	OE-HT	F-NT	OVX-NT	OE-NT
Control	30.8	26.0	23.4	28.9	20.8	16.6	19.2
Ridogrel	28.9	25.6	24.6	31.4	22.4	19.6	23.5

(b)

Figure 36: Aortic morphology with and without Ridogrel (a: wall thickness in cross-section, mm; b: wall area in cross-section, mm^2, c: wall to luminal ratio). All values were normalized by dividing the parameter by the weight of the rat in grams. Bars represent means ± SE, n = 4-13 (n, no. of animals).



	F-HT	M-HT	OVX-HT	OE-HT	F-NT	OVX-NT	OE-NT
Control	0.0019	0.0014	0.0012	0.0017	0.0013	0.0011	0.0012
Ridogrel	0.0019	0.0013	0.0014	0.0018	0.0014	0.0011	0.0015

(c)

Figure 36 (continued)

Table 17: Aortic wall thickness statistical results.

	F-HT-R	F-HT-V	M-HT-R	M-HT-V	OVX-HT-R	OVX-HT-V	OE-HT-R	OE-HT-V	F-NT-R	F-NT-V	M-NT-V	OVX-NT-R	OVX-NT-V	OE-NT-R	OE-NT-V
F-HT-R	-	NS	*	NS	*	*	*	NS	*	*	*	*	*	NS	*
F-HT-V	NS	-	NS	NS	NS	*	NS	NS	*	*	*	*	*	NS	*
M-HT-R	*	NS	-	NS	NS	NS	*	*	NS	NS	*	NS	*	NS	NS
M-HT-V	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	*	NS	*	NS	NS
OVX-HT-R	*	NS	NS	NS	-	NS	*	*	NS	NS	*	*	NS	NS	NS
OVX-HT-V	*	*	NS	NS	NS	-	*	*	NS	NS	*	NS	NS	NS	NS
OE-HT-R	*	NS	*	NS	*	*	-	NS	*	*	*	*	*	*	*
OE-HT-V	NS	NS	*	NS	*	*	NS	-	*	*	*	*	*	NS	*
F-NT-R	*	*	NS	NS	NS	NS	*	*	-	NS	*	NS	NS	NS	NS
F-NT-V	*	*	NS	NS	NS	NS	*	*	NS	-	*	NS	*	NS	NS
M-NT-V	*	*	*	*	*	*	*	*	*	*	-	*	*	*	*
OVX-NT-R	*	*	NS	NS	*	NS	*	*	NS	NS	*	-	NS	NS	*
OVX-NT-V	*	*	*	*	NS	NS	*	*	NS	*	*	NS	-	NS	NS
OE-NT-R	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	*	NS	NS	-	*
OE-NT-V	*	*	NS	NS	NS	NS	*	*	NS	NS	*	*	NS	*	-

* indicates P<0.05, NS indicates P>0.05 (no significant difference), R = Ridogrel treated, V= vehicle treated

Changes in aortic wall area in response to Ridogrel or vehicle-control treatments are shown in Fig. 36b and Table 18. The observed sexual dimorphism in wall area with HT remained despite Ridogrel treatment (one-way ANOVA, $P<0.001$). Ridogrel administration resulted in larger aortic areas in OE-NT compared to OVX-NT ($P<0.002$); otherwise, there were no significant differences in the NT groups with Ridogrel versus vehicle ($P>0.05$). All HT groups exhibited increases in aortic area compared to their respective NT counterparts ($P<0.02$). Wall area did not differ between F-HT and M-HT with Ridogrel, which was similar to F-HT and M-HT groups that received the vehicle-control ($P>0.05$). However, OE-HT exhibited a larger wall area than M-HT only when Ridogrel was administered ($P<0.05$). Both Ridogrel and vehicle-treated F-HT and OE-HT animals showed increased aortic wall areas compared to OVX-HT ($P<0.05$) indicating estrogen's effect on aortic wall area in hypertension was not inhibited and may be enhanced by TXB_2 receptor and synthase inhibition with Ridogrel (Table 18).

Table 18: Aortic wall area statistical results.

	F-HT-R	F-HT-V	M-HT-R	M-HT-V	OVX-HT-R	OVX-HT-V	OE-HT-R	OE-HT-V	F-NT-R	F-NT-V	M-NT-V	OVX-NT-R	OVX-NT-V	OE-NT-R	OE-NT-V
F-HT-R	-	NS	NS	NS	NS	NS	NS	NS	NS	*	*	*	*	NS	*
F-HT-V	NS	-	NS	NS	NS	*	NS	NS	*	*	*	*	*	*	*
M-HT-R	NS	NS	-	NS	NS	NS	NS	NS	NS	*	*	*	*	NS	*
M-HT-V	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	*	NS	*	NS	*
OVX-HT-R	NS	NS	NS	NS	-	NS	NS	NS	NS	*	*	*	*	NS	*
***OVX-HT-V**	NS	*	NS	NS	NS	-	NS	NS	NS	NS	*	NS	NS	NS	NS
OE-HT-R	NS	NS	NS	NS	NS	NS	-	NS	*	*	*	*	*	*	*
OE-HT-V	NS	NS	NS	NS	NS	NS	NS	-	*	*	*	*	*	NS	*
F-NT-R	NS	*	NS	NS	NS	NS	*	*	-	NS	*	NS	NS	NS	NS
F-NT-V	*	*	*	NS	*	NS	*	*	NS	-	*	NS	*	NS	NS
M-NT-V	*	*	*	*	*	*	*	*	*	*	-	*	NS	*	*
OVX-NT-R	*	*	*	NS	*	NS	*	*	NS	NS	*	-	NS	*	NS
OVX-NT-V	*	*	*	*	*	NS	*	*	NS	*	NS	NS	-	*	NS
OE-NT-R	NS	*	NS	NS	NS	NS	*	NS	NS	NS	*	*	*	-	*
I OE-NT-V	*	*	*	*	*	NS	*	*	NS	NS	*	NS	NS	*	-

* indicates P<0.05, NS indicates P>0.05 (no significant difference), R = Ridogrel treated, V= vehicle treated

Aortic wall area: lumen area ratios with Ridogrel and vehicle -control treatments are shown in Fig. 36c. The observed sexual dimorphism in aortic wall: lumen area ratio remained following aortic coarctation even with Ridogrel administration (one-way ANOVA, $P<0.001$). No significant differences were observed among the wall: lumen ratios in any of the treatment groups with administration of Ridogrel ($P>0.05$, when Ridogrel treatment was compared to vehicle-control treatment for each group, Table 19). In the Ridogrel and vehicle-control groups, both F-HT and OE-HT exhibited elevated ratios compared to M-HT and OVX-HT aortae ($P<0.02$). No differences were observed in ratios of M-HT and OVX-HT aortae ($P>0.05$). There was a significant difference between the ovariectomized groups, however. Without Ridogrel OVX-HT did not differ from OVX-NT, but with Ridogrel, the wall: lumen ratio of OVX-HT rats was significantly higher than that of OVX-NT rats ($P<0.03$). In the absence of ovarian steroids (OVX), Ridogrel increased the aortic ratio with the induction of HT; however, when estrogen or testosterone was present (F, M and OE), Ridogrel had no effect (Table 19).

Table 19: Aortic wall: lumen area ratio statistical results.

