THE RELATIVE CONTRIBUTIONS OF COX-1 AND COX-2 TO VASCULAR BIOSYNTHESIS OF PROSTACYCLIN AND THROMBOXANE

A Thesis

by

PAMELA A. OSBORN

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

MASTER OF SCIENCE

Chair of Committee,	John N. Stallone
Committee Members,	Randolph H. Stewart
	Farida Sohrabji
	Shannon Washburn
Head of Department,	Glen Laine

May 2014

Major Subject: Veterinary Physiology and Pharmacology

ABSTRACT

The relative contributions of cyclooxygenase-1 (COX-1) versus COX-2 to vascular biosynthesis of prostacyclin (PGI_2) and thromboxane (TxA_2) and the role of estrogen in the regulation of COX function were studied in the thoracic aorta of age-matched 14-16 week old female (F) and male (M) Sprague Dawley rats. We hypothesized that COX-1 and COX-2 contribute differentially to the synthesis of PGI_2 and TxA_2 in the presence of estrogen. Ovary intact (Int-F), ovariectomized (OvX-F) and OvX+estrogen-replaced (OvX+ER-F) rats were studied. Rats were sacrificed 14 days post-op, and 3mm aortic rings were incubated in Krebs-Henseleit-bicarbonate buffer (KHB), containing either vehicle (basal), vasopressin (VP, 10^{-6} M), VP+SC-560 (SC, selective COX-1 inhibitor, 10^{-7} M), or VP+NS-398 (NS, selective COX-2 inhibitor, 10^{-5} M). The KHB was analyzed by specific radioimmunoassays (RIA) for 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) and TxB_2 (stable metabolite of TxA_2). Basal PGI_2 was similar in M and all F groups (P=0.19). VP-stimulated PGI_2 in M (4,528 ± 745) and OvX-F (4,785 ± 773) was reduced similarly by SC and NS (P>0.05). Also, VP-stimulated PGI_2 in Int-F (15,352 ± 2,209) and OvX-ER-F (13,053 ± 3,086) was reduced significantly by SC and NS (P<0.05). Basal TxA_2 was similar in all groups (P>0.05). VP-stimulated TxA_2 in M (30 ± 4) and OvX-F (31 ± 7) was reduced similarly by SC and NS (P>0.05). VP-stimulated TxA_2 in OvX-ER-F (71 ± 7) was reduced markedly more by NS than by SC (P<0.05). In conclusion, these data suggest that: 1) in the absence of estrogen in OvX-F and M, both PGI_2 and TxA_2 are derived equally from COX-1 and

COX-2; and 2) in Int-F and OvX-ER-F, estrogen markedly enhances PGI_2 and TxA_2 production, primarily by upregulating COX-2 function.

Furthermore, the mechanism by which TxA_2 inhibits PGI_2 synthesis was investigated in M rats. It was hypothesized that TxA_2 inhibits PGI_2 through a PKC-mediated nitrosylation on PGI synthase. Aortic rings were prepared and incubated in KHB, containing either vehicle (VP, 10^{-6} M), Calphostin C (CAL; PKC inhibitor, 10^{-6}M), or Furegrelate (FUR; TP receptor antagonist; 50μM), Ridogrel (RID; combined TP receptor antagonist and TxS inhibitor; 10^{-5}M), or Dazoxiben (DAZ; 50μM). The KHB was analyzed by specific RIA for 6-keto-$PGF_{1\alpha}$ and TxB_2. TxA_2 release was significantly attenuated by DAZ, RID, and FUR but was markedly enhanced by CAL 228% ($0.001<P<0.05$). Despite Ridogrel and Furegrelate significantly decreasing TxA_2 release, neither of these inhibitors significantly attenuated PGI_2 release in the aorta ($P>0.05$). However, PGI_2 release was enhanced 158% in the presence of DAZ ($0.001<P<0.05$). It is possible that Calphostin may inhibit a PKC-mediated homologous desensitization loop to increase TxA_2 release. DAZ effects clearly show that TxS inhibitors increase PGI_2 release, however, it is puzzling that Ridogrel did not have the same effect. While TxA_2 does seem to have a depressing effect of PGI_2, it is clear that PKC-mediated nitrosylation of PGI synthase is not the mechanism by which TxA_2 inhibits PGI_2 release.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Stallone, for accepting me into his lab, and for all of his knowledge, direction and overall support throughout the course of my time at Texas A&M University. I would like to thank my committee members, Dr. Stewart, Dr. Washburn and Dr. Sohrabji for their guidance and patience throughout the course of this research. Also, Lisa Perkins, her kindness, emotional support, and many mornings of coffee and donuts made it possible to complete this process.

Finally, I would like to thank my husband, whose patience and love allowed me to achieve this goal. I am blessed to be able to spend the rest of my life with you.

DEDICATION

This thesis is dedicated to my parents, who are two of the most loving and compassionate people I have ever, and will ever know. Their love, support, and encouragement are unwavering.

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NOMENCLATURE

AA	Arachidonic acid
COX	Cyclooxygenase
CHD	Coronary heart disease
CVD	Cardiovascular disease
DAG	Diacylglycerol
ERT	Estrogen replacement therapy
ER	Estrogen receptor
F	Female
M	Male
NSAID	Non-steroidal anti-inflammatory drug
OvX	Ovariectomy
PKC	Protein kinase C
PGI_2	Prostacyclin
TxA_2	Thromboxane
VSMC	Vascular smooth muscle cells

CHAPTER I

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) comprise a therapeutic class of drugs, which exhibit anti-inflammatory, analgesic and antipyretic properties. In 1899, Bayer introduced the first mass-produced drug to treat pain, inflammation and fever - acetylsalicyclic acid, known as aspirin. Aspirin, one of the most widely used and marketed drugs in the pharmaceutical industry, has been celebrated in recent years as being cardioprotective when taken daily. The novel use of NSAIDs as prevention from certain cardiovascular diseases (CVD) could potentially change the fate of millions of people. The World Health Organization (WHO) estimates that CVD is the single largest contributor to global mortality, and will continue to be so for decades to come (2). In 2008, CVDs accounted for 17 million deaths, contributing to 48% of all deaths from non-communicable diseases. It is expected that by 2015, CVDs will be responsible for 20 million deaths (30% of all deaths) worldwide (27). Thus, aspirin and other NSAIDs could play an important role in the future of CVD prevention.

A subgroup of NSAIDs, the COX-2 selective inhibitors (COXIBS), appear to have a paradoxical deleterious effect on the cardiovascular system, and several of these were removed from the market by the FDA due to an increased risk of thrombosis associated with their use. The use of NSAIDs, and their effects on the cardiovascular system, is much more complicated than first acknowledged. The effects that NSAIDs have on the cardiovascular system is based on the inhibition of two functionally

antagonistic prostanoid mediators produced in the vascular wall (the vasodilator, prostacyclin (PGI_2) and the vasoconstrictor, thromboxane (TxA_2)) therefore disrupting the normal levels in the body. The specific effects of NSAIDs on the balance of prostanoids in the cardiovascular system have yet to be clearly elucidated.

Normal homeostasis within the vascular system relies upon the balance of PGI_2 vs. TxA_2 production. Therapeutic use of NSAIDs to inhibit the enzymes cycloxygenase (COX)-1 and/or COX-2, the rate-limiting enzyme for prostanoid synthesis, causes an alteration in the homeostatic balance between levels of PGI_2 and TxA_2. The imbalance theory is widely quoted and describes a negative shift in the release of vascular prostanoids, resulting in platelet aggregation and thrombosis. However, the principal studies upon which this theory is based are flawed, because urinary metabolites of PGI_2 do not provide an accurate measurement of vascular prostacyclin production.

Sexual dimorphism in prostanoid synthesis exists, but is largely ignored in vascular physiology. Estrogen enhances the biosynthesis of thromboxane through upregulation of COX-2 and thromboxane synthase expression. Therefore, the relative roles of COX-1 *vs.* COX-2 in the biosynthesis of PGI_2 and TxA_2 by the vascular wall were investigated by using specific COX-1 and COX-2 inhibitors. The thoracic aortas of both male and female rats were studied in order to elucidate sex differences that may exist due to the presence or absence of estrogen.

CHAPTER II

LITERATURE REVIEW

2.1 Cyclooxygenase and Arachidonic Acid Metabolism

The first NSAID discovered, aspirin, was produced over a hundred years ago from a component in willow bark into acetylsalicylic acid for the treatment of arthritis pain. However, the mechanism of action of NSAIDs was not discovered until the 1960's when Sir John Vane determined that the mechanism of action was inhibition of the enzyme cyclooxygenase (COX), which plays a central role in the metabolism of arachidonic acid and the synthesis of prostanoids. The metabolism of arachidonic acid (AA) into its functional components follows a well-established pathway. Arachidonic acid, a fatty acid present in phospholipids of cell membranes, is released from the cell membrane by the action of phospholipase A_2 (PLA_2) or diacylglycerol (DAG) lipase in response to a number of stimuli including mitogens, neurotransmitters, ATP, hormones, growth factors, and physical stimuli such as pressure and turbulent flow.

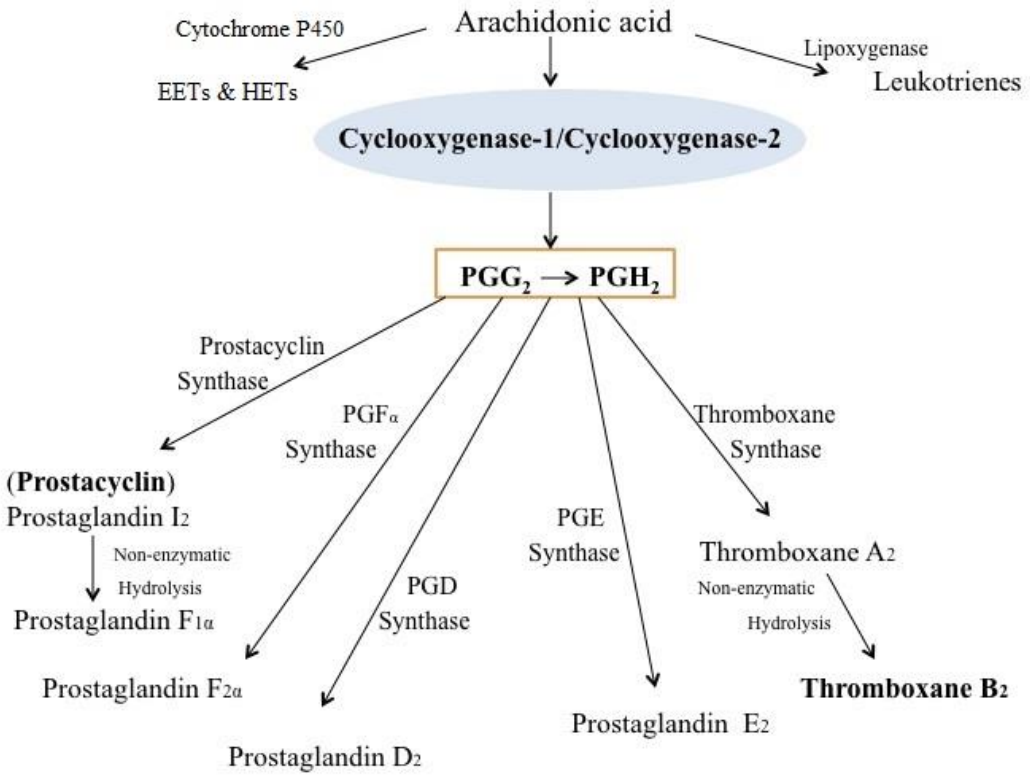

Figure 1: The Metabolism of Arachidonic Acid.
The metabolism of arachidonic acid by the two isoforms of the cyclooxygenase enzyme, and the specific synthases that produce the various prostanoid products.

The rate-limiting step in the metabolism of arachidonic acid involves the enzyme cyclooxygenase, which is responsible for the sequential biosynthesis of the prostanoid endoperoxide precursors: beginning with the conversion of arachidonic acid to prostaglandin G_2 (PGG_2) and then the conversion of PGG_2 to prostaglandin H_2 (PGH_2) (Figure 1). PGH_2 is further metabolized into prostaglandin I_2, $F_{2\alpha}$, D_2, E_2 and thromboxane A_2 (TxA_2) by specific synthase enzymes that are present within the nuclear and plasma membranes. The prostanoids produced have varying physiological effects and each tissue or organ has a specific profile of prostanoid production, dependent upon

the relative levels of the specific synthase enzymes. Vane discovered that inhibition of the COX enzyme by aspirin caused a decrease in the various prostanoids produced in the body responsible for pain, inflammation, fever, and platelet aggregation.

2.2 Synthesis, Metabolism and Function of Prostanoids

The various prostanoids synthesized from arachidonic acid metabolism act at specific receptors throughout the body to exert their effects via specific intracellular pathways. Prostacyclin (PGI_2), is synthesized from PGH_2 by prostacyclin synthase, which belongs to the cytochrome P450 superfamily of enzymes and is highly expressed in endothelial cells, vascular smooth muscle cells (VSMC), neurons, and intestinal epithelial cells (65). PGI_2 is a potent vasodilator and inhibitor of platelet aggregation, as the preferential ligand to act at a G-protein coupled receptor, the IP receptor, found on cell surfaces throughout the body, including VSM and endothelium. Binding of the IP receptor activates adenylyl cyclase, and thereby the accumulation of cAMP, which activates an intracellular effector protein kinase A (PKA) to phosphorylate downstream targets (19, 21).

$PGF_{2\alpha}$ production is mediated by PGF_{α} synthase and the $PGF_{2\alpha}$ ligand binds the FP receptor, a G-protein coupled receptor, which increases intracellular calcium concentration. FP activation by $PGF_{2\alpha}$ occurs during parturition in the female reproductive system, in the kidney to cause natriuresis and diuresis, and in the eye to regulate intraocular pressure (67).

The most abundant prostanoid produced in the body, PGE_2, is involved in several physiological pathways, including gastric secretion and motility, inflammation, and body temperature, and also plays a role in the regulation of the kidney and blood pressure. The production of PGE_2 is mediated by three known PGE synthases: one cystolic, cPGES, and two membrane bound, mPGES-1, and mPGES-2. PGE_2 activates various receptor subtypes (EP1, EP2, EP3, EP4, and multiple splice variants of these) that are coupled to diverse signaling pathways which induce specific physiological effects (56).

Another prostanoid, PGD_2, is synthesized from PGH_2 by PGD synthase and acts at two separate PGD receptors (DP_1 and DP_2) in the central nervous system, endothelial cells of the vascular wall, and commonly in immune cells. PGD_2 is the common prostaglandin involved in allergic reactions, and in regulation of sleep. When activated, the DP_1 and DP_2 receptors elevate intracellular calcium levels via two separate pathways.

Metabolism of PGH_2 into TxA_2 by thromboxane A_2 synthase (TxS) was first discovered as the major product of platelets, and later found to be a minor product of endothelial cells and VSM. TxA_2 has a very short half-life, during which it binds to a G-protein receptor known as the T-prostanoid (TP). Although TxA_2 is the preferential ligand at the TP receptor, PGH_2 and other PGI_2 can also bind TP (20). Upon activation of the TP receptor, intracellular activation of phosopholipase C (PLC) occurs to stimulate two secondary messengers, IP_3 and DAG that activate protein kinase C (PKC). PKC serves to activate several downstream targets, which cause vasoconstriction in the vasculature and cause stimulation of platelet aggregation (23, 26, 58). Activation of the

TP receptor can also lead to impaired endothelial nitric oxide-mediated vasorelaxation, further leading to increases vascular tone and constriction (45).