	F-HT-R	F-HT-V	M-HT-R	M-HT-V	OVX-HT-R	OVX-HT-V	OE-HT-R	OE-HT-V	F-NT-R	F-NT-V	M-NT-V	OVX-NT-R	OVX-NT-V	OE-NT-R	OE-NT-V
F-HT-R	-	NS	*	*	NS	*	NS	NS	*	NS	*	*	*	*	*
F-HT-V	NS	-	NS	NS	NS	*	NS	NS	NS	*	*	*	*	NS	*
M-HT-R	*	NS	-	NS	NS	NS	*	*	NS	NS	*	NS	NS	NS	NS
M-HT-V	*	NS	NS	-	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS
OVX-HT-R	NS	NS	NS	NS	-	NS	*	NS	NS	NS	*	*	NS	NS	NS
OVX-HT-V	*	*	NS	NS	NS	-	*	*	NS	NS	*	NS	NS	NS	NS
OE-HT-R	NS	NS	*	NS		*	-	NS	*	*	*	*	*	*	*
OE-HT-V	NS	NS	*	NS	NS	*	NS	-	*	NS	*	*	*	*	*
F-NT-R	*	NS	NS	NS	NS	NS	*	*	-	NS	*	*	NS	NS	NS
F-NT-V	NS	*	NS	NS	NS	NS	*	NS	NS	-	*	NS	NS	NS	NS
M-NT-V	*	*	*	*	*	*	*	*	*	*	-	*	NS	*	*
OVX-NT-R	*	*	NS	NS	*	NS	*	*	*	NS	*	-	NS	*	NS
OVX-NT-V	*	*	NS	NS	NS	NS	*	*	NS	NS	NS	NS	-	NS	NS
OE-NT-R	*	NS	NS	NS	NS	NS	*	*	NS	NS	*	*	NS	-	NS
OE-NT-V	*	*	NS	NS	NS	NS	*	*	NS	NS	*	NS	NS	NS	-

*indicates P<0.05, NS indicates P>0.05 (no significant difference), R = Ridogrel treated, V= vehicle treated

3.9 Effects of Aortic Coarctation on Aortic Collagen and Elastin

3.9.1 Collagen and Elastin Density

Aortic collagen density following aortic coarctation is shown in Figs. 37 and 38.The effects of Ridogrel on aortic collagen and elastin will be addressed in Section 3.9.2 below. Significant differences in collagen density of the thoracic aortae were observed (one-way ANOVA, $P<0.004$). If all groups of rats were compared excluding male rats, significant differences were still present (one-way ANOVA, $P<0.003$). Aortic collagen density was similar among NT groups of rats. F-HT increased in collagen content with aortic coarctation compared to the F-NT control ($P<0.016$). In females, ovariectomy abolished collagen responses to HT, since collagen density in OVX-HT remained unchanged after coarctation compared to OVX-NT aortae. Estrogen replacement in OE-HT rats restored the response to coarctation lost with OVX, resulting in an increase in collagen ($P<0.016$). Collagen density was significantly higher in F-HT than in M-HT or OVX-HT ($P<0.04$), but F-HT and OE-HT exhibited similar densities ($P>0.05$). See Tables 20 and 21.

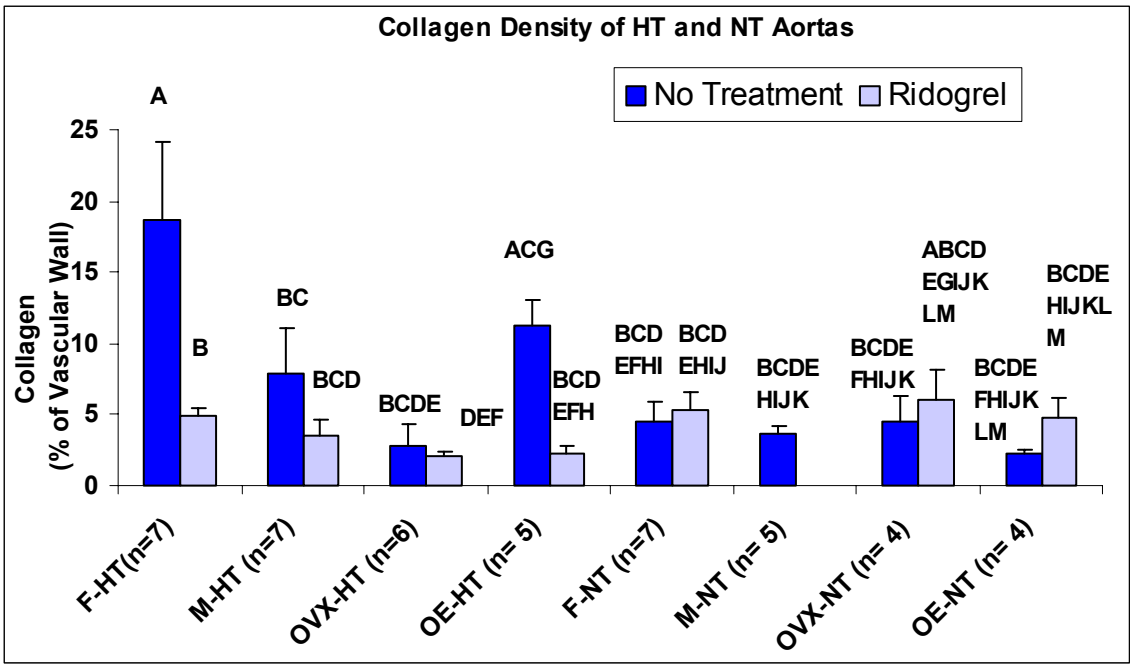


Figure 37: Collagen density following aortic coarctation-induced hypertension. Normotensive-control males were not administered Ridogrel. Bars represent means ± SE, n = 4-7 (n, no. of animals). A-M: Bars without common script are significantly different, $P<0.05$.

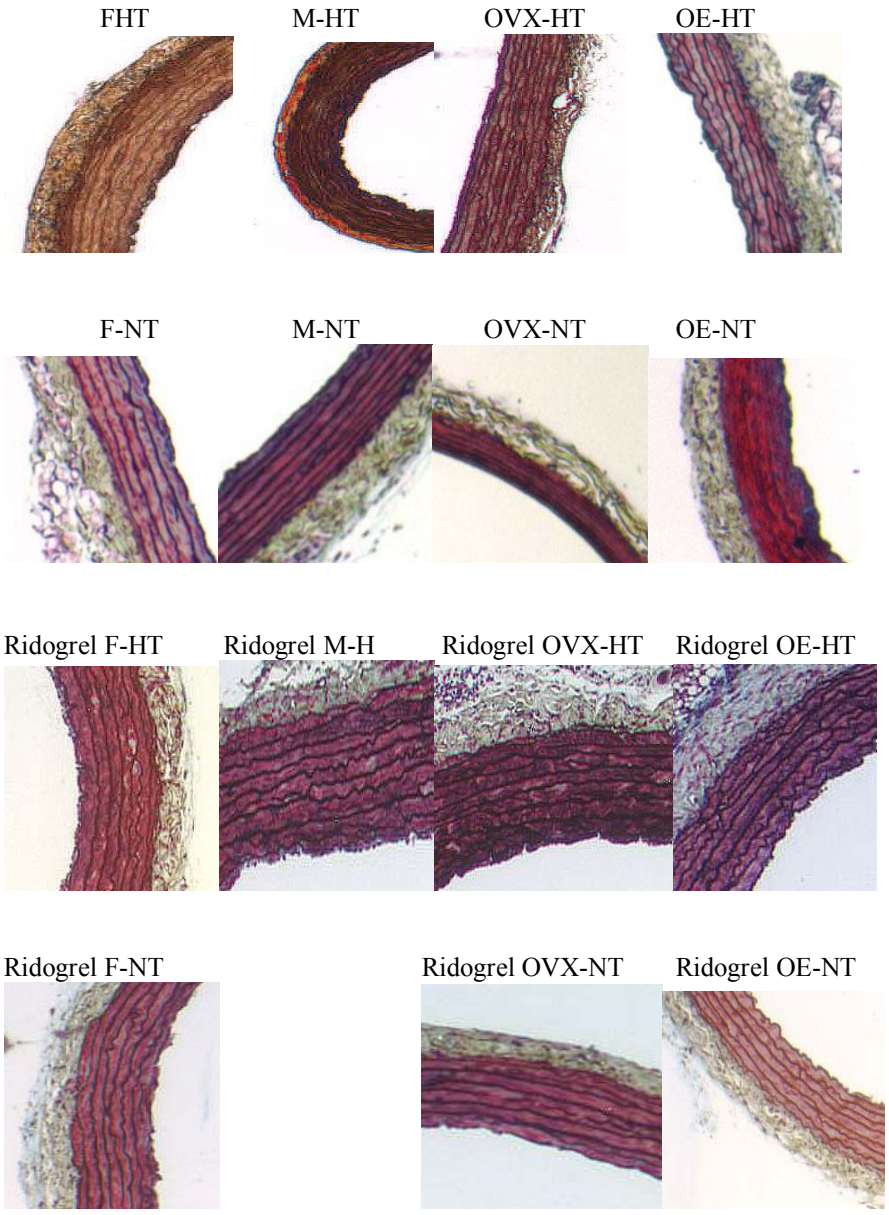


Figure 38: Movat's stain for elastin and collagen density. Elastin stains black to dark purple and collagen stains yellow. Note the yellow appearance of F-HT and OE-HT aortae compared to their respective controls (F-NT and OE-NT) and the other hypertensive aortae: M-HT and OVX-HT. Aortae of Ridogrel-treated F-HT and OE-HT rats exhibited much less collagen staining than F-HT and OE-HT not treated with Ridogrel.

Table 20: Statistical results of aortic collagen density.

	F-HT-R	F-HT-V	M-HT-R	M-HT-V	OVX-HT-R	OVX-HT-V	OE-HT-R	OE-HT-V	F-NT-R	F-NT-V	M-NT-V	OVX-NT-R	OVX-NT-V	OE-NT-R	OE-NT-V
F-HT-R	-	*	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS	NS	NS
F-HT-V	*	-	*	*	*	*	*	NS	*	*	*	NS	*	*	*
M-HT-R	NS	*	-	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS
M-HT-V	NS	*	NS	-	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
OVX-HT-R	*	*	NS	*	-	NS	NS	*	*	NS	*	*	NS	*	NS
OVX-HT-V	NS	*	NS	NS	NS	-	NS	*	NS	NS	NS	NS	NS	NS	NS
OE-HT-R	NS	*	NS	NS	NS	NS	-	*	NS	NS	NS	*	NS	NS	NS
OE-HT-V	*	NS	*	NS	*	*	*	-	*	*	*	NS	*	*	*
F-NT-R	NS	*	NS	NS	*	NS	NS	*	-	NS	NS	NS	NS	NS	NS
F-NT-V	NS	*	NS	NS	NS	NS	NS	*	NS	-	NS	NS	NS	NS	NS
M-NT-V	NS	*	NS	NS	NS	NS	NS	*	NS	NS	-	NS	NS	NS	NS
OVX-NT-R	NS	NS	NS	NS	*	NS	*	NS	NS	NS	NS	-	NS	NS	NS
OVX-NT-V	NS	*	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	-	NS	NS
OE-NT-R	NS	*	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS	-	NS
OE-NT-V	NS	*	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	-

* indicates $P<0.05$, NS indicates $P>0.05$ (no significant difference), R = Ridogrel treated, V= vehicle treated

Table 21: Aortic elastin and collagen densities and collagen: elastin ratio of HT and NT aortae.

Aorta Elastin Density, Collagen Density, and Collagen: Elastin Ratio Mean ± SE				
Group	Ridogrel ? (+/-)	Elastin Density (% of Wall)	Collagen Density (% of Wall)	Collagen: Elastin (ratio)
Female Hypertensive n=4-7	+	32.1 ± 3.9^{a}	5.0 ± 0.5^{a}	0.16 ± 0.02^{a}
	-	53.3 ± 2.3^{b}	18.7 ± 5.5^{b}	0.35 ± 0.10^{ab}
Male Hypertensive n=4-7	+	33.4 ± 3.9^{ac}	3.5 ± 1.1^{ac}	0.11 ± 0.04^{ac}
	-	37.3 ± 3.5^{acd}	7.8 ± 3.2^{acd}	0.26 ± 0.09^{abcd}
OVX Hypertensive n=4-6	+	27.6 ± 2.4^{ace}	2.2 ± 0.2^{ce}	0.08 ± 0.01^{cde}
	-	46.9 ± 3.7^{df}	2.8 ± 1.5^{acde}	0.06 ± 0.03^{cef}
OE Hypertensive n=5	+	24.0 ± 2.1^{aeg}	2.3 ± 0.5^{cdef}	0.10 ± 0.03^{acdefg}
	-	34.4 ± 3.5^{cdeh}	11.3 ± 1.8^{bdg}	0.36 ± 0.09^{bdh}
Female Normotensive Control n=4-7	+	32.5 ± 6.3^{acdeghi}	5.4 ± 1.2^{acdeh}	0.19 ± 0.06^{abcdeghi}
	-	39.6 ± 3.7^{acdehij}	4.6 ± 1.3^{acdefhi}	0.11 ± 0.02^{acdefgij}
OVX Normotensive Control n=4	+	34.5 ± 3.5^{adcehijk}	6.1 ± 2.1^{abcdeghij}	$0.17 \pm 0.06^{abcdeghij}$
	-	$41.9 \pm 7.3^{acdefhijkl}$	4.5 ± 1.8^{acdefhijk}	$0.12 \pm 0.06^{abcdefgijk}$
OE Normotensive Control n=4	+	$30.2 \pm 2.9^{acdeghijklm}$	4.8 ± 1.5^{acdefhijkl}	$0.17 \pm 0.02^{abcdefghijkl}$
	-	$46.2 \pm 9.0^{abcdfhijklmn}$	2.2 ± 0.4^{cdefijk}	$0.06 \pm 0.02^{cdefgjklm}$
Male Normotensive Control n=4	+	N/A	N/A	N/A
	-	$47.2 \pm 7.1^{bcdfhijkln}$	3.7 ± 0.6^{acdefhijkl}	$0.09 \pm 0.02^{cdefgijklm}$

Data are reported as means ± SE; n, no. of animals. A-N: Within columns only for each experimental treatment, mean values without common superscript are significantly different, $P<0.05$.

Aortic elastin density following aortic coarctation is shown in Figs. 38 and 39, and in Tables 21 and 22. Significant differences in elastin density of the thoracic aortae were seen (one-way ANOVA, $P<0.05$). When all female groups were compared (excluding all male rats), significant differences persisted (one-way ANOVA, $P<0.04$). Aortic elastin density was similar among all NT groups of rats. Among the HT groups, F-HT increased in elastin content with aortic coarctation compared to the F-NT control ($P<0.05$). OVX-HT also increased elastin content compared to OVX-NT ($P<0.02$). Estrogen replacement in OE-HT rats did not increase elastin ($P>0.05$), indicating elastin content is not dependent upon estrogen. M-HT elastin content did not differ from M-NT, either. Among the HT groups, F-HT had the highest elastin content ($P<0.05$).