Numerous studies in both human and rodents have shown that the gastric mucosa produces PGE_2, PGI_2, PGD_2, and $PGF_{2\alpha}$, with the latter two in minimal amounts (39, 57). Prostaglandins PGI_2 and PGE_2 are secreted by the mucosa and act to protect the lining of the stomach by stimulating mucus and bicarbonate secretion, regulating acid secretion and stimulating vasodilation to increase blood flow (28). Loss of prostaglandin synthesis in the stomach can lead to disruption of the homeostatic pH gradient that protects the gastric mucosal lining, causing ulceration. Additionally, loss of prostaglandins during ischemia-reperfusion injury of the stomach disrupts the maintenance of blood flow, causing further injury (75). NSAIDs, particularly aspirin and indomethacin, are documented to cause the most gastric damage due to their inhibitory effect on prostaglandin synthesis by COX-1 (60).

2.3 Cyclooxygenase-2 Discovery

Following Vane's discovery of the mechanism of action of aspirin, research focused on the effects of NSAIDs on the production of prostanoids. A breakthrough in prostanoid research came in the early 1990's when a mitogen-inducible form of the COX enzyme, now known as COX-2, was identified (79). Numerous follow-up studies confirmed that COX-2 expression was commonly induced at the site of inflammation and in the presence of growth factors, bacterial endotoxins, cytokines, and by hormonal

regulators such as estradiol (36, 55, 66). More recently, the expression of COX-2 mRNA and protein was found to be constitutive in the systemic vascular wall (43).

The discovery of the COX-2 isoform opened the doors to numerous hypotheses addressing the production of the various prostanoids. Because COX-2 was initially found mainly in various forms of inflammation, Needleman and colleagues hypothesized that COX-2 expression was an inducible isoform of the COX enzyme upregulated during inflammation to produce pro-inflammatory prostanoids such as PGE_2 and PGI_2, while the expression of COX-1 was constitutive, and played a role in normal cell physiology, and functioned to produce anti-inflammatory prostanoids. In support of this hypothesis, a study in 1993 of the basal release of eicosanoids from murine macrophages showed that only low levels of COX-1 were expressed in untreated cells, but COX-2 was also present in cells treated with endotoxin (50). Many studies confirming similar results led to the widespread belief that COX-1 was constitutively expressed and produced "housekeeping" prostanoids, while the induction of COX-2 expression during inflammation produced unfavorable prostanoids such as PGI_2 and PGE_2, which are known to potentiate inflammation.

The importance of the constitutively produced prostanoids is evident in retrospective studies of NSAID use. Meta-analyses of case-control studies show that the use of non-aspirin NSAIDs increases the risk of upper gastrointestinal tract bleeding/perforation four-fold compared with non-users (33). In an overview of random control trials examining the GI toxicity of aspirin, the odds ratio for gastric ulcers was 4.7 and odds ratio for all GI bleeding was 2.8 (61). It is important to note that aspirin,

which acts by acetylating a serine residue on the COX enzyme, is a selective, irreversible inhibitor of COX-1, with an IC_{50} of 0.3μg/ml for COX-1 versus 50μg/ml for COX-2 (50). These studies provide evidence that prostaglandin production via COX-1 is necessary for GI maintenance. Based on the evidence that COX-1 inhibition caused a disturbance in GI prostanoid synthesis, researchers believed it would be favorable to be able to block COX-2 expression selectively, while preserving the function of COX-1-derived prostanoids in order to avoid the deleterious effects associated with the existing NSAIDs on the market.

2.4 COX-2 Selective NSAIDs

After the identification and purification of COX-2, pharmaceutical companies focused on the development of more selective drugs targeting the COX-2 enzyme to avoid potential GI side-effects associated with COX-1 inhibition and to target the inhibition of pro-inflammatory prostaglandins. The first COX-2 selective NSAID (known commonly as coxibs) was originally marketed in 1999, when G.D. Searle and Pfizer (now Pfizer Inc.) introduced celecoxib (Celebrex®). Merck followed shortly thereafter with the COX-2 selective drug, rofecoxib (Vioxx®). Both celecoxib and rofecoxib had instant success in the pharmaceutical market, with sales exceeding one billion dollars in the first 15 months of sales (59). It is important to note that coxibs are different compared to traditional NSAIDs in that coxibs are COX-1 sparing drugs. Coxibs highly inhibit COX-2 (standard inhibition is ~80%) without affecting COX-1

(less than ~10% inhibition), yet NSAIDs and coxibs are similar in that they both inhibit COX-2 significantly (76).

Coxibs were praised as 'wonder drugs' after results from multiple studies, including the Vioxx Gastrointestinal Outcome Research (VIGOR) clinical trial, confirmed that significantly fewer patients treated with rofecoxib experienced gastrointestinal symptoms compared to naproxen (7). Additionally, a systematic review of randomized controlled trials has shown that COX-2 selective inhibitors produced significantly fewer gastroduodenal ulcers (RR 0.26; 95% CI 0.23, 0.30) and clinically important ulcer complications (RR 0.39; 95% CI 0.31, 0.50) than non-selective NSAIDs (63). The results seen in these trails were attributed to coxibs' ability to spare COX-1.

However, not long after the release of the first COX-2 selective NSAIDs, concerns were raised regarding adverse effects of the new drugs on the cardiovascular system. The VIGOR trial, which compared naproxen, a COX-1 inhibitor, with rofecoxib, a selective COX-2 inhibitor, had some surprising results in that the patients receiving rofecoxib had a fivefold increase in myocardial infarction in addition to the twofold reduction in severe gastrointestinal side effects (6). Due to these findings, the United States Food and Drug Administration (FDA) called for the removal of rofecoxib (Merck), in 2004, and valdecoxib (Pfizer), in 2005, from the market (32). However, the results of the VIGOR trial have been widely disputed based on multiple issues with the trial that were discussed or presented after the conclusion. Many disapproved of the conclusions drawn from the VIGOR trials based on the short duration of the trial, small sample size, and lack of suitable control. Indeed, there is evidence that naproxen can

provide sustained inhibition of platelet COX-1 when given at twice-daily doses, offering support to the view that the VIGOR results may have been biased (74). From these studies, the belief that selective inhibition of COX-2 causes deleterious cardiovascular side-effects was born. In order to understand why an increased risk of cardiovascular events would be caused by selective COX-2 inhibition, it is necessary to understand the origins and effects of prostanoids in the vascular system.

2.5 Role of Prostanoids in the Vascular System

Homeostasis within the vascular system relies on the balance of two functionally antagonistic prostanoids, TxA_2 and PGI_2. In normal physiological conditions, PGI_2, which acts as a vasodilator and inhibits platelet aggregation, is the major product of the vascular endothelium and vascular smooth muscle (VSM). TxA_2 is produced in much smaller amounts by the endothelium, VSM, and platelets, and acts antagonistically to PGI_2, causing vasoconstriction and platelet aggregation. Thus, vascular homeostasis and its key components (tone and hemostasis) depend upon the balance between PGI_2 and TxA_2. Because only one COX isoform was known for years, it was originally believed that TxA_2 and PGI_2 were both products of the same COX-1. After the discovery of COX-2, the prevailing view of the origins of TxA_2 and PGI_2 shifted.

It was previously believed that COX-2 expression was induced during inflammatory states, but there is now clear evidence to show that COX-2 is constitutively expressed in tissues such as the aortic endothelium (35). Turnover of COX-2 mRNA is rapidly regulated by post-transcriptional mechanisms; therefore

immunohistochemistry of COX-2 enzyme levels is likely an inaccurate measurement of the actual amount present or active in tissues. In addition, COX-2 is more active at lower substrate and hydroperoxide concentrations, therefore it is likely that a large amount of PGI_2 is synthesized by relatively small amounts of COX-2 in the vasculature (26). TxA_2 is historically a product of COX-1 in the platelets, but more recent studies suggest that the vascular endothelium synthesizes TxA_2 through either COX-1 or COX-2. Due to differences in COX expression and regulation, it is difficult to identify the origin of vascular prostanoid synthesis by each COX isoform.

PGI_2 is commonly found at sites of inflammation, in addition to its role in many housekeeping functions including renal blood flow regulation and gastric acid secretion. It was historically believed that in endothelial cells, COX-1 was the source of PGI_2. In 1999, the first study was published showing the effects of a COX-2 selective inhibitor drug on vascular prostanoid levels. The results were puzzling, as they showed the use of celecoxib depressed urinary PGI-M levels. This was the first time that PGI_2 was reported to be derived from COX-2 (47). Although these results were debated due to the unclear origin of urinary PGI-M metabolites, in 2007, the American Heart Association (AHA) published its acceptance of PGI_2 as the main product of COX-2 in endothelial cells (3). However, contrary to these aforementioned data that COX-2 is the main source of PGI_2, functional studies in humans have shown that PGI_2 levels are abolished by low doses of a selective COX-1 inhibitor, aspirin (42). Indeed, as recent as 2012, studies have continued to reverse those early reports that PGI_2 originates from COX-2 (37). Taken together, these studies provide strong evidence that both COX-1 and COX-2 are

involved in the biosynthesis of PGI_2. It is now known that, due to differences in the regulation of expression, it is possible for both COX-1 and COX-2 to function independently within the same cell (10, 14, 18, 43, 49).

TxA_2 is produced in minor amounts, but nevertheless acts antagonistically to PGI_2 to cause platelet aggregation and vasoconstriction. Upon platelet activation by thrombin, release of TxA_2 serves to increase platelet coagulation and vascular tone. In studies using TP receptor deficient mice, increased bleeding time was observed as an abnormal vascular response due to delayed platelet aggregation (70). Additional roles of TxA_2 in the vascular system include neovascularization and atherogenesis. It has long been known that TxA_2 synthesis is a product of COX-1 expression in the platelets; however, recent studies have shown that COX-2 expression in vascular smooth muscle (VSM) and endothelial cells can also produce TxA_2 (4, 43, 59).

2.6 The Imbalance Theory

Although PGI_2 is produced in much larger amounts than TxA_2, the balance between these two mediators is very important to the maintenance of vascular health. The imbalance theory states that a negative shift in the balance between PGI_2 and TxA_2 can lead to the pathogenesis of cardiovascular diseases such as coronary artery disease and stroke. A decrease in the major prostanoid released, PGI_2, may cause a shift in the balance from dominance by the anti-aggregatory, vasodilatory PGI_2 to the pro-aggregatory, vasoconstrictor TxA_2, increasing the deleterious influences of the latter. In theory, COX-1 synthesizes the majority of TxA_2 while COX-2 produces antagonistically

functioning PGI_2. The imbalance theory is commonly the scapegoat for an increased risk of thrombosis with coxib use, because of the belief that PGI_2 is mainly derived from COX-2 activity.

The unexpected deleterious effects of coxibs on the cardiovascular system started an ongoing dilemma within the medical community. A highly desirable effect of coxibs was the reduced the risk of GI disturbances through the sparing of COX-1 function. Conversely, an unforeseen drawback of selectively inhibiting COX-2 was the increased risk of harmful cardiovascular effects by altering the PGI_2/TxA_2 balance toward TxA_2. Many have claimed these effects can be explained by the imbalance theory, resulting from the increase in TxA_2 and the decrease in PGI_2 levels. However, there are several studies which suggest that the imbalance theory is flawed on the basis that urinary PGI_2 metabolite levels do not accurately reflect measurements of vascular PGI_2 synthesis (24). Therefore, the studies that originally proposed that coxibs cause an increased risk of thrombosis due to a selective decrease in PGI_2 without depressing TxA_2 levels are not valid. Although the imbalance theory may not accurately depict the underlying mechanisms, the fact that COX-2 selective drugs produce an increased risk of thrombotic events is indisputable. In light of this, other theories describing the possible mechanisms responsible for increased cardiovascular risk with the use of coxibs have arisen.

2.7 Thromboxane and Prostacyclin Pathway Interaction

The actions of TxA_2 and PGI_2, and their respective receptors' are known to interact through several mechanisms to alter the release and/or production of these mediators in the cardiovascular system. Activation of the TP receptor appears to mobilize calcium in endothelial cells and cause the release of the antagonistic mediator, PGI_2 (34). PGI_2 release modulates the effects of TxA_2 in the vasculature, by limiting TxA_2 production (80). Furthermore, PGI_2 receptor (IP) activation reportedly depresses TxA_2 response by down regulating expression of the TP receptor (29). These interactions may be due to heterodimerization of the TP and IP receptors that result in cross-talk between the two prostanoid receptors in endothelial cells (19). Another mechanism of cross-talk between TxA_2 and PGI_2 includes the independent promotion or repression, of prostanoid receptor expression due to the overlapping genetic response elements for the TP and IP receptors (29). It has been speculated that TxA_2 depresses PGI_2 synthesis through activation of the TP receptor and subsequent activation of intracellular protein kinase C (PKC), which causes nitrosylation of a tyrosine residue on the PGIS enzyme, reducing its activity and the synthesis of PGI_2. If correct, this intriguing theory would be the first evidence that TxA_2 negatively regulates PGI_2 synthesis in the vascular wall.

2.8 Sexual Dimorphism in Cardiovascular Function

Sexual dimorphism is a factor that contributes to the incidence of cardiovascular disease, but that is often overlooked. Many epidemiological studies reveal that the incidence of cardiovascular disease is greater in men than in premenopausal women,

suggesting that sex steroids, especially estrogen, may protect women from such pathophysiological states. Carotid atherosclerosis in postmenopausal women is notably more prevalent than in premenopausal women (54% vs 25%, respectively) suggesting that changes that occur in female body during menopause may have deleterious effect on the cardiovascular system (69). Additionally, postmenopausal women have an incidence of coronary heart disease (CHD) similar to that of men. These findings suggest that the presence of circulating ovarian steroid hormones exert a protective effect on the cardiovascular system. On the other hand, more recent epidemiological studies in women and experimental studies in animals reveal that CVD that are primarily vascular in origin occur more frequently in females and are exacerbated in the presence of estrogen (31, 43).

2.9 Role of Estrogen in the Cardiovascular System

Beginning in 1992, a series of studies were published which concluded that estrogen reduces the risk for CHD by a substantial amount, some claiming a 35% reduction, others 40% to 60% (30, 31). However, more recent clinical trials in conjunction with recent epidemiological studies challenge these earlier studies and suggest that estrogen is not protective against CHD. The Heart and Estrogen/Progestin Replacement Study (HERS) was the first randomized, double-blind, placebo-controlled study that evaluated postmenopausal women with established CHD treated with estrogen replacement therapy (ERT). The HERS study found no difference in cardiac outcome between estrogen-treated and placebo-treated women after 4.1 and 6.8 years follow up,

therefore repealing the initial proposal that estrogen plays a protective role in women (77, 78). The HERS study was widely quoted, and somewhat misinterpreted, in the years following. In 2001, at the recommendation of the *Journal of the American Heart Association*, the American Heart Association (AHA) advised against the prescription of ERT in women with preexisting cardiovascular disease. However, these warnings were taken out of context and caused confusion in the medical community leading many to believe that all women on ERT had an increased risk for CVD (48). One problem with the AHA making recommendations from the conclusions of the HERS Study, is that that the sample of women included only represented a small population of postmenopausal women with established CHD. The current dogma is that in postmenopausal women, the loss of female sex steroids exerts a deleterious effect on the cardiovascular system, while the existence of sex steroids in premenopausal women has a protective effect.