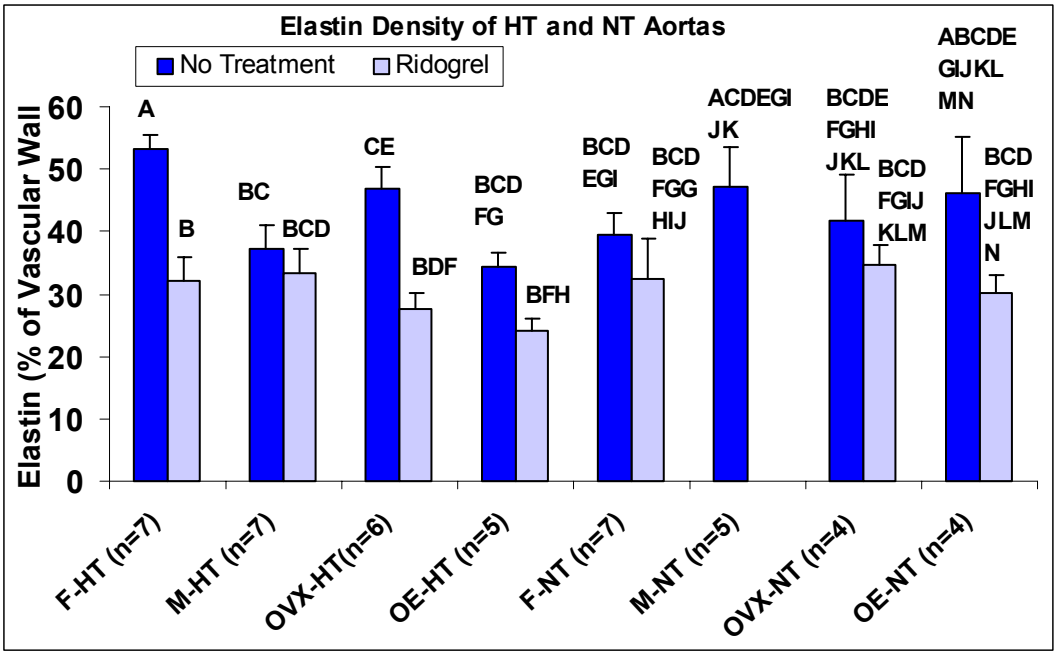


Figure 39: Elastin density following aortic coarctation or sham surgery. Bars represent means ± SE, n = 4-7 (n, no. of animals). A-N: Bars without common script are significantly different, $P<0.05$.

Table 22: Statistical results of aortic elastin density.

	F-HT-R	F-HT-V	M-HT-R	M-HT-V	OVX-HT-R	OVX-HT-V	OE-HT-R	OE-HT-V	F-NT-R	F-NT-V	M-NT-V	OVX-NT-R	OVX-NT-V	OE-NT-R	OE-NT-V
F-HT-R	-	*	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	NS	NS
F-HT-V	*	-	*	*	*	*	*	*	*	*	NS	*	*	*	NS
M-HT-R	NS	*	-	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS
M-HT-V	NS	*	NS	-	*	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
OVX-HT-R	NS	*	NS	*	-	*	*	NS	NS	*	*	NS	NS	NS	*
OVX-HT-V		*	*	NS	*	-	*	*	*	NS	NS	*	NS	*	NS
OE-HT-R	NS	*	*	*	*	*	-	*	NS	*	*	*	NS	NS	*
OE-HT-V	NS	*	NS	NS	NS	*	*	-	NS	NS	NS	NS	NS	NS	NS
F-NT-R	NS	*	NS	NS	NS	*	NS	NS	-	NS	NS	NS	NS	NS	NS
F-NT-V	NS	*	NS	NS	*	NS	*	NS	NS	-	NS	NS	NS	NS	NS
M-NT-V	*	NS	NS	NS	*	NS	*	NS	NS	NS	-	NS	NS	*	NS
OVX-NT-R	NS	*	NS	NS	NS	*	*	NS	NS	NS	NS	-	NS	NS	NS
OVX-NT-V	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS
OE-NT-R	NS	*	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	-	NS
OE-NT-V	NS	NS	NS	NS	*	NS	*	NS	NS	NS	NS	NS	NS	NS	-

* indicates P<0.05, NS indicates P>0.05 (no significant difference), R = Ridogrel treated, V= vehicle treated.

Aortic collagen: elastin density ratios following aortic coarctation .are shown in Figs. 38 and 40, and in Tables 21 and 23. Aortic collagen: elastin ratio was significantly different among the treatment groups (one-way ANOVA, P<0.015). Significant differences were present among the female groups even without the influence of the male groups (one-way ANOVA, P<0.006). The ratio was similar among all NT groups of rats (P>0.05). In, the HT groups, F-HT increased in collagen: elastin ratio with aortic coarctation compared to F-NT (P<0.05). In F, ovariectomy abolished the response to HT with respect to collagen: elastin ratio since the ratio in OVX-HT remained unchanged after coarctation compared to OVX-NT aortae (P>0.05).

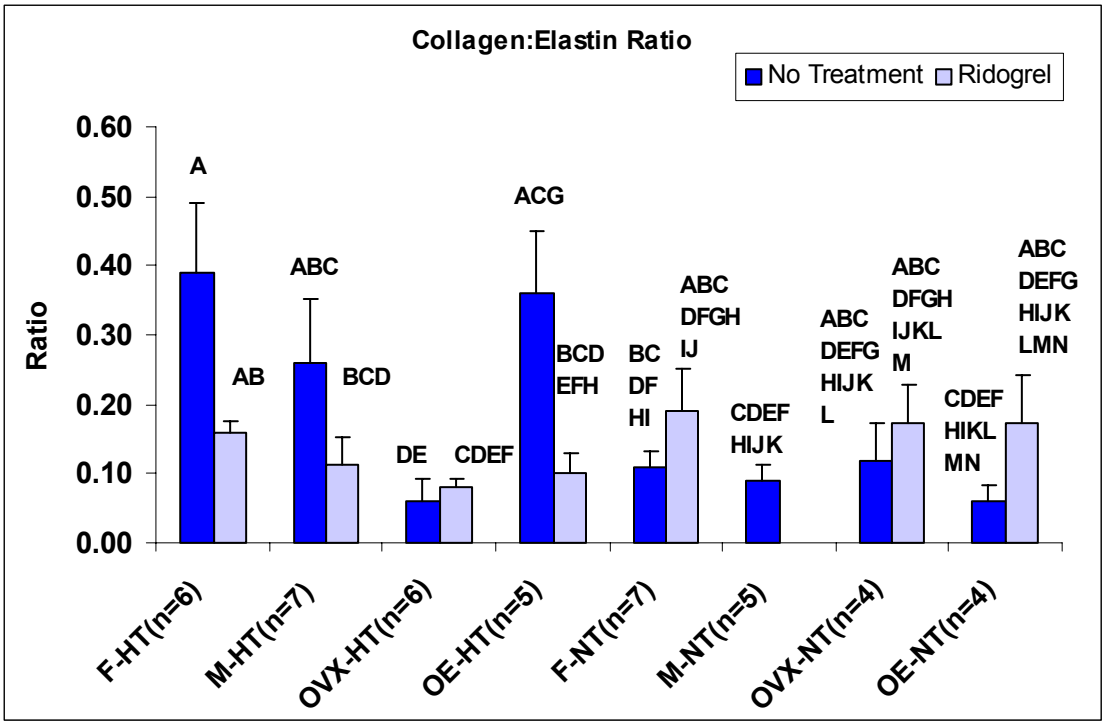


Figure 40: Collagen: elastin ratio following aortic coarctation or sham surgery. Bars represent means ± SE, n = 4-7 (n, no. of animals). A-N: Bars without common script are significantly different, P<0.05.

Table 23: **Statistical results of the aortic collagen: elastin ratio.**

	F-HT-R	F-HT-V	M-HT-R	M-HT-V	OVX-HT-R	OVX-HT-V	OE-HT-R	OE-HT-V	F-NT-R	F-NT-V	M-NT-V	OVX-NT-R	OVX-NT-V	OE-NT-R	OE-NT-V
F-HT-R	-	NS	NS	NS	*	*	NS	*	NS	NS	*	NS	NS	NS	*
F-HT-V	NS-	-	*	NS	*	*	*	NS	NS	*	*	NS	NS	NS	*
M-HT-R	NS	*	-	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS
M-HT-V	NS	NS	NS	-	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
OVX-HT-R	*	*	NS	NS	-	NS	NS	*	NS	*	NS	NS	NS	NS	NS
OVX-HT-V	*	*	NS	*	NS	-	NS	*	*	*	NS	*	NS	NS	NS
OE-HT-R	NS		NS	NS	NS	NS	-		NS	NS	NS	NS	NS	NS	NS
OE-HT-V	*	NS	*	NS	*	*	*	-	NS	*	*	NS	NS	NS	*
F-NT-R	NS	NS	NS	NS	NS	*	NS	NS	-	NS	NS	NS	NS	NS	*
F-NT-V	NS	*	NS	NS	*	*	NS	*	NS	-	NS	NS	NS	NS	NS
M-NT-V	*	*	NS	NS	NS	NS	NS	*	NS	NS	-	NS	NS	NS	NS
OVX-NT-R	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	-	NS	NS	NS
OVX-NT-V	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS
OE-NT-R	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS
OE-NT-V	*	*	NS	NS	NS	NS	NS	*	*	NS	NS	NS	NS	NS	-

* indicates P<0.05, NS indicates P>0.05 (no significant difference), R = Ridogrel treated, V= vehicle treated

Estrogen replacement in OE-HT rats restored the response to coarctation with an increase in collagen: elastin ratio ($P<0.05$). Collagen: elastin ratio was significantly higher in F-HT, M-HT and OE-HT compared to OVX-HT ($P<0.01$).

3.9.2 The Effects of Ridogrel on Collagen and Elastin Density in Aortic Coarctation

See Figs. 37,38, and 39 and Tables 20, 21, 22, and 23 for elastin density, collagen density, and collagen: elastin ratio with the administration of Ridogrel, the thromboxane synthase inhibitor/thromboxane-endoperoxide receptor antagonist. Ridogrel treatment during the 14 days following aortic coarctation had no effect on collagen, elastin or collagen: elastin ratios in the NT groups ($P>0.05$). Ridogrel was not administered to M-NT rats.

Ridogrel administration abolished any differences in collagen density (one-way ANOVA, $P>0.05$). Ridogrel significantly reduced collagen density in F-HT aortae ($P<0.05$), so that the density was similar to F-NT ($P>0.05$). Ridogrel had no effect on collagen density in M-HT or OVX-HT when compared to same groups that received only vehicle ($P>0.05$). Estrogen supplementation restored the effects of Ridogrel on collagen density in OE-HT in that the collagen density was significantly less than OE-HT receiving vehicle ($P<0.05$), and similar to OE-NT treated with Ridogrel or vehicle ($P>0.05$). Even with the administration of Ridogrel, F-HT still exhibited elevated collagen density compared to OVX-HT treated with Ridogrel ($P<0.02$). Ovariectomy effectively abolished any changes in collagen density of the aorta whether or not the rats were hypertensive or treated with Ridogrel.

Ridogrel administration abolished any differences in elastin density (one-way ANOVA, $P>0.05$) and this effect remained even if all males were excluded from analysis (one-way ANOVA, $P>0.05$). Ridogrel did not affect elastin density in any of the NT groups ($P>0.05$). Elastin content of M-HT aortae was not affected by Ridogrel ($P>0.05$). Ridogrel treatment did prevent hypertension-induced increases in elastin density in F-HT, OVX-HT or OE-HT aortae ($P<0.02$).

As with elastin density, Ridogrel administration abolished any differences in collagen: elastin ratios (one-way ANOVA, $P>0.05$) and this effect remained even if only the female groups were compared (one-way ANOVA, $P>0.05$). Ridogrel administration did not affect collagen: elastin ratios in any of the NT groups ($P>0.05$, Fig. 40). F-HT exhibited a similar ratio with Ridogrel or vehicle treatment ($P>0.05$), as did M-HT and OVX-HT. Conversely, OE-HT exhibited a much smaller ratio with Ridogrel treatment than with vehicle treatment ($P<0.004$). In the HT groups treated with Ridogrel, F-HT had a higher ratio than OVX-HT ($P=0.014$). Estrogen replacement did not restore the elevated ratio in OE-HT Ridogrel-treated rats, indicating again that collagen density is likely estrogen- and TXB_2-dependent while elastin density may only be TXB_2- (but not estrogen)-dependent.

CHAPTER IV

SUMMARY AND DISCUSSION

This study investigated the effects of estrogen on CP function and the development of aortic coarctation-induced hypertension. The central hypothesis tested was that ovarian steroid hormones upregulate CP function in the female rat aorta during the development of aortic coarctation-induced hypertension, thereby altering the functional and structural responses of the aorta to a greater extent in female than in male rats. If so, then CP-mediated changes in vascular reactivity, vascular structure, and systemic blood pressure during the development of aortic coarctation-induced hypertension would play a larger role in female than in male rats. Indeed, significant sexual dimorphism in the development of aortic coarctation-induced hypertension was identified at 12-14 days following aortic coarctation. There was a significant increase in MAP in both male and female rats; however, MAP increased to a much greater extent in female than in male rats. Ovariectomy abolished, and estrogen-replacement restored, the observed sex differences in MAP.

Confirming the greater role of CP in females during the development of aortic-coarctation-induced hypertension, release of the CP TXA_2 *in vitro* was markedly higher in F-HT than in M-HT or OVX-HT. *In vivo* blockade of the thromboxane/endoperoxide receptor acutely (with SQ 29,548) or chronically (with Ridogrel which also blocked thromboxane synthase) resulted in a significantly lower MAP in F-HT than in M-HT or OVX-HT. Physiological estrogen replacement restored CP function and hypertension in OE-HT rats to levels indistinguishable from those of F-HT rats. Expression of the

enzyme responsible for TXA_2 production, thromboxane synthase, was elevated to a much greater extent in both endothelial and vascular smooth muscle cells in F-HT than in M-HT or OVX-HT aortae, explaining, at least in part, the enhanced release of TXA_2 and the greater response to CP antagonism observed in F-HT and OE-HT. Upregulation of the CP pathway also resulted in morphologic changes in the aortae of F-HT rats; but perhaps most strikingly, it also resulted in increased collagen and elastin density with an elevated collagen: elastin ratio in the aortic wall. Upregulation of the TXA_2 pathway and the resultant morphologic changes identified in aortae from F-HT and OE-HT rats are consistent with the higher MAP present in these rats compared to either M-HT or OVX-HT rats.

4.1 Effects of Aortic Coarctation-Induced Hypertension on Mean Arterial Blood Pressure

The present study revealed that MAP was significantly higher in female than in male rats 12 to 14 days following aortic coarctation. The elevation in blood pressure in female rats was abolished by OVX, but was restored by estrogen replacement, revealing that estrogen plays a key role in the development of this form of HT in female rats.

Similar to the findings in the male rat in the present study, previous investigators reported that arterial pressure peaked at 4-6 days post-coarctation and then declined to a stable MAP of approximately 160 mm Hg by 14 days (64; 148). A peak and subsequent decline in MAP was not observed in female or OVX rats; instead, a steady increase in MAP was measured over the 14 day period. Indeed, female rats exhibited a lower MAP during the first week following aortic coarctation compared to males; however, MAP

increased dramatically during the second week. In addition to the estrogen-dependent contribution of CP to the elevated MAP, female rats may also produce more ANG II due to the effects of estrogen on the renin angiotensin system. Hypertensive women receiving estrogen replacement therapy develop elevations in plasma renin activity, and in plasma ANG I and ANG II levels (258). At the molecular level, estrogens may stimulate angiotensinogen synthesis 2-fold (34; 136) more in females than in males and estrogen-responsive elements (DNA sequences that bind the estrogen-estrogen receptor dimer) have been identified in the 5' flanking region of the rat angiotensinogen gene (7). Functionally, when estradiol is administered to ovariectomized rats, there is also a significant increase in the vasopressor response to angiotensin II (compared to ovariectomized controls) (219).

Previous studies of aortic coarctation-induced hypertension have not examined arterial blood pressure in female rats, with the exception of two short-term investigations. A single study of the acute effects of estrogen 45 minutes after partial acute aortic coarctation revealed that in OVX rats, estrogen increases the sensitivity of the carotid arteries to ANG II-induced vasoconstriction (220). This finding is consistent with those of the present study that MAP was higher in females than in males, and was reduced by OVX but restored by estrogen replacement. The effects of partial aortic coarctation also have been investigated in pregnant and virgin female rats for a duration of 10 to 11 days (96). Increases in mean arterial pressure were identified only in pregnant rats. The most likely reason for the lack of a significant increase in arterial pressure in virgin female rats is that only partial rather than total aortic occlusion was performed, unlike the present investigation.