Estrogen may exert either beneficial or detrimental effects on the vasculature depending on the estrogen receptors (ER) present. There are both membrane-bound and cytosolic target receptors in the endothelium cells (45). Both cell types contain α and β receptors, the two classical types of ER. Activation of an ER stimulates different signaling pathways and changes in expression of target genes, leading to various effects, such as protein synthesis and cell proliferation (16). In human studies, estrogen directly increases the expression of the PGI_2 receptor (IP) within the vasculature through an ERα transcriptional mechanism (72). Recent evaluation of the role of ERα and ERβ on the balance of PGI_2/TxA_2 in cultured human umbilical vein endothelial cells (HUVEC) revealed multiple mechanisms by which estrogen exerts cardioprotective effects. After

exposure to estradiol, production of PGI_2 was increased in a dose-dependent manner, while expression of both COX-1 and PGIS genes and their proteins were increased by 20% and 50%, respectively. These effects were dependent upon the activation of ERα by 17β-estradiol (E_2). In this study, TxA_2 was unaltered by exposure to E_2. In similar studies, PGI_2 production was also increased in response to an increase in E_2 and COX-1 (1). These data reveal that through ERα, estradiol enhances PGI_2 production by increasing both COX-1 and PGIS expression, shifting prostanoid balance in favor of the beneficial PGI_2 (68). Although ERα is known to have protective effects in the vasculature, ERβ has been rarely documented to have an effect on prostanoid production.

In studies investigating the effects of E_2 on cultured endothelial cells, E_2-induced activation of protein kinase C (PKC) resulted in increased expression of COX-2 (30). Similarly, studies in intact arterial vessels revealed that COX-2 mRNA and protein expression are attenuated in the absence of estrogen in ovariectomized (OvX) female rats and are enhanced in intact female (Int) and OvX + E_2-replaced rats (1, 43). Additionally, it was reported that serum estradiol levels coincide with COX-2 expression throughout the estrus cycle, with levels of estradiol and COX-2 expression peaking during proestrus and both plummeting during diestrus (54). Together, the results from these studies suggest that estrogen exerts its protective effect on the vasculature by activating ERα, thereby increasing gene expression of IP, PGIS, and both COX-1 and COX-2 causing an increase in PGI_2 production.

In contrast, recent studies have revealed that estrogen can also potentiate thromboxane pathway expression in endothelial cells and VSM, enhancing constrictor function. Estrogen potentiated the release of and the reactivity to TxA_2 by upregulating mRNA and protein expression of COX-2, TxS, and TP in the female rat aorta. These results were attenuated in ovariectomized female (OvX-F) rats and restored in OvX-ERT rats (43). These results were one of the few recent studies to reveal that estrogen can exert deleterious effects, despite the largely accepted dogma that estrogen plays a beneficial role in cardiovascular function.

Our lab has previously reported that a more complex relationship exists between the TxA_2 and PGI_2 biosynthesis pathways, and that the presence of TxA_2 may negatively regulate PGI_2 production. The inhibition of the TxA_2 synthesis pathway using Dazoxiben (Daz, TxS inhibitor) enhanced production of PGI_2. This evidence suggests that TxA_2 may attenuate PGI_2 synthase (PGIS). A possible mechanism of action for this may be protein kinase C (PKC) nitration of a tyrosine residue on PGIS (12).

2.10 Statement of Objectives and Investigation Rationale

2.10.1 Objectives

The prostanoid biosynthesis profiles of the major COX isozymes in the vascular wall remain uncertain. PGI_2 (the main prostanoid produced by the endothelium and VSM) was initially believed to be the main product of the COX-2 isoform; however, it was also synthesized also by COX-1 in more recent studies (37). Similarly, TxA_2 was initially believed to be produced mainly by COX-1 in platelets; however, more recent

studies revealed that estrogen potentiates COX-2 expression in the vasculature and may be responsible for increased TxA_2 production in the female rat aorta (43). If the COX isoforms present in the endothelium and VSM determine the production of PGI_2 and TxA_2, then inhibition of a specific COX enzyme (COX-1 or COX-2) should yield a specific ratio of PGI_2/TxA_2. The ratio of PGI_2/TxA_2 production in the vascular wall plays an important role in the vascular homeostasis; therefore, it is important to understand the relative roles of COX-1 and COX-2 in this balance. The experiments in this thesis were designed to clarify the roles of COX-1 vs. COX-2 in the production of PGI_2 and TxA_2 and the vascular wall.

2.10.2 Rationale

The existing "imbalance theory" dominates the view regarding the roles of vasodilator prostacyclin (PGI_2) versus vasoconstrictor thromboxane (TxA_2) in vascular homeostasis and the pathogenesis of vascular dysfunction and thrombosis. In theory, vascular tone and hemostasis are dependent upon a greater ratio of PGI_2-derived via COX-2 than platelet-derived TxA_2 via COX-1(24). Thus, a selective COX-2 inhibitor would alter this balance in favor of TxA_2 synthesis, producing vasoconstriction, platelet aggregation, and thrombosis. Concern over the cardiovascular risks associated with the vascular effect of selective COX-2 inhibitors forced the Food and Drug Administration (FDA) to remove multiple COX-2 selective NSAIDs from the market, citing the imbalance theory as the likely cause for such risks (41). However, recent reports suggest

that the assumptions upon which the imbalance theory is based are incorrect, and therefore the validity of the theory is now in question (22, 24).

Based on the measurements of 2,3-dinor-6-keto $PGF_1\alpha$ ((PGI-M), the major urinary metabolite of PGI_2), it was proposed that endothelial COX-2 was the primary mediator of PGI_2 formation; however, this theory was challenged recently on the basis that the use of urinary metabolites of PGI_2 as a means of measuring prostacyclin from vascular tissues is inaccurate. Because PGI_2 acts locally at its site of production, and that other, non-endothelial sources of PGI_2 exist throughout the body that could contribute to levels measured in the urine, urinary metabolites provide an inaccurate measurement of vascular production of PGI_2 (22, 53). Several methods of analysis using direct measurement from the vasculature suggests that in the normal state, endothelial cells in human coronary arteries, coronary arterioles, carotid arteries, aorta, and other vascular sources express high levels of the COX-1 enzyme, but not the COX-2 enzyme [1]. In human studies using low doses of aspirin, a selective COX-1 inhibitor (35-75mg/day), vascular levels of PGI_2 and TxA_2 were both abolished. These studies show that, unlike previous reports, the COX-1 enzyme generates PGI_2 in the vasculature. Therefore, selective inhibition of COX-2 may not affect the local levels of PGI_2 in the vasculature, and should not increase the likelihood of thrombotic events (71). Taken together, these findings suggest that the assumption that COX-2 selective inhibitors tip the scales in favor of prothrombotic TxA_2 formation is flawed on the basis that significant amounts of PGI_2 appear to be derived from COX-1 in the endothelium.

The importance of sex differences in vascular physiology is often overlooked, especially as it pertains to the balance of prostanoid activity. The incidence of cardiovascular disease is higher in men than women, as demonstrated by a variety of earlier epidemiological studies. Several recent studies have shown that estradiol (E_2), the most potent estrogen in humans, exerts its effects on prostanoid biosynthesis by upregulation of COX-2 in the endothelium and vascular smooth muscle (43). Therefore, estrogen may play a protective role in the vasculature in females as it potentiates PGI_2 release from the endothelium. Our lab previously reported that TxA_2 exerts an inhibitory effect on PGI_2 production, an effect found to be greater in male than female; however, the mechanism of action is unknown. This research was designed to determine the role of PGI_2/TxA_2 cross-talk in prostanoid balance in the vascular wall.

Many of the studies that have examined the roles of COX-1 and COX-2 in the vasculature were performed solely in males. The roles of sex and the sex hormones in the production of prostanoid synthesis have been commonly disregarded in vascular physiology and pharmacology. Estrogen is now known to increase the expression of both COX-1 and COX-2 in the vasculature (43, 52). Therefore, the effects of sex and the sex steroids in balancing prostanoid synthesis may play a larger role in the cardiovascular system than previously believed. The proposed research was expected elucidate the role of estrogen in regulating the balance between COX-1 and COX-2 and the production of TxA_2 and PGI_2 in the vascular wall. Thus, the overall hypothesis to be tested is as follows (Figure 2):

Inhibition of COX-1 will reduce TxA_2 to a greater extent than PGI_2 production, whereas COX-2 inhibition will reduce PGI_2 production to a greater extent than TxA_2 production in the rat aorta. Furthermore, estrogen replacement will enhance synthesis of COX-2 derived prostanoids, but have no effect on COX-1 prostanoid products.

Figure 2: Hypothesis.

2.10.3 Specific Aims

The specific aims of this project are to determine:

1) the relative roles of COX-1 vs. COX-2 in the production of TxA_2 and PGI_2 by the female vs. male rat aorta

2) the role of estrogen in the production of vascular prostanoids PGI_2 and TxA_2 in the female rat aorta

3) cross-talk between PGI_2 and TxA_2 pathways, specifically the mechanism by which TxA_2 inhibits PGI_2 synthesis in the vascular wall

CHAPTER III

EXPERIMENTAL DESIGN AND METHODS

3.1. Project I

3.1.1 Experimental Animals and Maintenance

Age-matched (14-16 week old) male and female Sprague-Dawley rats (Harlan, Houston, TX) were used in all experiments. The rats were housed in the Laboratory Animal Resources and Research facility (LARR) with controlled temperature (22–24°C), relative humidity (~50%), and 12-h:12-h light-dark cycle. The rats were provided with a 16% protein phytoestrogen-free rat chow and tap water ad libitum. All experiments were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). The rats were randomly divided into four treatment groups: Males (M), intact females (Int-F), ovariectomized females (OVX-F), and OVX-estrogen-replaced (OvX-ER-F) females.

3.1.2 Ovariectomy and Estrogen Replacement Therapy

Bilateral ovariectomy surgery (OvX Sx) was performed on 14-week-old females using standard methods. Half of the OvX rats received estrogen replacement therapy using 17β-estradiol (3 x 0.05 mg/60-day release pellets, Innovative Research; Sarasota, FL). Previous studies have shown that this dose produces physiological plasma levels of 17β-estradiol that are similar to Int-F rats (64).

3.1.3 Animal Sacrifice, Tissue Preparation, and Experimental Set Up

Rats were sacrificed 14-16 days post-OvX or post OvX+ER by rapid decapitation, in accordance with American Veterinary Medical Association and Texas A&M University IACUC guidelines. At the time of decapitation, trunk blood was collected for RIA of plasma 17β-estradiol. The thoracic aorta was removed gently without stretching and cleaned of all adipose and connective tissue, then sectioned into aortic rings 3mm in length. Krebs-Henseleit-Bicarbonate (KHB) buffer was prepared daily and kept chilled (4°C), gassed (95% O2-5% CO_2), and composed of (in mM) 118.0 NaCl, 25.0 $NaHCO_3$, 10.0 glucose, 4.74 KCl, 2.5 $CaCl_2$, 1.18 $MgSO_4$, and 1.18 KH_2PO_4 (pH 7.40; osmolality, 292 ± 1 mosmol/kgH_2O).

The aortic rings were kept in KHB at 4°C throughout dissection and during a 45-minute stabilization period. Two aortic rings each were placed in a 12 x 75 μm plastic culture tube containing 2 mL pre-incubation KHB, either vehicle (basal), SC-560 (a selective COX-1 inhibitor, 10^{-7} M), or NS-398 (a selective COX-2 inhibitor, 10^{-5}) for a pre-incubation period of 30-minutes to gradually warm the rings to 37°C. The concentration of each inhibitor used was formulated to be ten times the IC_{50} provided from Caymen Chemicals (IC50 = SC-560: 9nM, NS-398:0.15μM). The pre-incubation buffer was removed and replaced with 1mL incubation buffer, either KHB vehicle (basal), vasopressin (VP; 10^{-6} M), VP+SC-560 (10^{-7} M), or VP+NS-398 (10^{-5} M) and incubated at 37°C and gassed continuously for 45 minutes. The treatment buffer was removed and stored at -80°C until RIA of TxB_2 and 6-keto-$PGF_{1\alpha}$ was performed.

Prostanoid release was normalized by dry tissue weight of the aortic rings and expressed as pg/mg dry tissue wt/45 minutes.

3.1.4 Plasma Estrogen Measurement

Following decapitation, trunk blood was collected into 13 x 100-mm glass culture tubes coated with 150 U of EDTA, centrifuged (10,000 *g* for 5 min), and the resultant plasma separated and stored at -80°C until RIA of 17β-estradiol was performed. The estradiol concentrations were measured in duplicate using a double-antibody RIA (Diagnostic Products; Los Angeles, CA) and validated for measurements of rat plasma (43). Plasma 17β–estradiol concentrations were expressed as pg/mL plasma.

3.1.5 Vaginal Smears

Vaginal smears were performed on Int-F each day for two weeks leading up to the day of sacrifice in order to determine phase of the estrous cycle. The smears were dried and stained with crystal violet then analyzed under a microscope for the presence of phase-specific cell types. The phase of the estrus cycle was determined based on the histological profile observed. The proestrus stage was identified by a predominance of nucleated epithelial cells; the estrus stage was identified by anucleated cornified cells; the diestrus stage was identified by a mix of all cell types: nucleated epithelial cells, anucleated cornified cells, and a small presence of leukocytes; the metestrus stage was identified by the overwhelming presence of rounded leukocytes (46).

3.1.6 Radioimmunoassay Measurement of Thromboxane A_2 and Prostacyclin

Specific RIAs for 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and TxB_2 (a stable metabolite of TxA_2) were used to measure prostanoid concentrations in the tissue incubation media. 100μL of sample was incubated with [3H] 6-keto-$PGF_{1\alpha}$ and with antiserum overnight at 4°C. The charcoal-dextran method was used to separate bound and free fractions of PGI_2. 100μL of sample was also used for RIA of TxB_2 using a commercial kit from Caymen Chemicals. Bound radioactivity from each RIA was counted by liquid scintillation spectroscopy. The limit of detection of the PGI_2 RIA was 3.90 pg/mL and 1.95 pg/mL for TxB_2.

3.1.7 Experimental Design

The experiments were designed to determine: 1) The relative roles of COX-1 versus COX-2 in the production of PGI_2 vs. TxA_2 by the vascular wall, and: 2) The effects of estrogen on the production of COX-1-derived vs. COX-2-derived prostanoids and 3) The cross-talk effects of TxA_2 on PGI_2 release.

3.1.8 Data Analysis

The data were expressed as the mean ± standard error with "n" indicating the number of animals in each experimental group. A two-way analysis of variance (sex vs. treatment) with repeated measures was used to detect significant differences among the experimental groups, Int-F (intact female), OvX-F (ovariectomized female), OvX-ER-F (ovariectomized, estrogen-replaced female), and M (male), and the treatments, basal,

VP, VP+SC-560, VP+NS-389, followed by a post hoc Tukey test to identify differences among the means of the experimental groups. The difference between COX-1 and COX-2 inhibition (Δ VP by SC-560 and NS-398) was evaluated by a two-way analysis of variance (sex vs. treatment) with repeated measures followed by a post hoc Tukey test. A P value of ≤ 0.05 was considered statistically significant.

3.2 Project II

3.2.1 Experimental Animals and Maintenance

14-16 week old male Sprague-Dawley rats (Harlan, Houston, TX) were used in all experiments. The rats were housed in the Laboratory Animal Resources and Research facility (LARR) with controlled temperature (22–24°C), relative humidity (~50%), and 12-h:12-h light-dark cycle. The rats were provided 16% protein phytoestrogen-free rat chow and tap water ad libitum. All experiments were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC).