Sex differences in CP release and/or action in female versus male rats that are ovarian steroid hormone (probably estrogen) dependent, have been identified (10; 11; 68; 145). Reactivity to the thromboxane/endoperoxide receptor agonist U-46619 is greater in female than in male rat aortae and vascular reactivity to vasopressin and phenylephrine are enhanced in female aortae due to the greater release of CP in female than in male rats (10; 11; 68; 145). In chronically-coarcted male rats (4 weeks after coarctation), a persistent enhanced vascular reactivity to CP and a concomitant modulatory effect of nitric oxide are present, suggesting that these two mechanisms, working in direct opposition, are responsible for the stable elevated MAP observed in male rats in this form of hypertension (232). In females, 17β-estradiol induces rapid release of nitric oxide and increases nitric oxide synthase expression in endothelial cells (93; 125; 216); therefore, nitric oxide release may prevent a more dramatic early rise in blood pressure in female rats, unlike male rats, during the first week following aortic coarctation. The later rise in MAP in females is likely due to the increased expression of thromboxane synthase, resulting in enhanced release of TXA_2, as observed in the present study. The elevated MAP and resultant damage to the endothelium, may also decrease nitric oxide production, exacerbating the hypertensive effects of TXA_2. An additional mechanism also may operate through the impaired nitric oxide synthesis, and is related to the rise in ANG II levels that occurs with aortic coarctation (13; 14; 82; 95). ANG II stimulates the formation of reactive oxygen species (79; 206), which in turn inactivate the nitric oxide pathway (13; 14; 82). Thus in female rats, the enhanced production of renin and ANG II, resulting from the effects of both aortic coarctation and estrogen, may impair the production of nitric oxide to such an extent that estrogen-stimulated elevations in CP

such as TXA_2 likely result in greater increases in systemic blood pressure than occur in male rats. In this manner, the equilibrium between nitric oxide, vasodilator prostanoids, and vasoconstrictor prostanoids likely present in normotension may have been altered, resulting in greater elevations in systemic blood pressure in females than in males. In addition, long-term, estrogen-dependent changes in arterial wall composition may also contribute to the continued rise in MAP seen 12 to 14 days after aortic coarctation in F-HT and OE-HT rats, since estrogen treatment increases aortic stiffness in OVX rats (252). Similarly, sex differences in heart rate in response to aortic coarctation also were present during the first 4 days, but not later, when MAP was significantly different; suggesting that a change in baroreceptor set point may have occurred in F-HT and OE-HT rats, resulting in higher MAP without any changes in heart rate.

4.2 Effects of Constrictor Prostanoid Pathway Inhibitors on Aortic Coarctation-Induced Hypertension

To determine the role that CP play in aortic coarctation-induced HT, the effects of acute and chronic inhibition of CP receptor function were investigated. Acute antagonism of the TXA_2/PGH_2 receptor with SQ 29,548 resulted in a substantially greater decrease in MAP in F-HT and OE-HT rats than in M-HT, rats 14 days after aortic coarctation. TXA_2/PGH_2 receptor blockade had little or no effect in OVX-HT rats. The decrease in MAP seen in M-HT was similar to that in previous reports (149), but much less than occurred in F-HT or OE-HT rats. F-HT and OE-HT rats exhibited about a 3-fold greater decrease in blood pressure compared to M-HT, confirming the greater role that the CP were believed to play in female than in male rats during the development of HT.

Chronic inhibition of TXA_2 synthase and the TXA_2 /PGH_2 receptor with Ridogrel over the 2-week period following aortic coarctation did not affect MAP in any NT rats, nor in M-HT or OVX-HT rats in the present study. This is similar to previous reports that MAP of M-HT was unaffected by Ridogrel (52; 201). MAP was significantly reduced in F-HT and this effect was abolished by ovariectomy. The hypotensive effect of Ridogrel was restored by estrogen replacement in OE-HT rats. These findings support the central hypothesis of the present study that the ovarian steroids (likely estrogen) activate the CP pathway to induce much larger increases in MAP following aortic coarctation than in rats without estrogen (M, OVX). The present findings are also consistent with studies in women with pulmonary hypertension and preeclampsia, in which TXA_2 production appears to be an important mediator of increases in blood pressure (12; 38; 210; 295). Estrogen may also have influenced the heart rate (and baroreflex) responses of F-HT and OE-HT to Ridogrel, because both F-HT and OE-HT had slower heart rates than any of the NT groups on days 4 and 6.

4.3 Effects of Aortic Coarctation-Induced Hypertension and Ovarian Steroid Hormones on Aortic Prostanoid Release

Striking sex differences in vascular prostanoid release were observed in both NT and HT rats in the present study. While basal release of TXB_2 in the incubation studies was similar in the aortae of F-NT and M-NT rats, basal release increased 4-fold in F-HT and but only 2-fold in M-HT, compared to their respective NT controls. Further, ANG II stimulated greater release of TXB_2 in F than in M, both in normotension and

hypertension, suggesting greater CP-potentiated aortic responsiveness to ANG II in the presence of ovarian steroid hormones.

In the continuous pressure and flow studies, aortae from both OE and F rats produced similar amounts of TXB_2, which were greater than OVX rat aortae; however, the higher TXB_2 production achieved statistical significance only in F-HT rats, likely due to greater variability in TXB_2 production in the OE-HT aortae. Despite the lack of overall significance in the OE-HT aortae, most OE-HT aortae produced at least 3-fold more TXB_2 than OVX-HT at any pressure or ANG II dose, providing compelling evidence that estrogen does indeed play a role in the regulation of vascular tone through TXB_2.

Previous studies have identified differences in CP release and/or action that are dependent upon ovarian steroid hormones, probably estrogen (68; 145). Vasopressin and PE-induced contractions of the rat aorta exhibit sexual dimorphism that is CP dependent, at least in part (67; 68; 145; 248). Vascular reactivity to the thromboxane/endoperoxide receptor agonist U-46619 is also greater in female than in male rat aortae (68; 145). The increased responsiveness to ANG II identified in OVX-NT rats replaced with 17β estradiol (219) supports the present findings in the ANG II-dependent aortic coarctation model, where the presence of estrogen increased CP output, especially in the presence of ANG II stimulation.

Further evidence for the upregulation of CP synthesis in hypertension in the female rat has been identified in the present study. Increases in the expression of thromboxane synthase in the aortae of F-HT and OE-HT compared to M-HT and OVX-HT were identified using immunohistochemistry. RT PCR identified dramatic parallel

increases in TXS mRNA in the endothelium and moderate but parallel increases in vascular smooth muscle. These findings are entirely consistent with the increased release of TXB_2 measured in isolated aortae and the increased MAP present *in vivo* in F-HT and OE-HT rats. Estrogen, then, appears to upregulate CP production and the renin-angiotensin system, to result in greater MAP in F-HT and OE-HT rats in aortic coarctation-induced hypertension.