3.2.2 Animal Sacrifice and Experimental Set Up

Rats were sacrificed by rapid decapitation, in accordance with American Veterinary Medical Association and Texas A&M University IACUC guidelines. The thoracic aorta was removed gently, without stretching, and placed in chilled (4°C), gassed (95% O2-5% CO_2) Krebs-Henseleit-bicarbonate buffer (KHB). The aorta was cleaned of all adipose and connective tissue, and then sectioned into rings measuring 3mm in length. KHB buffer was prepared daily and composed of (in mM): 118.0 NaCl,

25.0 $NaHCO_3$, 10.0 glucose, 4.74 KCl, 2.5 $CaCl_2$, 1.18 $MgSO_4$, and 1.18 KH_2PO_4 (pH 7.40; osmolality, 292 ± 1 mosmol/kgH_2O).

The aortic rings were kept in KHB at 4°C throughout dissection and during a 45-minute stabilization period. Two aortic rings were placed in each 12 x 75 plastic culture tube containing 2 mL pre-incubation KHB (either vehicle, Calphostin C (protein kinase C inhibitor; 10^{-6} M), Ridogrel (TxS inhibitor/TP receptor antagonist; 10μM), Furegrelate (TxS inhibitor; 50 μM) or Dazoxiben (TxS inhibitor; (50μM)). The rings were gradually warmed to 37°C during a 30-minute pre-incubation period and gassed constantly with 95% O2-5% CO_2. The pre-incubation buffer was removed and replaced with 1mL incubation buffer, (either vehicle vasopressin (VP; 10^{-6} M), or a combination of VP (10^{-6}M) + Calphostin C (10^{-6} M), VP + Ridogrel (10μM), or VP + Furegrelate (50μM), or VP + Dazoxiben (50μM)), and incubated at 37°C and gassed continuously for 45 minutes. The treatment buffer was removed and stored at -80°C until RIA of TxB_2 and 6-keto-$PGF_{1\alpha}$ was performed. Prostanoid release was normalized by dry tissue weight of the aortic rings and expressed as pg/mg dry tissue wt/45 minutes.

3.2.3 Radioimmunoassay Measurement of Thromboxane A_2 and Prostacyclin

Specific RIAs for 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and TxB_2 (a stable metabolite of TxA_2) were used to measure the concentration in the samples collected from experimentation. 100μL of sample was incubated with [3H] 6-keto-$PGF_{1\alpha}$ and with antiserum overnight at 4°C. The charcoal-dextran method was used to separate bound

and free fractions. Bound radioactivity was counted by liquid scintillation spectroscopy. The limit of detection of the RIA is 3.90 pg/mL for PGI_2 and 1.95 pg/mL for TxB_2.

3.2.4 Experimental Design

The experiments were designed to determine the mechanism by which TxA_2 inhibits PGI_2 synthesis in the vascular wall. We hypothesized that inhibition of PKC with Calphostin C would increase PGI_2 production similarly to Dazoxiben and Ridogrel, TxS synthase inhibitor and combined TxS inhibitor and TP receptor antagonist., respectively. Furegrelate, a TP receptor antagonist should produce similar results to Calphostin C and Ridogrel, but should not increase PGI_2 production as dramatically.

3.2.5 Data Analysis

The data were expressed as the mean ± standard error with "n" indicating the number of animals in each experimental group. A one-way analysis of variance was used to detect significant differences among the treatments, VP, VP+Calphostin, VP+Furegrelate, VP+Ridogrel, VP+Dazoxiben followed by a post hoc Tukey test to identify differences among the means of the experimental groups. A P value of ≤ 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

4.1 Plasma Estrogen Concentrations

Plasma 17β–estradiol concentration was averaged 6.0 ± 2.1 pg/ml in Int-F rats and 24.5 ± 5.9 pg/ml in OvX-ER-F rats. OvX reduced plasma estradiol levels to 0.5 ± 0.14 pg/mg, similar to levels found in M (0.59 ± 0.11 pg/ml). 17β–estradiol concentrations were significantly higher in Int-F and OvX-ER-F than in OvX-F or M rats (P=0.01, P=0.03). OvX-ER-F rats had significantly higher 17β–estradiol concentrations compared to Int-F (P=0.01). There were no significant differences in 17β–estradiol concentrations between OvX-F and M rats (P≥0.05) (Table 1).

Treatment Group	Plasma 17-β-estradiol (pg/ml)
Int-F	6.0 ± 2.1^{a}
OvX-ER-F	24.5 ± 5.9^{b}
OvX-F	0.50 ± 0.14^{c}
M	0.59 ± 0.11^{c}

Table 1: Plasma 17β–estradiol Levels.
The average plasma 17β–estradiol levels from each group. Data are reported as the means ± SE of plasma estrogen levels in pg/ml.

4.2 Effects of COX-1 versus COX-2 Inhibition on PGI_2 Release

4.2.1 Prostaglandin I_2

4.2.1.1 Basal and VP-Stimulated PGI_2 Release

Basal release of PGI_2 from aortic rings was similar in all groups (Int-F (3,663 ± 530 pg/mg ring wt/45 min); OvX-F (2,726 ± 436 pg/mg); OvX-ER-F (4,312 ± 341 pg/mg); M (2,967 ± 850 pg/mg)) (P≥0.05) (Fig. 2).

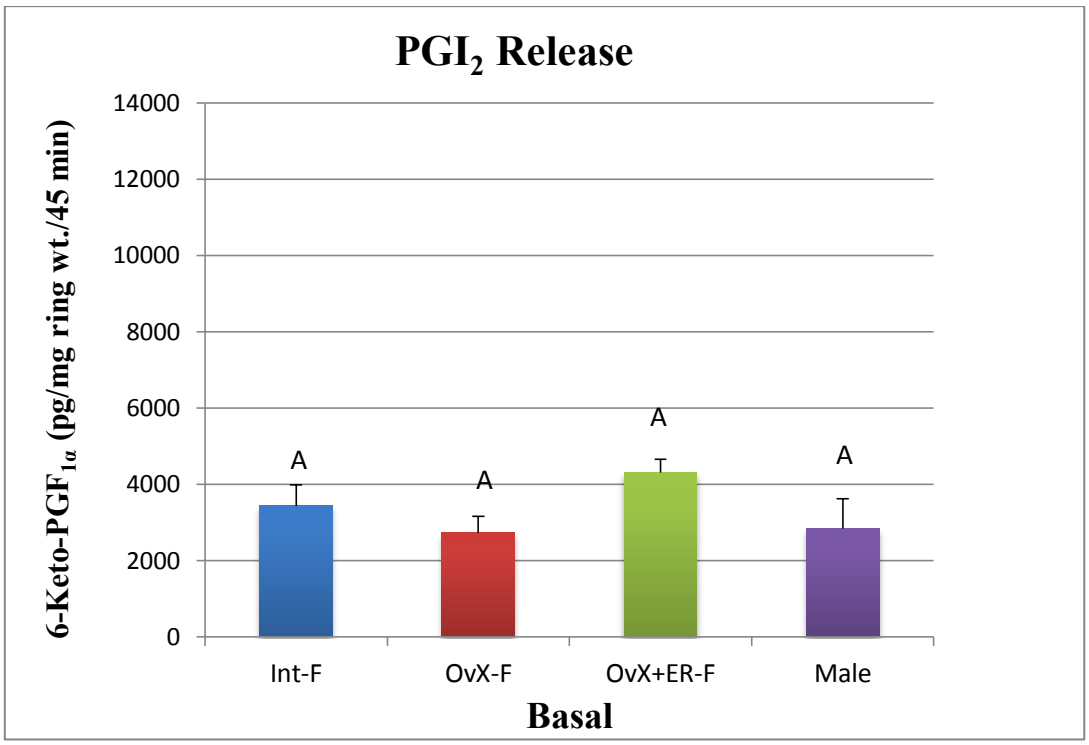

Figure 3: Basal Release of PGI_2.
Basal release of PGI_2 (measured as the stable metabolite 6-keto-$PGF_{1\alpha}$) from rat thoracic aortic rings. Bars are means ± S.E. Int-F, intact female; OvX-F, ovariectomized-females; OvX-ER-F, ovariectomized-estrogen replaced female; M, male rats. PGI_2 release did not differ significantly among any of the experimental groups (P≥0.05).

In response to VP (10^{-6}M), PGI_2 release markedly increased in Int-F (15,352 ± 2,209 pg/mg) and OvX-ER-F (13,053 ± 3,086 pg/mg). This was markedly greater than in OvX-F (4,785 ± 773 pg/mg) or M (4,528 ± 745 pg/mg). Stimulation with VP produced was only significantly increased from basal in Int-F and OvX-ER-F ($.001 \leq P \leq 0.05$) (Figure 3: Basal release of PGI2). VP stimulated 6-keto-PGF_2 419% above basal in Int-F and 303% in OvX-ER-F, whereas stimulation in OvX-F (176%) and M (159%) was increased markedly less (Figure 4, Table 2).

Group	PGI_2 (pg/mg ring wt./45 min)	
	Basal	VP (10^{-6} M)
Int-F (n=7)	3,663 ± 530[a]	15,352 ± 2,209[b]
OvX-F (n=8)	2,726 ± 436[a]	4,785 ± 773[a]
OvX-ER-F (n=7)	4,312 ± 341[a]	13,053 ± 3,086[b]
Male (n=6)	2,844 ± 776[a]	4,528 ± 745[a]

Table 2: Basal and VP-Stimulated Release of PGI_2.
Basal and VP-stimulated release of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) in each group (n, number of animals per group).

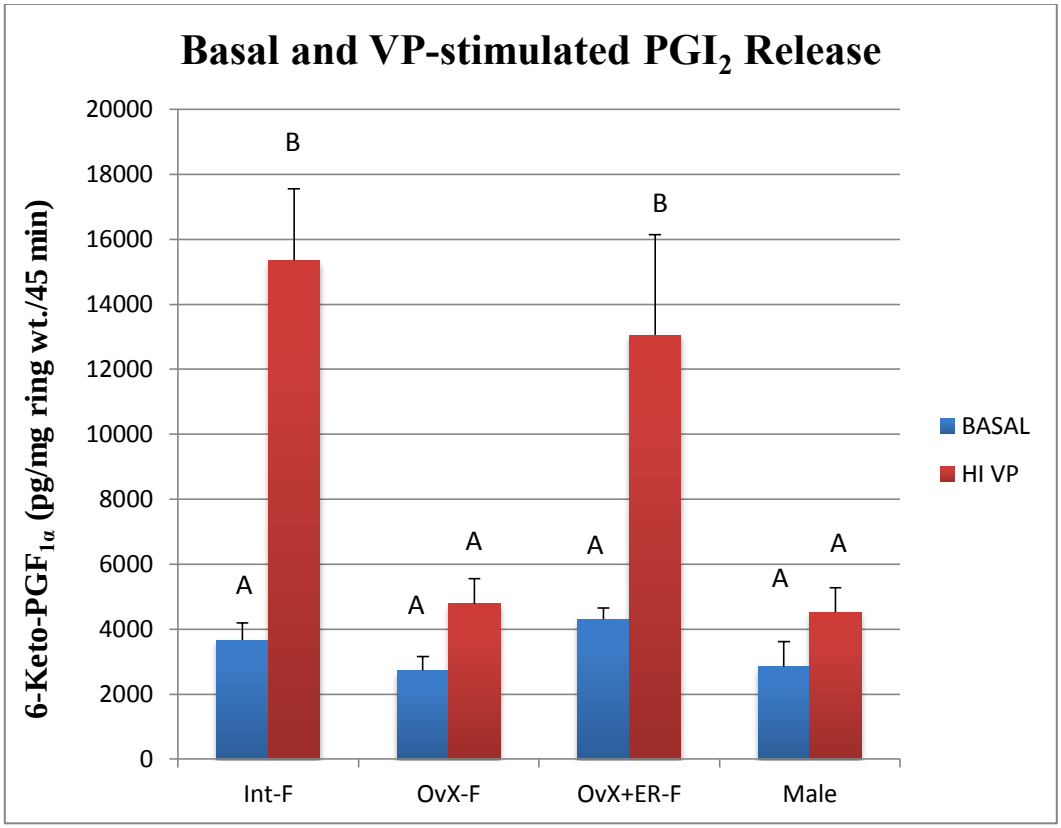

Figure 4: Basal and VP-stimulated PGI_2 Release.
Basal and Vasopressin-stimulated release of PGI_2 (measured as the stable metabolite 6-keto-$PGF_{1\alpha}$) from thoracic aortic rings of Int-F, OvX-F, OvX-ER-F, and M rats. Bars are means ± S.E. Stimulation with VP caused a significant increase in release of PGI_2 in Int-F and OvX-ER-F ($.001 \leq P \leq 0.05$). VP stimulated PGI_2 release was significantly greater in Int-F and OvX-ER-F than in OvX-F and M ($.001 \leq P \leq 0.05$).

4.2.1.2 Effects of COX-1 Inhibition on VP-Stimulated PGI_2 Release

Aortic rings from each group were pre-treated with the COX-1 inhibitor (SC-560; 10^{-7}M) and the effects of VP (10^{-6}M) on PGI_2 release was assessed. SC-560 reduced the maximal response to VP by 71% in Int-F (15,352 ± 2,209 to 4,421 ± 809 pg/mg), 74% in OvX-F (4,785 ± 773 to 1,264 ± 369 pg/mg), 62% in OvX-ER-F (13,053 ± 3,086 to 4,916 ± 923 pg/mg), and 61% in M (4,528 ± 745 to 1,768 ± 507 pg/mg). SC-560 significantly

decreased PGI_2 in Int-F and OvX-ER-F to near or below basal concentrations (Figure 5).

4.2.1.3 Effects of COX-2 Inhibition on VP-Stimulated PGI_2 Release

Aortic rings from each group were pre-treated with the COX-2 inhibitor NS-398 (10^{-5}M) and the effects of VP (10^{-6}M) on PGI_2 release was assessed. NS-398 significantly reduced the maximal response to VP by 83% in Int-F (15,352 ± 2,209 to 2,498 ± 790 pg/mg), and 83% in OvX-ER-F (13,053 ± 3,086 to 2,282 ± 266 pg/mg), whereas the maximal response was decreased by 65% in OvX-F (4,785 ± 773 to 1,672 ± 364 pg/mg) and 48% in M (4,528 ± 745 to 2,335 ± 713 pg/mg) (Fig.5). NS-398 abolished the response to VP, and reduced PGI_2 release to nearly 50% of basal concentrations in Int-F (basal=3,663 vs. NS-398=2,498 pg/mg) and OvX-ER-F (basal=4,312 vs. NS-398=2,282 pg/mg) (Fig. 4).

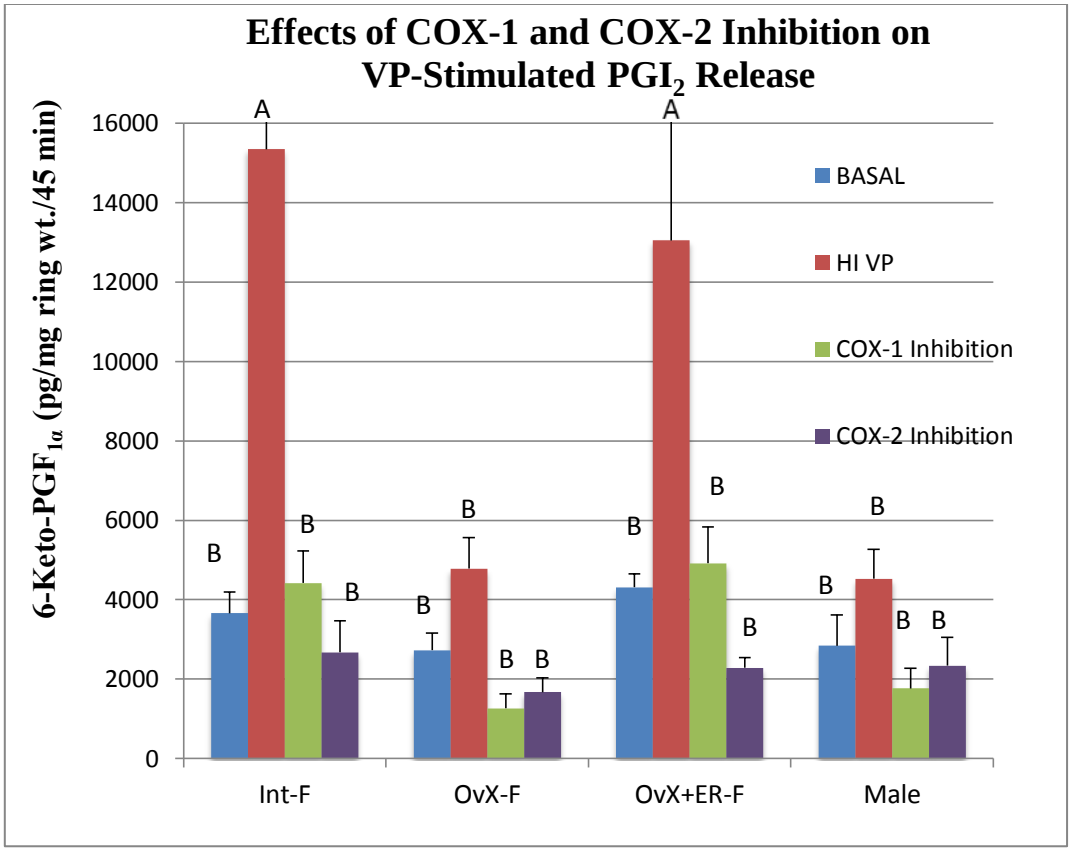

Figure 5: Effects of COX-1 and COX-2 Inhibition on VP-Stimulated PGI_2 Release. Release of PGI_2 (measured as the stable metabolite 6-keto-$PGF_{1\alpha}$) in response to VP-stimulation (10^{-6}M) versus COX-1 inhibition with SC-560 (10^{-7}M) + VP-stimulation (10^{-6}M), or COX-2 inhibition with NS-398 (10^{-5}M) + VP-stimulation (10^{-6}M) from aorta in rats. Bars are means ± S.E. Both SC-560 and NS-398 significantly inhibited VP-stimulated PGI_2 release ($.001 \leq P \leq 0.05$). Inhibition by SC-560 and NS-398 did not significantly differ in OvX-F and M ($P \geq 0.05$).

4.3 Effects of COX-1 versus COX-2 Inhibition on PGI_2 Release

The effects of specific COX-1 and COX-2 inhibitors, SC-560 and NS-398, respectively, on PGI_2 release, were investigated in the aorta of M and F rats. Release of PGI_2 was similar in the presence of SC-560 in all groups ($P<0.05$). Likewise, release of PGI_2 was similar in the presence of NS-398 in all groups. Inhibition of PGI_2 release by

SC-560 and NS-398 did not differ in OvX-F (74% and 65%) or M (61% and 48%) rat aorta. (Table 3, Figure 4).

Group (n≥6)	High VP (10^{-6}M) (pg/mg ring wt/45 min)	SC-560 (10^{-7}M) (pg/mg ring wt/45 min)	NS-398 (10^{-5}M) (pg/mg ring wt/45 min)
Int-F	15,352 ± 2,209[a]	4,421 ± 809[b]	2,498 ± 790[b]
OvX-F	4,785 ± 773[b]	1,264 ± 369[b]	1,672 ± 364[b]
OvX-ER-F	13,053 ± 3,086[a]	4,916 ± 923[b]	2,282 ± 266[b]
M	4,528 ± 745[b]	1,768 ± 507[b]	2,335 ± 713[b]

Table 3: Effects of COX-1 and COX-2 Inhibition on PGI_2 Release.
The average release of $PGF_{2\alpha}$ (stable metabolite of PGI_2) ± standard error from each group in response to high VP-stimulation (10^{-6}M), high VP+SC-560 (COX-1 inhibitor 10^{-7}M), or high VP+NS-398 (COX-2 inhibitor; 10^{-5}M). Mean values without common script are significantly different (.001≤P≤0.05).

4.3.1 Thromboxane A_2

4.3.1.1 Basal and VP-Stimulated TxA_2 Release

Basal release of TxA_2 from aortic sections was similar in all groups (Int-F (22.4 ± 3.0 pg/mg ring wt/45 min, OvX-F (14.3 ± 3.7 pg/mg), OvX-ER-F (23.7 ± 3.8 pg/mg), and M (18.02 ± 3.4 pg/mg) (P=0.31)). VP-stimulated TxA_2 release was increased significantly in Int-F by 261% (22.4 ± 3.0 to 58.5 ± 9.8 pg/mg) and in OvX-ER-F by 298% (23.7 ± 3.8 to 70.6 ± 7.3 pg/mg) (P<0.05). (Table 4, Figure 5).

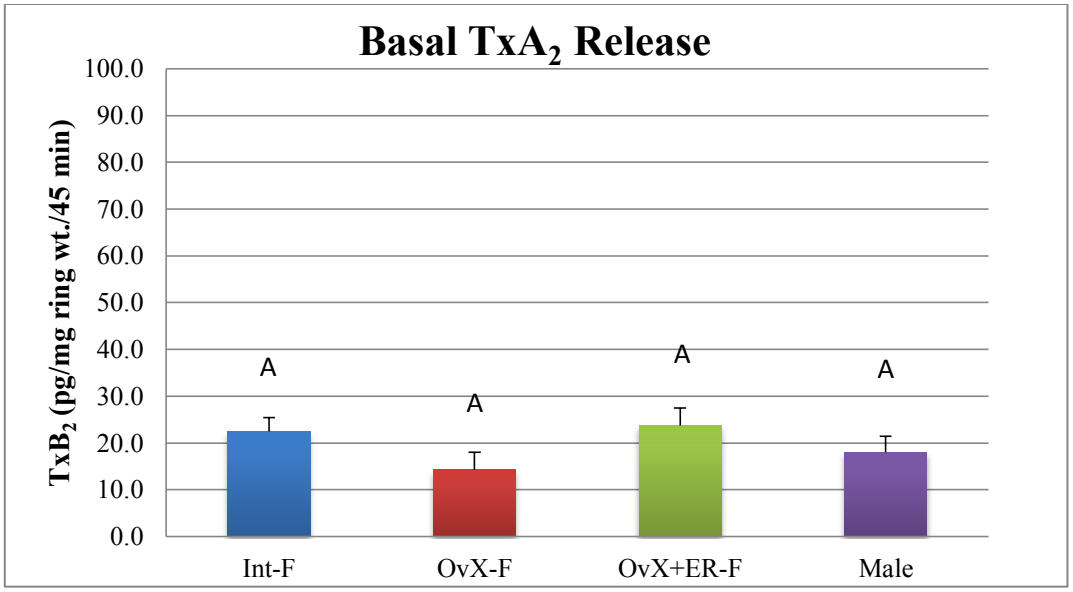

Figure 6: Basal TxA_2 Release.
Basal release of TxA_2 (measured as the stable metabolite TxB_2) from aorta in rats. Bars are means ± S.E. Mean values did not differ significantly between groups ($P \geq 0.05$).

Group	TxA_2 (pg/mg ring wt./45 min)	
	Basal	VP (10^{-6} M)
Int-F (n=6)	22.4 ± 3.0^a	58.5 ± 9.8^b
OvX-F (n=6)	14.3 ± 3.7^a	30.7 ± 7.3^a
OvX-ER-F (n=7)	23.7 ± 3.8^a	70.6 ± 7.3^b
Male (n=6)	18.0 ± 3.4^a	30.3 ± 4.2^a

Table 4: Basal and VP-Stimulated Release of TxA_2.
Basal and VP-stimulated release of TxA_2 (measured as stable metabolite $Tx\beta_2$) from Int-F, OvX-F, OvX-ER-F, and Male rat aortas. Values are means ± SE (n, no. of animals). Mean values without common script are significantly different ($.001 \leq P \leq 0.05$). VP stimulation increased TxA_2 release significantly in Int-F and OvX-ER-F ($P<0.05$).

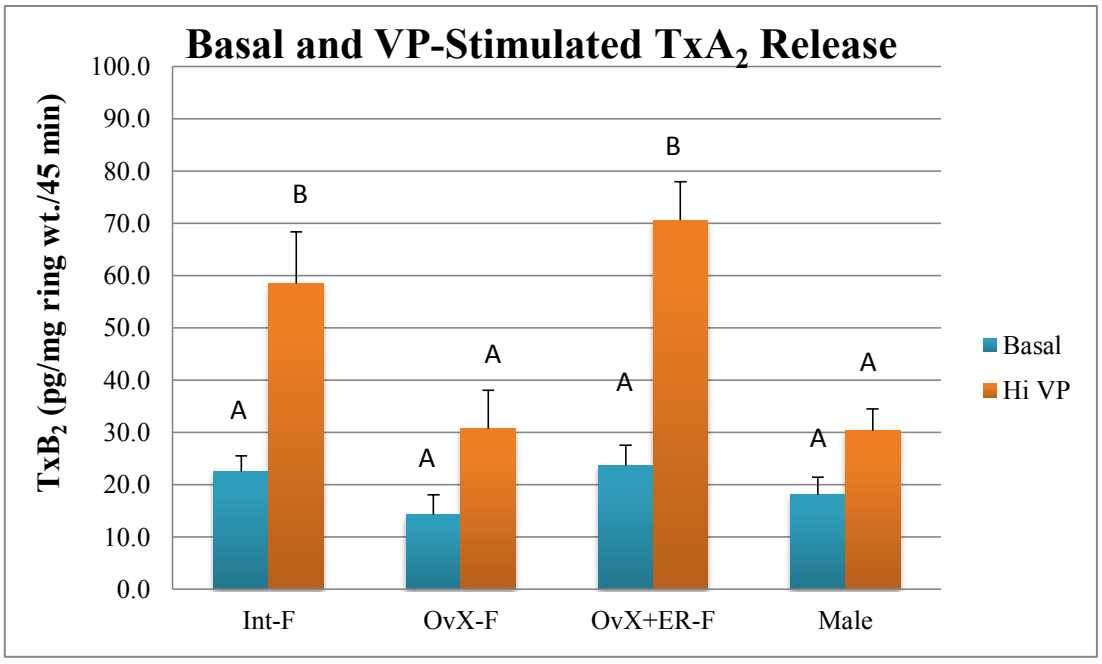

Figure 7: Basal and VP-Stimulated TxA_2 Release.
Release of TxA_2 (measured as the stable metabolite TxB_2) in response to VP-stimulation (10^{-6}M) from aorta in rats. Bars are means ± S.E. VP stimulation in Int-F and OvX-ER-F increased TxA_2 release significantly more than OvX-F and M ($P<0.05$). Mean values without common script are significantly different ($.001 \leq P \leq 0.05$).

4.3.1.2 Effects of COX-1 Inhibition on VP-Stimulated TxA_2 Release

In the presence of COX-1 inhibitor SC-560 (10^{-7}M), TxA_2 release in response to VP was significantly reduced by 73% in Int-F (15.9 vs. 58.5 pg/mg), 60% in OvX-F (12.2 vs. 30.7 pg/mg), 61% in OvX-ER-F (34.7 vs. 70.3 pg/mg) and 82% in M (5.6 vs. 30.3 pg/mg). In OvX-ER-F and M, inhibition of TxA_2 with SC-560 was significantly different ($P<0.05$) (Figure 6).

4.3.1.3 Effects of COX-2 Inhibition on VP-Stimulated TxA_2 Release

In the presence of COX-2 inhibitor NS-398 (10^{-5}), TxA_2 release in response to VP was reduced significantly by 89% in Int-F (6.4 vs. 58.5 pg/mg ring wt./45 min.), 65% in OvX-F (10.7 vs. 30.7 pg/mg), 86% in OvX-ER-F (9.6 vs. 70.3 pg/mg), and 79% in M (6.2 vs. 30.3 pg/mg). There was no differences between groups in response to COX-2 inhibition ($P>0.05$) (Figure 7).

4.3.1.4 TxA_2 Release in Response to COX-1 versus COX-2 Inhibition

The effects of specific COX-1 and COX-2 inhibitors, SC-560 and NS-398, respectively, on TxA_2 release, were investigated in the aorta of M and F rats. Release of TxA_2 was similar in the presence of SC-560 in all groups. Release of TxA_2 was similar in the presence of NS-398 in all groups. Inhibition of TxA_2 release by SC-560 and NS-398 did not differ in in Int-F (73% and 89%), OvX-ER-F (61% and 86%), OvX-F (60% and 65%) or M (82% and 79%) rat aorta. ($P>0.05$) (Table 5, Figure 7).

Group (n≥6)	TxA_2 (pg/mg ring wt./45 min) High VP (10^{-6}M)	SC-560 (10^{-7}M)	NS-398 (10^{-5}M)
Int-F	58.5±9.8[a]	15.9±5.1[c,d]	6.4±2.3[c,d]
OvX-F	30.7±7.3[b]	12.2±3.6[c,d]	10.7±3.7[c,d]
OvX-ER-F	70.3±7.3[a]	34.7±8.3[c]	9.6±1.8[d]
M	30.3±4.2[b]	5.6±1.1[d]	6.2±1.9[c,d]

Table 5: Effects of COX-1 and COX-2 Inhibition on TxA_2 Release.
The average TxA_2 release from each group in response to high VP-stimulation (10^{-6}M), high VP+SC-560 (COX-1 inhibitor 10^{-7}M), or high VP+NS-398 (COX-2 inhibitor; 10^{-5}M).

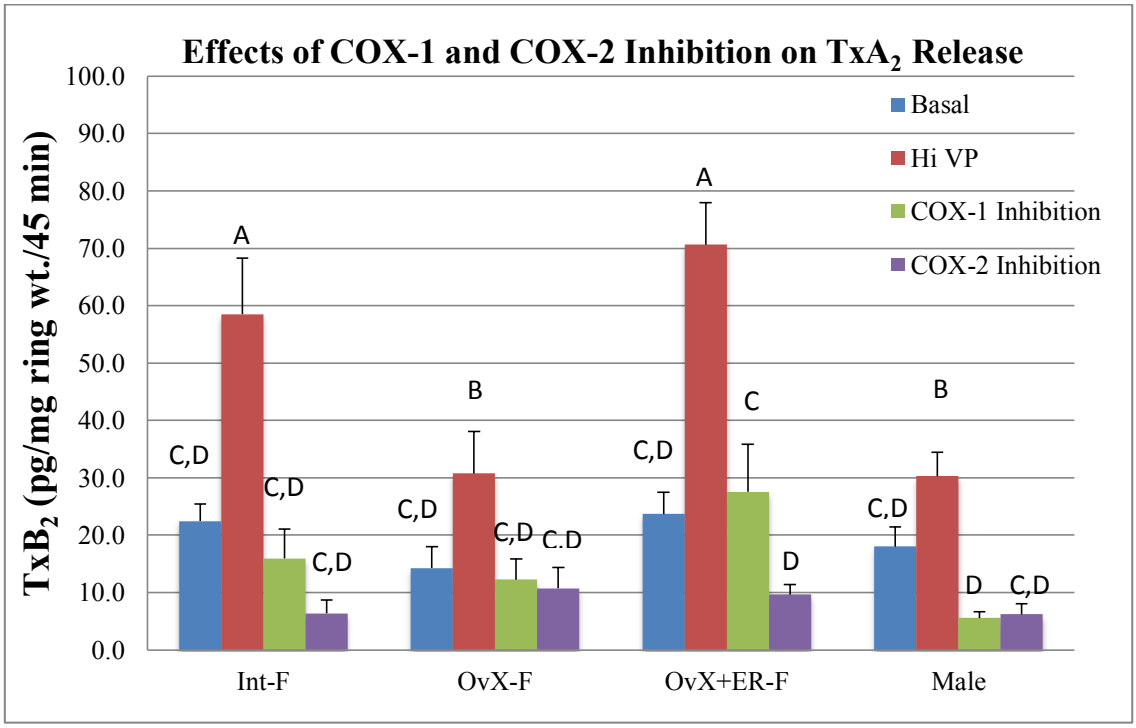

Figure 8: Effects of COX-1 and COX-2 Inhibition on VP-Stimulated TxA_2 Release.
Release of TxA_2 (measured as the stable metabolite 6-keto-$PGF_{1\alpha}$) in response to COX-2 inhibition with NS-398 (10^{-5}M) + VP-stimulation (10^{-6}M) from aorta in rats. Bars are means ± S.E. Mean values without common scripts are significantly different (.001≤P≤0.05).