Release of the vasodilator prostanoid prostacyclin (PGI_2), was also analyzed in the present incubation and pressure/flow studies. Release of $PGF_{1\alpha}$ (stable metabolite of PGI_2) increased more in F-HT than in M-HT rats following aortic coarctation. In the aortic ring incubation experiments, basal $PGF_{1\alpha}$ production increased 5-fold in F-HT compared to F-NT, but only 3-fold in M-HT compared to M-NT ($P<0.05$). In the presence of ANG II, F-HT again exhibited a 2-fold greater release of $PGF_{1\alpha}$ than M-HT. In the continuous pressure/flow experiments on thoracic aortae, $PGF_{1\alpha}$ release was elevated at all pressures (100 and 150 mm Hg) in F-HT compared to F-NT. This response to HT was abolished by ovariectomy (OVX-HT production was similar to OVX-NT) and restored by estrogen replacement (OE-HT was greater than OE-NT). With ANG II (0.1 nmol) stimulation, production of $PGF_{1\alpha}$ was at least 2-fold higher in F-HT and OE-HT than in OVX-HT. In both F and OE, there was likely an upregulation of the PGI_2 pathway in parallel with the vasoconstrictor (TXA_2/PGH_2) pathway. This concurrent upregulation of vasodilator as well as vasoconstrictor prostanoids may function to balance vascular tone and maintain homeostasis. However, oxygen free radical production (increased by ANG II) antagonizes the production of PGI_2 (156), which may contribute to the pathogenesis of vascular diseases where ANG II is increased (79; 206). Although the

relative amounts of PGI_2 and TXA_2 differ in normotensive states, PGH_2 may also contribute to the balance between vasoconstriction and vasodilation. The relative concentrations and binding affinities of each prostanoid for its receptor was not investigated in the present studies, and may also contribute to the balance between vasoconstriction and vasodilation in the arterial vasculature. Therefore, in hypertensive states in which ANG II is not increased, increased production of both TXA_2 and PGI_2 in the female may result in continued homeostatic balance of platelet aggregation and vascular tone, because these two prostanoids exert opposing effects on platelets and vascular smooth muscle (156; 173; 183; 224). However, in cases where ANG II production is increased, such as in aortic coarctation (a renin-angiotensin dependent form of hypertension), the release and/or action of constrictor and dilator prostanoids may be imbalanced and thus contribute to increased vascular tone and higher systemic blood pressure in female rats.

Preeclampsia and other placental insufficiency syndromes in humans are characterized by an overproduction of TXA_2 in the placenta and by a deficit in production of PGI_2 in the fetoplacental tissue, which may account for the enhanced vasoconstriction and platelet hyperactivity seen in hypertensive pregnancies (295). Similarly, in pulmonary hypertension and Raynaud's disease, an imbalance between TXA_2 and PGI_2 with the overproduction of TXA_2 has been reported (38; 218). Furthermore, inhibitors of thromboxane synthase (Dazoxiben, CGS-13080) have been used successfully in the treatment of primary pulmonary hypertension and Raynaud's disease (15; 210). In aortic coarctation rats in the present study, MAP did not increase as soon as rapidly in F-HT as in M-HT rats. Since ANG II production does not peak until 5 to 9 days after aortic

coarctation (64), a marked increase in CP and free radical production and subsequent inhibition of vasodilator activity probably does not occur until that time.

4.4 Effects of Aortic Coarctation-Induced Hypertension on the Pressure-Diameter Relationship of the Thoracic Aorta in Response to Angiotensin II

The pressure/diameter responses of NT and HT rat aortae were recorded *in vitro* during constant flow/pressure experiments (150 mm Hg). In these experiments, the aorta functioned best as a model for humoral events and the structural remodeling that occurs throughout the arterial vascular tree, because its function as a conduit vessel precluded functional pressure-diameter changes during testing *in vitro*. Thus, only minor changes in pressure occurred with ANG II administration during continuous flow, and changes in compliance were more readily apparent than changes in resistance.

Initial pressurization of the aorta from 0 to 150 mm Hg resulted in significantly smaller increases in diameter in F-HT and OE-HT than in other groups. This likely reflects morphologic remodeling that occurs with aortic coarctation-induced hypertension in the presence of estrogen in F-HT and OE-HT rats.

ANG II-stimulation of the aorta *in vitro* resulted in apparent vasodilation of all groups (both HT and NT) at the lowest dose of ANG II (0.005 nmol). At subsequent higher doses of ANG II (0.02 and 0.1 nmol), much smaller changes in diameter resulted, and none differed significantly among the various experimental groups (one-way ANOVA, $P>0.05$). The vasodilation that occurred in response to ANG II in the HT as well as NT aortae was likely the result of several factors. ANG II stimulation in conjunction with pressurization to 150 mm Hg and continuous flow may have stimulated

not only direct and CP-mediated contraction of the aortic wall, but also the release of vasodilators such as nitric oxide and vasodilator prostanoids. The release of vasodilators may have resulted in the maintenance of equilibrium of tensile and shear stresses so that no significant changes in aortic diameter occurred, even though release of vasoconstrictors such as TXB_2 occurred. In this regard, greater release of TXB_2 and $PGF_{1\alpha}$ were measured in the perfusates of F-HT and OE-HT aortae than in OVX-HT aortae in response to ANG II ($P<0.05$). Changes in pressure were not marked and changes in diameter in any group were minimal, probably due to the function of the aorta as a conduit artery, in which only small changes in diameter occur with humoral activation (45). The sensitivity of the rat aorta to vasoconstrictor and vasodilator agonists, *in vitro*, is often lower than that of smaller resistance vessels; however, it is well established that the functional properties of the rat aorta are more similar to peripheral resistance vessels than those of other large artery models such as the rabbit aorta (78). This was reflected in the release of prostanoids during the *in vitro* pressurization experiments. Further investigations with small resistance vessels may be warranted to determine active responses in the model of complete aortic coarctation in the F rat.

4.5 Effects of Aortic Coarctation-Induced Hypertension on Aortic Morphology and Extracellular Matrix

To determine the roles that hypertension, ovarian steroid hormones, and CP play in the regulation of aortic morphology, the effects of aortic coarctation, ovariectomy, and estrogen replacement therapy on aortic composition were examined. Prostanoids have been identified previously as important regulators of cell proliferation, differentiation and

apoptosis (143; 199; 238); therefore, their effects on vascular morphology were investigated in the present studies. With the induction of hypertension, wall thickness increased in F-HT aortae. Ovariectomy abolished HT-mediated increases in aortic wall thickness seen in F-HT, while estrogen-replacement restored hypertension-related increases in thickness in the OE-HT aorta. Wall area also increased in the presence of ovarian steroid hormones and in response to hypertension. Interestingly, M-HT had similar increases in wall thickness, area and wall: luminal ratios as F-HT, indicating a possible role for androgens as well as estrogens in the response to aortic coarctation.

Ridogrel administration did not alter hypertension-mediated changes in aortic morphology in the present studies. Wall thickness was not affected by Ridogrel and wall area was affected only in OE-NT aortae. Wall: luminal ratio did not change with Ridogrel, with the exception of OVX aortae. Aortic wall: luminal ratio of OVX-HT aortae was elevated following Ridogrel administration, compared to OVX-NT. These findings strongly suggest that the morphologic changes seen in the aorta after coarctation and the induction of hypertension are mediated through the sex steroid hormones, likely estrogen, and do not involve the CP (TXA_2/PGH_2).

In contrast, extracellular production of elastin and collagen were markedly affected by CP and ovarian steroid hormones. Collagen density increased markedly in F-HT aortae compared to F-NT control or M-HT aortae. Ovariectomy abolished HT-mediated increases in collagen, while estrogen replacement restored increases in collagen in OE-HT. Elastin content increased in all HT groups, but the greatest increase was seen in F-HT. The collagen: elastin ratio exhibited significant sexual dimorphism. The ratio increased with the induction of hypertension in F-HT but not in M-HT, and this effect

was abolished by ovariectomy (OVX-HT) and restored by estrogen replacement in OE-HT.

Ridogrel administration clearly prevented increases in elastin in F-HT, OVX-HT and OE-HT rat aortae. Ridogrel also inhibited hypertension-induced increases in collagen production in estrogen-replete rats (female and OE). Collagen production in OVX aortae was unaffected by the induction of hypertension or by treatment with Ridogrel. Ovariectomy abolished any hypertension-mediated increases in elastin seen with aortic coarctation. It is unknown whether the ovarian steroid hormones (estrogen) act directly to alter extracellular matrix production or through TXA_2; however, it seems likely that estrogen acts together with the CP to increase elastin and collagen density in the aorta. Aortic elastin and collagen contents in the M were unaffected by hypertension or Ridogrel administration. This is similar to previous findings in age-matched M spontaneously hypertensive rats and their normotensive Wistar-Kyoto counterparts at 3 to 6 months of age, in that hypertensive and normotensive aortae had similar elastin and collagen densities (260). Similarly, in an additional study, male rats administered ANG II for 2 weeks developed a significant increase in media area (as seen in male rats in the present study) but no change in elastin or collagen density (25).