4.4 Effects of Calphostin C, Ridogrel, Furegrelate, and Dazoxiben on Prostanoid Release

4.4.1 Prostacyclin I_2

VP-stimulated release of PGI_2 (4,016 ± 523 pg/mg ring wt./45 min.) was not significantly affected by pretreatment with the PKC inhibitor, Calphostin C (3,164 ± 593 pg) in male rat aortic sections. Similarly, Ridogrel, a combined thromboxane synthase inhibitor and thromboxane receptor antagonist, and Furegrelate, a thromboxane synthase inhibitor, did not significantly affect the release of PGI_2. (Figure 9, Table 6). Dazoxiben, a TxS inhibitor, increased the release of PGI_2 significantly ($0.001 \leq P \leq 0.05$) (Figure 10).

4.4.2 Thromboxane A_2

VP-stimulated release of TxA_2 (20.1 ± 2.5 pg/mg ring wt./45 min.) was significantly increased by 228% in the presence of the PKC inhibitor, Calphostin C (46.0 ± 8.0 pg), in male rat aortic rings ($P=0.005$). Conversely, Ridogrel, a combined TxS inhibitor and TP receptor antagonist (5.2 ± 0.8 pg), and Furegrelate, a TxS inhibitor (6.5 ± 0.7 pg), both attenuated the release of TxA_2 in aortic rings in a similar manner (74% and 68% reduction, respectively) ($P \leq .0001$) (Table 6, Figure 11). Dazoxiben significantly attenuated the release of TxA_2 in male aortic rings ($0.001 \leq P \leq 0.05$) (Figure 12).

Group	6-ketoPGF$_{1\alpha}$ (pg/mg ring wt./45 min)	TxB$_2$ (pg/mg ring wt./45 min)
VP (10^{-6}M)	4,016±523[a]	20.1±2.5[b]
Calphostin C (10^{-6}M)	3,164±593[a]	46.0±8.0[c]
Ridogrel (10^{-5}M)	3,263±513[a]	5.2±0.8[d]
Furegrelate (50µM)	4,657±552[a]	6.5±0.7[d]

Table 6: Effects of Calphostin, Ridogrel and Furegrelate on PGI$_2$ and TxA$_2$ Release. VP-Stimulated release of 6-keto-PGF$_{1\alpha}$ (stable metabolite of PGI$_2$) and TxB$_2$ (stable metabolite of TxA$_2$) in response to Calphostin C (10^{-6}M), Ridogrel (10^{-5}M), or Furegrelate (50µM) in the aorta of male rats.

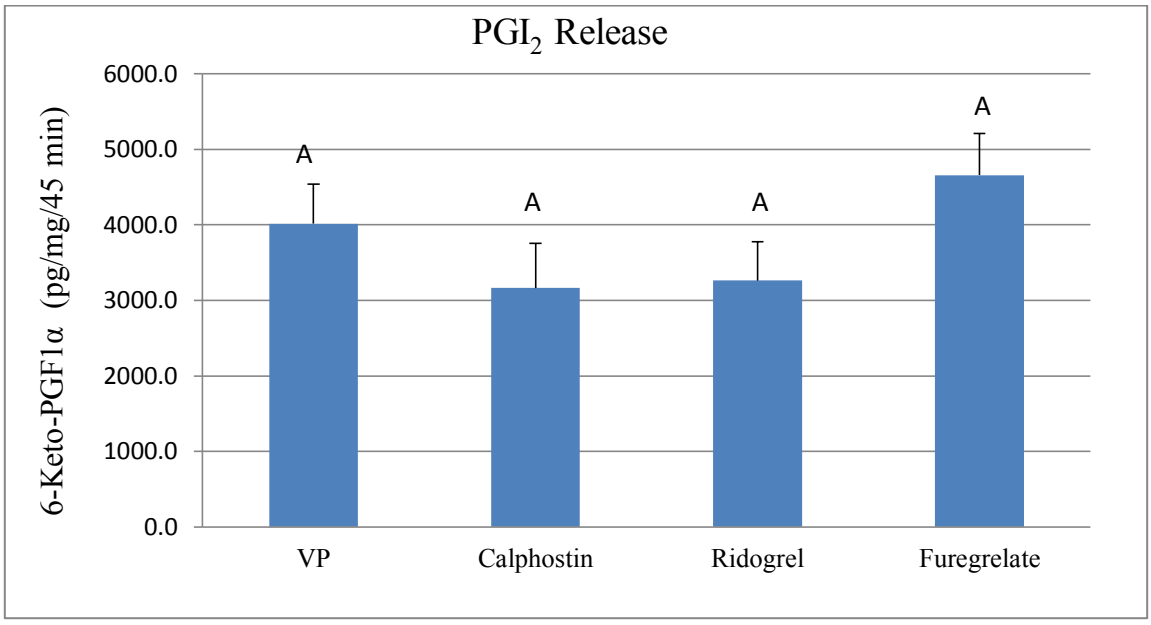

Figure 9: Effects of Calphostin, Ridogrel, and Furegrelate on PGI$_2$ Release. VP-stimulated release of 6-keto-PGF$_{1\alpha}$ (stable metabolite of PGI$_2$) in response to Calphostin C (10^{-6}M), Ridogrel (10^{-5}M), or Furegrelate (50µM) in the aorta of male rats.

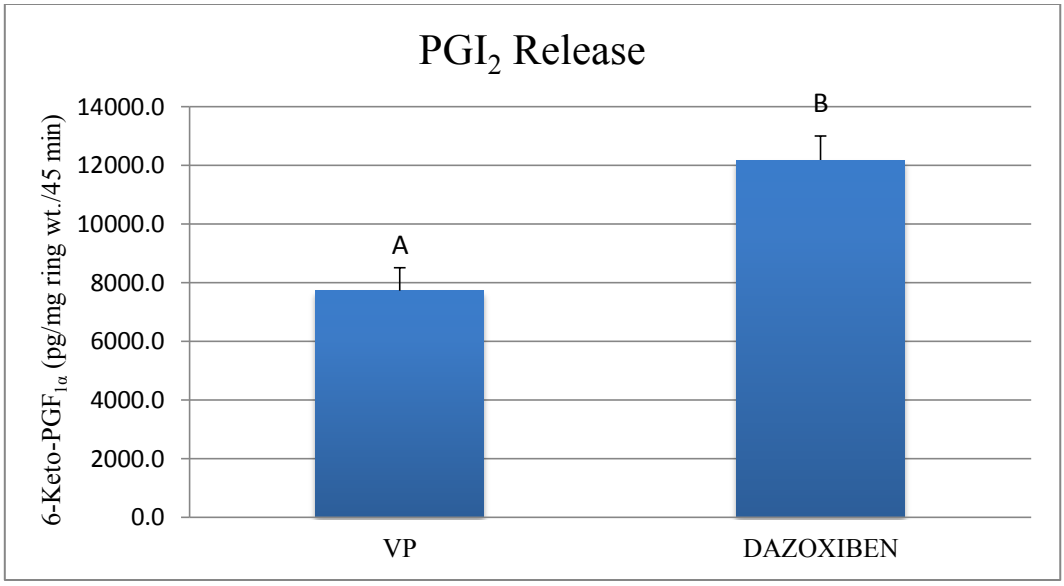

Figure 10: Effects of Dazoxiben (50 µM) on PGI_2 Release.
VP-stimulated release of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) in response to Dazoxiben (50 µM) in the aorta of male rats. DAZ significantly increased the release of PGI_2 ($.001 \leq P \leq 0.05$). Bars are means ± S.E.

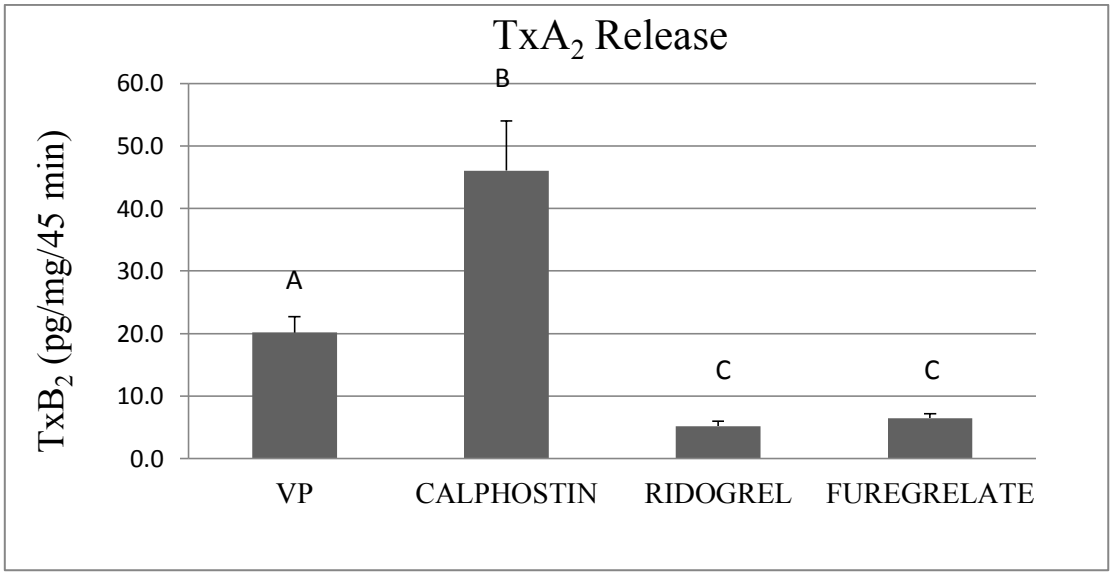

Figure 11: Effects of Calphostin, Ridogrel, and Furegrelate on TxA_2 Release.
VP-Stimulated release of TxB_2 (stable metabolite of TxA_2) in response to Calphostin C (10^{-6}M), Ridogrel (10^{-5}M), or Furegrelate (50µM) in the aorta of male rats. Bars are means ± S.E. (Mean values without common script are significantly different ($.001 \leq P \leq 0.05$).

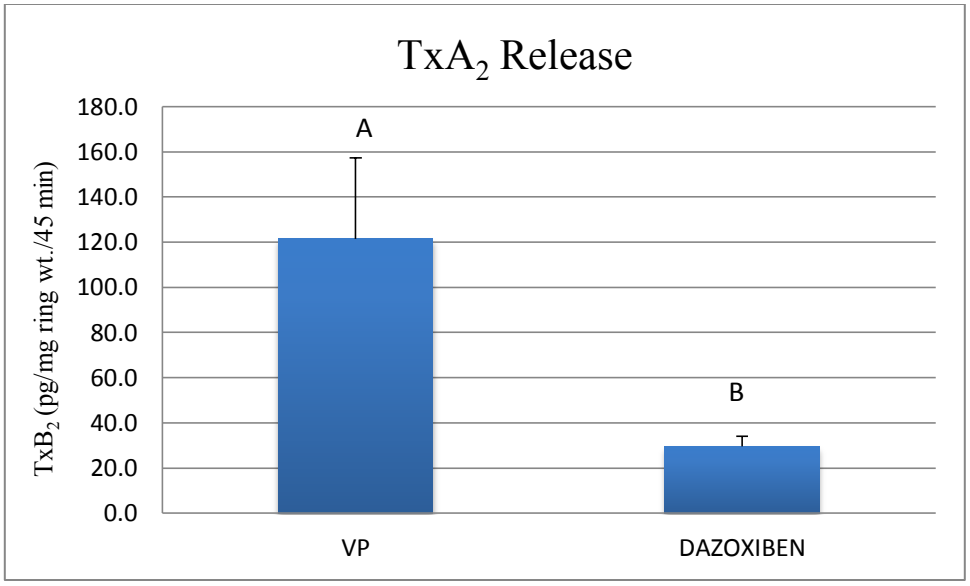

Figure 12: Effects of Dazoxiben (50μM) on TxA_2 Release.
VP-stimulated release of TxA_2 in response to Dazoxiben (50 μM) in the aorta of male rats. DAZ significantly decreased the release of TxA_2 ($.001 \leq P \leq 0.05$). Bars are means ± S.E.

CHAPTER V

DISCUSSION AND SUMMARY

This study investigated the effects of selective COX-1 and COX-2 inhibitors and specific prostanoid pathway inhibitors on the production of vascular prostanoids, specifically thromboxane and prostacyclin, in the aortas of male and female Sprague-Dawley rats. The central hypothesis tested was that selective inhibition of COX-1 would reduce the release of TxA_2 to a greater extent than PGI_2 in the aorta, whereas selective COX-2 inhibition would decrease the release of PGI_2 more than TxA_2. We further hypothesized that estrogen would enhance the production of prostanoids derived from COX-2, but not those derived from COX-1. Furthermore, it was hypothesized that TxA_2 has an inhibitory effect on PGI_2 synthesis, mediated by a TP receptor mediated signaling pathway that inhibits PGI_2 synthase. Indeed, the selective inhibition of COX-1 and COX-2 in male and female rat aortas revealed that a clear sexual dimorphism exists in the synthesis of PGI_2 and TxA_2 through the COX-1 and COX-2 pathways. VP-stimulated TxA_2 release was significantly increased in aortas from both female and male rats; however, VP-stimulated PGI_2 release was significantly increased only in female rats.

Selective inhibition of COX-1 with SC-560 and inhibition of COX-2 with NS-398 significantly reduced VP-stimulated release of PGI_2 in female rats, but not in male rats. There was no difference in the inhibition of PGI_2 by SC-560 or NS-398 between any groups. Selective inhibition of COX-1 with SC-560 and inhibition of COX-2 with

NS-398 significantly reduced VP-stimulated TxA_2 release in the aortas of both male and female rats. Furthermore, inhibition of VP-stimulated TxA_2 release with NS-398 was greater than with SC-560 in estrogen-replaced OvX-ER-F females while inhibition with SC-560 was similar among all groups.

This study also investigated the mechanism by which TxA_2 depresses PGI_2 synthesis. Although Dazoxiben, TxS inhibitor proved a marked increase in PGI_2 release while depressing TxA_2 release, inhibition of PKC (with Calphostin C) did not produce a significant increase in PGI_2 release. Similarly, there were no significant increases in PGI_2 release after treatment with a TP receptor antagonist (Furegrelate) or with the combined TP receptor antagonist and TxS inhibitor (Ridogrel).

5.1 Estrogen Levels

In order to determine the role of estrogen in the regulation of PGI_2 and TxA_2 release via the cyclooxygenase isoforms, estrogen-intact female rats (Int-F) , ovariectomized rats (OvX-F), and estrogen-replaced OvX-F rats (OvX-ER-F) were included in this study. Plasma 17β–estradiol levels were measured in all rats included in the present study. Ovariectomy reduced the plasma 17β–estradiol concentrations to a similar level found in M, whereas estrogen replacement in ovariectomized female rats restored plasma 17β–estradiol to similar concentrations found in Int-F. Estrogen levels in Int-F were lower than OvX-ER-F because the rats were sacrificed on random days within the estrus cycle. OvX-ER-F rats maintain high levels of circulating estrogen from the estradiol replacement treatment. It is normal for cycling Int-F to produce estrogen

levels in range 19 ± 5pg/mL plasma at the onset of estrus to 284 ± 25pg/mL plasma during proestrus, thus a discrepancy between Int-F and OvX-ER-F estrogen levels is to be expected (64). The concentrations of plasma 17β–estradiol measured in the present study were similar to age matched male and female animals in previous studies (8, 73).

5.2 Basal and VP-Stimulated Release of PGI_2 and TxA_2

In the present study, basal release of PGI_2 and TxA_2 was similar in all groups. In females, stimulation with vasopressin significantly increased the release of PGI_2 and TxA_2; however, in males TxA_2 was also increased. VP-stimulated release of PGI_2 was increased more in Int-F and OvX-ER-F compared to M and OvX-F. Similarly, VP-stimulated release of TxA_2 was increased more in Int-F and OvX-ER-F compared to M and OvX-F rats. This finding was not surprising, as it is consistent with previous studies that reported a three-fold increase in response to VP by the female rat aorta (4, 43, 68). The evidence from the present study and previous studies suggest that the sexual dimorphism in the systemic vasculature is dependent upon the presence of estrogen, a potent ovarian hormone in females. Although these studies reported that the mechanism responsible for the increase in prostanoid release is the effect of estrogen to upregulate COX-2, TxS and TP receptor expression, the studies did not investigate the relative contributions of COX-1 vs. COX-2 on prostanoid release.

5.3 Effects of Selective COX-1 and COX-2 Inhibition on PGI_2 Release from the Aorta

To determine the role of the different COX enzymes on the release of PGI_2 in female and male rat aorta, the selective COX-1 inhibitor SC-560 was used in Int-F, OvX-F and OvX-ER-F rats. COX-1 is well documented to be constitutively expressed throughout the body and serves as a housekeeping enzyme. SC-560 reduced VP-stimulated PGI_2 release similarly in Int-F by 56% and by 54% in M. These results demonstrate an explicit role for COX-1 in PGI_2 synthesis; however, they also suggest that estrogen does not affect the contribution of COX-1 to PGI_2 release. It is not surprising that estrogen affected COX-1 similarly in all groups because COX-1 is known as the constitutive isoform of the cyclooxygenase enzyme and was not altered by estrogen in previous studies (45).

The present study suggests COX-1 is a major contributor to PGI_2 synthesis in the rat aorta. Despite the dogma that COX-2 is solely responsible for PGI_2 synthesis, multiple studies have also reported a significant role for COX-1 synthesis of PGI_2 in the vasculature (37, 38, 44). It is crucial that COX-1 plays a role in the synthesis of PGI_2 in the vasculature to counterbalance the presence of other vasoconstricting prostanoids, such as TxA_2. This may be an important step toward disproving the theory that aspirin offers cardiovascular protection through selective COX-1 inhibition.

The COX-2 isoform is constitutively expressed in some tissues throughout the body such as kidney, lung, spinal cord, heart and in the rat aorta (43). Unlike COX-1, COX-2 is also an inducible form of the cyclooxygenase enzyme, and is known to be mediated by inflammatory agents, mitogens and hormones such as estrogen (19, 43).

The present study found that VP-stimulated PGI_2 release was significantly reduced by COX-2 inhibition with NS-398 in all groups by an average of 71% (76% in Int-F and 53% in M*)*. This indicates that COX-2 contributes significantly to the synthesis of PGI_2 in both the male and female rat aorta.

VP-stimulated PGI_2 release in female rats (Int-F and OvX-ER-F) was reduced to similar levels in estrogen-depleted rats (M and OvX-F) by COX-1 and COX-2 inhibition. This is significant because if COX-2 inhibition reduces the protective prostaglandins released by the vasculature similarly to COX-1 inhibition in females, non-selective NSAIDs may predispose females to cardiovascular risks. In estrogen-depleted rats (M and OvX-F), inhibition of either COX isoform reduced PGI_2 release similarly. Therefore, COX-1 selective NSAIDs (such as aspirin) reduce the synthesis of PGI_2 similarly to non-selective NSAIDs.

5.4 Effects of Selective COX-1 and COX-2 Inhibition on TxA_2 Release from the Aorta

The COX-1 isoform is widely known to be present in platelets as well as constitutively expressed in most cell types. Historically, COX-1 was believed to mediate TxA_2 synthesis in the platelets, causing platelet aggregation and vasoconstriction. However, more recent studies have revealed that the vascular wall is also a physiologically relevant source of TxA_2 (25, 43). In the present study, inhibition of VP-stimulated TxA_2 release with SC-560 caused a greater reduction in males than in ovariectomized, estrogen replaced females. Interestingly, inhibition with NS-398 significantly reduced TxA_2 release similarly in all groups, which strongly suggests that

both COX-1 and COX-2 contribute to the release of TxA_2 in both the male and female rat aorta, although COX-1 may play a larger role in TxA_2 release in males than females.

Aspirin, a widely used relatively specific COX-1 inhibitor, is recommended to reduce the risk of negative thrombotic events by reducing TxA_2 in the vasculature while preserving the synthesis of vasoprotective PGI_2. Recent studies have indicated that both TxA_2 and PGI_2 are significantly synthesized via COX-1 in the vasculature, which was confirmed in the present study. Additionally, this study also established that COX-1 and COX-2 both independently contribute to the production of a significant amount of PGI_2 and TxA_2 in the systemic vasculature. This suggests that the mechanism by which aspirin confers its cardioprotective effects is not by solely reducing TxA_2, but rather an unknown mechanism that alters the sensitive balance of PGI_2 and TxA_2 in the vasculature. Furthermore, it has been suggested that COX-2 selective inhibitors are systematically more potent in inhibiting the endothelial production of PGI_2 than the platelet production of thromboxane A_2 (51). This would support the theory that the use of COX-2 inhibitory drugs may increase the risk of thrombotic vascular events, however, the present study found no difference in the production of PGI_2 versus TxA_2 by COX-1 vs. COX-2. Thus, the apparently deleterious effects of COX-2-selective inhibitory drugs on thrombotic events may result from a different mechanism, independent of PGI_2 inhibition.

5.5 Effects of Calphostin, Ridogrel, Furegrelate, and Dazoxiben on PGI_2 and TxA_2 Release

Within the vascular system, PGI_2, TxA_2, and their respective receptors are known to interact and mediate the release of these vascular mediators. PGI_2 and TxA_2 are derived from the same intermediate PGH_2, via the action of their respective synthase enzymes, prostacyclin synthase and TxS. Because the balance between PGI_2 and TxA_2 is critical for maintaining homeostasis in the vasculature, altering the production of one of these mediators can cause significant effects. Previous studies reported that inhibition of TxS causes accumulation of the intermediate PGH_2, thus enhancing PGI_2 production (43). PGI_2 has been shown to depress TxA_2 production in the cardiovascular system, causing an overall vasodilatory effect (17). Conversely, TxA_2 has been shown to attenuate PGI_2 within endothelial cells of the vasculature, leading to increased vasoconstriction and platelet aggregation (43). Several mechanisms may be involved in pathway interaction between PGI_2 and TxA_2. Cross-talk between the two prostanoids and their receptors may be a result of receptor heterodimerization, overlapping genetic response elements for the receptors, or activation/inactivation of intracellular proteins involved within each pathway by the opposing prostanoid (5, 15).

The present study investigated the role of PKC, TxS and the TP receptor in the interactions between the PGI_2 and TxA_2 pathways. Because the balance of PGI_2 and TxA_2 is critical for maintaining homeostasis in the vasculature, altering the production of one of these mediators can cause significant effects. Specifically, the present study investigated the mechanism by which TxA_2 regulates PGI_2 in vivo.

PGI_2 and TxA_2 are derived from the same intermediate PGH_2, and their respective synthase enzymes, prostacyclin synthase and TxS. Previous studies reported that inhibition of TxS causes accumulation of the intermediate PGH_2, thus enhancing PGI_2 production (43). Furthermore, it has been suggested that another form of cross-talk between the pathways occurs by TxA_2 down-regulating PGI_2 release, because the TXS inhibitor Dazoxiben increases PGI_2 release in the rat aorta (45). It was hypothesized that through the downstream effects of PKC-mediated nitrosylation of tyrosine residues on PGI synthase, TxA_2 decreases PGI_2 production in the vascular wall. These mechanisms were investigated using Calphostin C, a PKC inhibitor, Ridogrel, a combined TxS inhibitor and TP receptor antagonist, Furegrelate, a TP receptor antagonist, and Dazoxiben, a TxS inhibitor. Interestingly, despite Ridogrel and Furegrelate significantly decreasing TxA_2 release, neither of these inhibitors significantly attenuated PGI_2 release in the aorta. Dazoxiben markedly attenuated TxA_2 release, which was not surprising. In previous studies using Dazoxiben on PGI_2 production in rat aorta (45) and human blood vessels (3), DAZ increased prostacyclin production. The authors suggested that inhibition of TxS made endoperoxides PGG_2 and PGH_2 more readily available for conversion to PGI_2 (13). However, the relative increase in PGI_2 is far greater in comparison to the amount of PGG_2 and PGH_2 that would be made available by TxS inhibition. One possible explanation for the extreme increase in PGI_2 is the chemical structure of Dazoxiben, which is very similar to inhibitors of long chain fatty acyl COA synthetase, which results in increased release of arachidonic acid from cells. Thus,

Dazoxiben could inhibit TxS and release more arachidonic acid from cells, resulting in the substantial increase in PGI_2.

Calphostin did not alter PGI_2 release either; however, it did cause a 228% increase in release of TxA_2. The mechanism by which Calphostin increased TxA_2 release is unclear. It is possible that PKC is involved in homologous desensitization (negative feedback loop) of TxA_2, therefore inhibition of PKC causes increased release of TxA_2. In a previous study, PKC knockout mice exhibited shorter times to occlusion and shorter bleeding times in tail bleeding experiments suggesting that PKC negatively regulates TxA_2, but may not affect PGI_2 (9).

The present study also used the TP receptor antagonist Furegrelate, and the combined TxS inhibitor and TP receptor antagonist, Ridogrel, to clarify the effects of TxA_2 on PGI_2 synthesis. Despite the observation that Ridogrel and Furegrelate both significantly decreased TxA_2, neither of these inhibitors significantly affected the release of PGI_2 in the aorta. However, TxS inhibitor, Dazoxiben (DAZ) showed inhibition of TxS and reduction of TxA_2 production enhanced the production of PGI_2. This is similar to previous studies which suggest that TxS inhibitors enhance the production of PGI_2 (43). Ridogrel and Furegrelate decreased TxA_2 levels to concentrations similar to those with the use of DAZ; however they did not have a similar effect on PGI_2. This discrepancy is puzzling, and may be of interest for future research.

Although the TP receptor antagonists did not show increase in PGI_2, the effects may be of clinical relevance. The balance of TxA_2 and PGI_2 is complex and sensitive. Blocking the TP receptor may potentially reduce the negative cardiovascular effects

while not altering the release of PGI_2 (11). This may be beneficial in situations where only mild effects of PGI_2 are desired, particularly PGI_2's vasodilatory effect may be desired, but its antiplatelet effect may not. The EC_{50} for inhibition of platelet aggregation by PGI_2 was found to be 1.3nM in isolated uterine artery, while the EC_{50} for vasodilation by PGI_2 was found to be 10^{-8}M in bovine coronary artery (40, 62). Therefore, it is unlikely that one could regulate the amount of PGI_2 to produce one effect without the other.

In conclusion, although for years the dogma was that the PGI_2/TxA_2 balance can be altered by COX-2 specific drugs to cause negative cardiovascular effects, more recent studies in addition to the present study, suggest otherwise. It was found that COX-1 and COX-2 both mediate significant production of both PGI_2 and TxA_2 similarly in the rat. Furthermore, when estrogen is present, a marked sexual dimorphism exists in the production of both TxA_2 and PGI_2. The production of both prostanoids was enhanced in the presence of estrogen. Further, there was no difference in the inhibition of the prostanoids when estrogen was not present. In the presence of estrogen, the selective COX-2 inhibitor seemed to attenuate TxA_2 production more than PGI_2. This suggests that in females, it is unlikely that COX-2 inhibitors would elevate the risk of thrombosis, and may be useful in determining the guidelines for NSAIDs usage in women. Furthermore, at the doses of COX-1 and COX-2 inhibitors used, the present study does not support the existing dogma that COX-2 selective inhibitors increase the risk of cardiovascular disease by tipping the balance between TxA_2/PGI_2 in favor of TxA_2.

REFERENCES

1. Akarasereenont P. Techatraisak K., Thaworn A., Chotewuttakorn S. The induction of cyclooxygenase-2 by 17B-estradiol in endothelial cells is mediated through protein kinase C. *Inflammation Research* 40: 460-465, 2000.

2. Alwan A. World Health Organization. *Promoting cardiovascular health in the developing world.* Washington (DC): National Academies Press (US), 2010.

3. Antman E.M. Bennett J.S., Daugherty A., Furberg C., Robert H., Taubert K.A. Use of nonsteriodal antiinflammatory drugs: an update for clinicians. A scientific statement from the American Heart Association. *Circulation* 115: 1634-1642, 2007.

4. Baltzer W.I. Kuo L. and Stallone J.N. Estrogen enhances constrictor prostanoids and blood pressure in aortic coarctation-induced hypertension in female rats. *FASEB J* 17: 1234, 2003.

5. Berthois Y. Pourreau-Schneider N., Gandilhon P., Mittre H., Tubiana N., and Martin P.M. Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. *Journal of Steriod Biochemistry* 25: 963-972, 1986.

6. Bombardier C., Laine L, Reicin A, et al. . Comparison of Upper Gastrointestinal Toxicity of Rofecoxib and Naproxen in Patients with Rheumatoid Arthritis. *The New England Journal of Medicine* 343: 1520-1528, 2000.

7. Bombardier C., Laine L., Reicin A., Shapiro D., R. Burgos-Vargas R., Davis B., Day R., Ferraz M.B., Hawkey C.J., Hochberg M.C., Kvien T.K., Schnitzer T.J.

Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. *New England Journal of Medicine* 343: 1520-1528, 2000.

8. Butcher R.L. Collins W.E., Fugo N.W. Plasma concentration of LH, FSH, Prolactin, Progesterone and Estradiol-17β throughout the 4-day estrous cycle of the rat. *Endocrinology* 94: 1704-1708, 1974.

9. Bynagari-Settipalli Y.S. Lakhani P., Jin J., Bhavaraju K., Rico M.C., Kim S., Woulfe D., Kunapuli S.P. Protein kinase C isoform ε negatively regulates ADP-induced calcium mobilization and thromboxane generation in platelets. *Atherosclerosis, Thrombosis, and Vascular Biology* 32: 1211-1219, 2012.

10. Caughey G.E. Cleland L.G., Penglis P.S., Gamble J.R., James M.J. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. *Journal of Immunology* 167: 2831-2838, 2001.

11. Cheng Y., Austin S.C., Rocca B., Koller B.H., Coffman T.M., Grosser T., Lawson J.A., and FitzGerald G.A. Role of Prostacyclin in the Cardiovascular Response to Thromboxane A2. *Science* 296: 539-541, 2002.