The remodeling that occurs with aortic coarctation-induced hypertension differs in the presence of ovarian steroids (specifically estrogen), especially with respect to collagen production. Collagen production is upregulated in F-HT and OE –HT, but is attenuated by inhibition of the CP pathway. These findings suggest that ovarian steroid hormones act to upregulate CP function directly or in concert to alter collagen production. TXA_2 has been identified as a potent mitogen and mediator of vascular

remodeling (156), and increases in collagen mRNA due to TXA_2 have been documented in male rats (26; 164; 165). If estrogen upregulates CP function in aortic coarctation-induced hypertension, then an increase in collagen production is reasonable, since TXA_2 stimulates collagen production. Indeed, the increased MAP seen 12 to 14 days after aortic coarctation in F-HT and OE-HT rats may be due, at least in part, to increased collagen expression in the arterial wall, resulting in increased aortic stiffness. Estrogen treatment is known to increase aortic stiffness in OVX rats (252) and this appears to involve increased collagen production. Previous investigations of aortic coarctation in female have not identified changes in extracellular matrix (96); however, these studies were short-term and only involved partial coarctation of the aorta. ANG II is reported to stimulate collagen production in arterial walls (121); thus, the resulting ANG II levels in these previous studies may not have been sufficient to stimulate collagen production in the aortic wall. The rats in the present studies underwent complete ligation of the aorta to induce the largest possible activation of the renin-angiotensin system. Complete ligation of the aorta resulted in a unique model of hypertension for study in female and OE rats, in which maximal ANG II levels likely resulted in pronounced interactions among estrogen, ANG II and TXA_2.

4.6 Conclusions

Numerous previous investigations established the concept that vascular TXA_2 production is important in the regulation of vascular tone only in hypertension and in males but not in females (33; 49; 96; 169; 175). However, recent findings have challenged this dogmatic concept and have established the importance of CP in the

regulation of vascular tone in the normal systemic vasculature and in females but not males (63; 68; 145). The findings of the present study confirm and extend the importance of CP in the regulation of tone in the normal female vasculature and establish the importance of endogenous estrogen and CP as mediators of increased vascular tone and hypertension in the aortic coarctation model. This model is dependent upon the renin-angiotensin system and induces hypertension only in the upper body. Other models of hypertension, such as the Spontaneously Hypertensive rat, do not involve the induction of CP systemically (49), although CP may be involved in the renal vasculature (23). Further, regional heterogeneity may exist in the modulatory influence of estrogen on vascular function and TXA_2 production, such that vascular responses may vary with anatomical location in the female rat (252).

In previous studies, ovariectomy attenuated the contractile responses of the rat aorta to vasopressin and PE by 25-30% (68; 145), while treatment of OVX animals with estrogen resulted in enhanced contractions to both vasopressin and PE in the rat aorta (194) and to arachidonic acid in the rabbit aorta (168) and. Contractile responses of female rat aortae to vasopressin and PE were attenuated by indomethacin and SQ 29,548 (68), indicating that constrictor prostanoids are involved in agonist-induced vasoconstriction in the female. Estrogen also enhances vasoconstriction by increasing vascular smooth muscle reactivity and binding sites to vasopressin in rat mesenteric arteries (243). Further, increased release of CP by the vasculature likely underlies the enhanced vascular reactivity to vasopressin seen in the female rat aorta (68; 145). The present study demonstrated that estrogen plays an important role in the regulation of vascular tone in the female, both in normotension and in aortic coarctation-induced

hypertension. Following OVX, expression of TXS enzyme and the release of CP declined concurrently in the OVX-HT vasculature, resulting in a smaller increase in MAP following aortic coarctation compared to female rats. The present research establishes, experimentally, the concept that epidemiologic studies have proposed; namely, that ANG II-induced hypertension is exacerbated in the female by estrogen-induced CP production. This change in vascular function during development of hypertension is exacerbated by the structural effect of estrogen to increase collagen density, reducing arterial compliance and further enhancing vascular tone.

Human hypertension may involve the interaction of estrogen, CP and ANG II, as in the aortic coarctation model of hypertension described in the present studies. ANG II has been implicated as an important component in essential hypertension in humans (107; 198; 258). Estradiol levels are higher in men and women with essential hypertension than in their normotensive counterparts, and estrogen elevates angiotensinogen gene expression and blood levels of ANG II as well as renin activity (92; 214; 258), suggesting that estrogen may play an important role in the development of HT. Previous studies of aortic coarctation-induced hypertension have identified the important role that CP (TXA_2 and PGH_2) play in the rat (20; 31; 55; 130; 148; 148) and essential hypertension in the human is characterized not only by elevated ANG II and estradiol, but also by enhanced release of local constrictor factors such as CP (186; 230; 262; 263). Taken together, the past and present findings suggest that ANG II, estrogen, and CP likely function in concert to exacerbate the development of hypertension through simultaneous changes in vascular function and structure to a substantially greater extent in the female than in the male

vasculature. Further, the findings of the present investigation may be important in the development of sex-specific treatment of essential hypertension in humans.

The present studies identify the functional and structural events that occur during the induction of HT in the female rat in a renin angiotensin-dependent model. The responses of the female vasculature differ from those of the male at the local humoral level, with a markedly greater release of CP. Sex differences in prostanoid release were observed both in static and perfused aortae *in vitro* and were verified at the cellular level by increases in TXA_2 synthase enzyme content (immunostaining) and at the molecular level by increased expression of TXA_2 synthase mRNA. This local humoral induction appeared to result in the elevation of systemic arterial pressure *in vivo,* and this effect of CP was confirmed by the hypotensive actions of the TXA_2/PGH_2 receptor antagonist SQ 29,548. The rise in MAP in female rats was likely the combined result of functional and structural events, including increased vascular tone (vasoconstriction), increased extracellular vascular matrix (increased collagen), increased vascular wall area, and altered baroreceptor set points in the systemic vasculature. F-HT and OE-HT rats developed more complications after aortic coarctation, including thromboembolism, weight loss, paresis, paralysis, and sudden death (in addition to higher MAP) than M-HT rats. These sex differences may reflect the altered balance of local vasoconstrictors to vasodilators that likely occurs in this form of HT. The aortic coarctation model differs from other models such as the Spontaneously Hypertensive Rat, in that induction of HT results from a transient elevation in plasma renin and ANG II levels (64). Hypertension persists even though plasma renin and ANG II levels return to baseline-precoarctation levels. There are, then, persistent changes in vascular structure and function, and perhaps

the baroreceptors, which result in the continued maintenance of HT, and this includes vascular wall remodeling as demonstrated by the present studies. In the female, the extracellular matrix was altered so that aortic stiffness may have increased (due to increased collagen density). Whether the vascular remodeling and alterations in baroreceptor set point may be reversed with reversal of HT is unknown. Regardless, the findings reported herein, supported by previous epidemiological studies, argue that the way in which we study, diagnose and treat hypertension should be approached differently in premenopausal women than in men.

It can be concluded from the present investigation that ANG II, estrogen, and CP work in concert to induce hypertension by enhancing vascular tone and to sustain this pathophysiological state through structural changes in the vascular wall. One or more factors may act as facilitators for the others, or they may act synergistically to induce a form of HT that is more malignant in the female than in the male rat. Estrogens have been shown to increase circulating angiotensinogen (34; 136) and to increase vasoconstrictor responses to ANG II (219). There may also be increased ANG II production, either systemically or locally at the endothelium, contributing to increased oxygen free-radical induced vasodilator inhibition, as well as increased collagen production, resulting in reduced vascular compliance. This proposed interaction of factors is consistent with the concurrent increases in MAP and collagen density in F-HT and OE-HT rats in this study; however, further experiments are required to fully determine the cellular and molecular interactions underlying this form of hypertension.

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SELECTED PUBLICATIONS:

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