12. Cosentino F. Eto M., De Paolis P., Van Der Loo B., Bachschmid M., Ullrich V., Kouroedov A., Delli Gatti C., Joch H., Volpe M., Lüscher T.F. . High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species. *Circulation* 107: 1017-1023, 2003.

13. Cowley A. Jones E., Hanley S. Effects of dazoxiben, an inhibitor of thromboxane synthetase, on forearm vasoconstriction in response to cold stimulation, and on human blood vessel prostacyclin production. *British Journal of Clinical Pharmacology* 15: 107-112, 1983.

14. Davidge S.T. Prostaglandin H synthase and vascular function. *Circulation Research* 89: 650-660, 2001.

15. DeWitt D.L. Day J.S., Sonnenburg W.K., and Smith W.L. Concentrations of prostaglandin endoperoxide synthase and prostaglandin I2 synthase in the endothelium and smooth muscle of bovine aorta. *Journal of Clinical Investigation* 72: 1882-1888, 1983.

16. Díaz N.A. and Valverde M.A. The estrogen trinity: membrane, cytosolic, and nuclear effects. *Physiology* 16: 251-255, 2001.

17. Eatman D. Stallone J.N., Rutecki G.W., and Whittier F.C. Sex differences in extracellular and intracellular calcium-mediated vascular reactivity to vasopressin in rat aorta. *European Journal of Pharmacology* 361: 207-216, 1998.

18. Felatou M. Huang Yu., Vanhoutte P.M. Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *British Journal of Pharmacology* 164: 894-912, 2011.

19. Félétou M., Huang, Y., Vanhoutte P.M. Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *British Journal of Pharmacology* 164: 894-912, 2011.

20. Feletou M. Vanhoutte P.M., Vanbeuren T.J. The TP-receptor: the common villain. *Journal of Cardiovascular Pharmacology* 55: 317-332, 2010.

21. Fetalvero K.M., Martin, K.A., Hwa, J. Cardioprotective prostacyclin signaling in vascular smooth muscle. *Prostaglandins & Other Lipid Mediators* 82: 109-118, 2007.

22. FitzGerald G.A. Brash A.R., Falardeau P. and Oates J.A. Estimated rate of prostacyclin secretion into the circulation of normal man. *The Journal of Clinical Investigation* 68: 1272-1276, 1981.

23. FitzGerald G.A. Pedersen A.K., Patrono C. Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. *Circulation* 67: 1174-1177, 1983.

24. Flavahan N.A. Balancing prostanoid activity in the human vascular system. *Trends in Pharmacological Sciences* 28: 106-110, 2007.

25. Fulton C.T. Stallone J.N. . Sexual dimorphism in prostanoidpotentiated vascular contraction: roles of endothelium and ovarian steroids. . *Am J Physiol Heart Circ Physiol* 283, 2002.

26. Funk C.D. FitzGerald G.A. COX-2 Inhibitors and Cardiovascular Risk. *Journal of Cardiovascular Pharmacology* 50: 470-476, 2007.

27. Fuster V. Global status report on noncommunicable dieseases 2010. In: *Deaths from CVD and diabetes*, edited by Organization WH, 2011.

28. Garner A. Flemström G., Allen A., Heylings J.R., McQueen S. Gastric mucosal protective mechanisms: roles of epithelial bicarbonate and mucus secretions. *Scand J Gastroenterol Suppl* 101: 79-86

1984.

29. Gleim S. Kasza Z., Martin K., Hwa J. Prostacyclin receptor/thromboxane receptor interactions and cellular responses in human atherothrombotic disease. *Current Atherosclerosis Reports* 11: 227-235, 2009.

30. GradyD. Rubin S.M., Petitti D.B., Fox C.S., Black D., Ettinger B., Ernster V.L., Cummings S.R. Hormone therapy to prevent disease and prolong life in postmenopausal women. *Annals of Internal Medicine* 117: 1016-1037, 1992.

31. Grodstein F Stampfer MJ, Manson JE, Colditz GA, Willett WC, Rosner B, Speizer FE, Hennekens CH. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. *New England Journal of Medicine* 335: 453-461, 1996.

32. Grosser T. Fries S., FitzGerald G.A. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *The Journal of Clinical Investigation* 116: 4-15, 2006.

33. Hernández-Díaz S. Rodríguez L.A. Association between nonsteroidal anti-inflammatory drugs and upper gastrointestinal tract bleeding/perforation: An overview of epidemiologic etudies published in the 1990s. *Archives of Internal Medicine* 160: 2093-2099, 2000.

34. Hunt J. Merritt J., MacDermot J., Keen M. Characterization of the thromboxane receptor mediating prostacyclin release from cultured endothelial cells. *Biochemical Pharmacology* 43: 1747-1752, 1992.

35. Kawka D.W. Ouellet M., Hetu P.O., Singer I.I., Riendeau D. Double-label expression studies of prostacyclin synthase, thromboxane synthase and COX isoforms in normal aortic endothelium *Biochem Biophys Acta* 1771: 45-54, 2007.

36. Khan K.N. Venturini C.M., Bunch R.T., Brassard J.A., Koki A.T., Morris D.L., Trump B.F., Maziasz T.J., Alden C.L. Interspecies differences in renal localization of cyclooxygenase isoforms: implications in nonsteroidal antiinflammatory drug-related nephrotoxicity. *Toxicologic Pathology* 26: 612-620, 1998.

37. Kirkby N.S. Lundberg M.H., Harrington L.S., Leadbeater P.D., Milne G.L., Potter C.M., Al-Yamani M., Adeyemi O., Warner T.D., Mitchell J.A. Cyclooxygenase-1, not cyclooxygenase-2, is responsible for physiological production of prostacyclin in the cardiovascular system. *Proceedings of the National Academy of Sciences of the United States of America* 110: 17597-17602, 2012.

38. Kirkby N.S. Zaiss A.K, Urquhart P., Jiao J., Austin P.J., Al-Yamani M., Lundberg M.H., MacKenzie L.S., Warner T.D., Nicolaou A., Herschman H.R., and Mitchell J.A. LC-MS/MS confirms that COX-1 drives vascular prostacyclin whilst gene expression pattern reveals non-vascular sites of COX-2 expression. *PLos One* 8: 1-9, 2013.

39. Knapp H.R. Oelz O., Sweetman B.J., Oates J.A. Synthesis and metabolism of prostaglandins E2, F2alpha and D2 by the rat gastrointestinal tract. Stimulation by a hypertonic environment in vitro. *Prostaglandins* 15: 751-757, 1978.

40. Kobzar G. Shelkovnikov S., Mardla V., Savitski G., Lopp M., Kanger T., Lille U. A 15-nonstereogenic carbocyclic analogue of prostacyclin: effects on human platelets and uterine artery. *Journal of Lipid Mediators and Cell Signaling* 10: 243-249, 1994.

41. Kweder S. Vioxx and drug safety. *Senate Committee on Finance*. Food and Drug Administration, 2004.

42. Kyrle P.A. Eichler H.G., Jager U., Lechner K. Inhibition of prostacyclin and thromboxane A2 generation by low-dose aspirin at the site of plug formation in man in vivo. *Circulation Research* 75: 1025-1029, 1987.

43. Li M. Kuo L., Stallone J.N. Estrogen potentiates constrictor prostanoid function in female rat aorta by upregualation of cyclooxygenase-2 and thromboxane pathway expression. *American Journal of Physiology Heart and Circulation Physiology* 294: 2444-2455, 2008.

44. Liu B. Luo W., Zhang Y., Li H., Zhu N., Huang D., Zhou Y. Involvement of cyclo-oxygenase-1-mediated prostacyclin synthesis in the vasoconstrictor activity evoked by ACh in mouse arteries. *Experimental Physiology* 97: 277-289, 2011.

45. Liu C.Q. Leung F.P., Wong S.L., Wong W.T., Lau C.W., Lu L., Yao X., Yao T., Huang Y. Thromboxane prostanoid receptor activation impairs endothelial nitric oxide-dependent vasorelaxations: The role of Rho kinase. *Biochemical Pharmacology* 78: 374-381, 2009.

46. Marcondes F.K., Bianchi, F.J. and Tanno, A.P. Determination of the estrous cycle phases of rats: some helpful considerations. *Brazilian Journal of Biology* 62, 2002.

47. McAdam B.F. Catella-Lawson F., Mardini I.A., Kapoor S., Lawson J.A., FitzGerald G.A. . Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: The human pharmacology of a selective inhibitor of COX-2. *Proceedings of the National Academy of Sciences of the United States of America* 96: 272-277, 1999.

48. Mendelsohn M.E. Karas R.H. The time has come to stop letting HERS take way the dogma. *Circulation* 104: 2256-2264, 2001.

49. Mikkola T. Turunen P., Avela K., Orpana A, Viinikka L., Ylikorkala O. 17 beta-estradiol stimulates prostacyclin, but not endothelin-1, production in human vascular endothelial cells. *Journal of Clinical Endocriniology and Metabolism* 80: 1832-1836, 1995.

50. Mitchell J.A. Akarasereenont P., Thiemermann C., Flower R.J., and Vane J.R. Selectivity of non steroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proceedings of the National Academy of Sciences of the United States of America* 90: 11693-11697, 1993.

51. Mitchell J.A. Lucas R., Vojnovic I., Hasan K., Pepper J.R., Warner T.D. Stronger inhibition by nonsteroid anti-inflammatory drugs of cyclooxygenase-1 in endothelial cells than platelets offers an explanation for increased risk of thrombotic events. *Journal of the Federation of American Societies for Experimental Biology* 20: 2468-2475, 2006.

52. Mitchell J.A. Warner T.D. . COX isoforms in the cardiovascular system: understanding the activities of non-steriodal anti-inflammatory drugs. *Nature Reviews Drug Discovery* 5: 75-86, 2006.

53. Miyata A. Hara S., Yokoyama C., Inoue H., Ullrich V., Tanabe T.,. Molecular cloning and expression of human prostacyclin synthase. *Biochemical and Biophysical Research Communications* 200: 1728-1734, 1994.

54. Nishijima M. Katori M., Yamamoto S. Induction of cyclooxygenase type-2 (COX-2) in rat endometrium at the peak of serum estradiol during the estrus cycle. *The Japanese Journal of Pharmacology* 69: 289-291, 1995.

55. Okahara K. Sun B., Kambayashi J. Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 12: 1922-1926, 1998.

56. Park J.Y. Pillinger M.H., Abramson S.B. . Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clinical Immunology* 119: 229–240, 2006.

57. Peskar B. On the synthesis of prostaglandins by human gastric mucosa and its modification by drugs. *Biochem Biophys Acta* 487: 307-314, 1977.

58. Pomposiello S. Yang X.P., Liu Y.H., Surakanti M., Rhaleb N.E., Sevilla M., Carretero O.A. Autacoids mediate coronary vasoconstriction induced by nitric oxide synthesis inhibition. *Journal of Cardiovascular Pharmacology* 30: 599-606, 1997.

59. Praveen P.N. Knaus E.E. Evolution of nonsteriodal anti-inflammatory drugs (NSAIDs): Cyclooxygenase (COX) inhibition and beyond. *Journal of Pharmacy & Pharmaceutical Sciences* 11: 81-110, 2008.

60. Rainsford K.D. Mechanisms of gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs. *Scandinavian Journal of Gastroenterology* 24, 1989.

61. Roderick P.J. Wilkes H.C., and Meade T.W. The gastrointestinal toxicity of aspirin: an overview of randomised controlled trials. *British Journal of Clinical Pharmacology* 35: 219-226, 1993.

62. Rosolowsky M. Campbell W.B. Role of PGI2 and epoxyeicosatrienoic acids in relaxation of bovine coronary arteries to arachidonic acid. *American Journal of Physiology* 264: 327-335, 1993.

63. Rostom A. Muir K., Dubé C., Jolicoeur E., Boucher M., Joyce J., Tugwell P., Wells, G.W. Gastrointestinal safety of cyclooxygenase-2 inhibitors: A cochrane collaboration systematic review. *Clinical Gastroenterology and Hepatology* 5: 818-828.e815, 2007.

64. Shaikh A.A. Estrone and estradiol levels in the ovarian venous blood from rats during the estrous cycle and pregnancy. *Biology of Reproduction* 5: 297-307, 1971.

65. Simmons D.L. Botting R.M., Hia T. Cyclooxygenase isozymes: The biology of prostaglandin synthesis and inhibition. *Physiological Reviews* 56: 387-437, 2004.

66. Simmons D.L. Levy D.B., Yannoni Y., Erikson R. L. . Identification of phorbol ester-repressible v-src-inducible gene. *Proceedings of the National Academy of Sciences of the United States of America* 86: 1178-1182, 1989.

67. Smyth E.M. Grosser T., Wang M., Yu Y., and FitzGerald G.A. Prostanoids in health and disease. *The Journal of Lipid Research* 50: 423-428, 2009.

68. Sobrino A. Oviedo P., Novella S., Laguna-Fernandez A., Bueno C., Garcı´a-Pe´rez M.A., Tarı´n J.J., Cano A. and Hermenegildo C. Estradiol selectively stimulates endothelial prostacyclin production through estrogen receptor-alpha. *Journal of Molecular Endocrinology* 44: 237-246, 2010.

69. Sutton-Tyrrell K. Lassila H.C., Meilahn E., Bunker C., Matthews K.A., Kuller L.H. Carotid atherosclerosis in premenopausal and postmenopausal women and its association with risk factors measured after menopause. . *Stroke* 29: 1116-1121, 1998.

70. Thomas D.W. Mannon R.B., Mannon P.J., Latour A., Oliver J.A., Hoffman M. et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *Journal of Clinical Investigation* 102: 1994-2001, 1998.

71. Tuleja E. Mejza F., Cmiel A., Szczeklik A. Effects of cyclooxygenases inhibitors on vasoactive prostanoids and thrombin generation at the site of microvascular injury in healthy men. *Arteriosclerosis, Thrombosis, and Vascular Biology* 23: 1111-1115, 2003.

72. Turner E.C. Kinsella B.T. Estrogen increases expression of the human prostacyclin receptor within the vasculature through an ERα-dependent mechanism. *Journal of Molecular Biology* 413: 899, 2011.

73. Voss M.R. Stallone J.N., Li M., Cornelussen R.N., Knuefermann P., and Knowlton A.A. . Gender differences in the expression of heat shock proteins: The effect of estrogen. . *Am J Physiol Heart Circ Physiol* 285: H687-H692, 2003

74. W.B. White. Cardiovascular effects of the cyclooxygenase inhibitors. *Hypertension* 49: 408-418, 2007.

75. Wallace J.L. Prostaglandins, NSAIDs, and gastric mucosal protection: Why doesn't the stomach digest itself? *Physiological Reviews* 88: 1547-1565, 2008.

76. Warner T.D. Mitchell J.A. COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs. *The Lancet* 371: 270-273, 2008.

77. Wells G Herrington DM. The Heart and Estrogen/Progestin Replacement Study: what have we learned and what questions remain? *Drugs Aging* 15: 419-422, 1999.

78. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin. *Journal for the American Medical Association* 288: 321-333, 2002.

79. Xie W. Robertson D.L., Simmons D.L. Mitogen-inducible prostaglandin G/H synthase: A new target for nonsteroidal antiinflammatory drugs. *Drug Development Research* 25: 249-265, 1992.

80. Yan Cheng Sandra C. Austin, Bianca Rocca,Beverly H. Koller, Thomas M. Coffman, Tilo Grosser, John A. Lawson, and Garret A. FitzGerald. Role of Prostacyclin in the Cardiovascular Response to Thromboxane A2. *Science* 296: 539-541, 2002.